Protein Kinase C-θ Phosphorylation of Moesin in the Actin-binding Sequence*

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Moesin, a member of the ezrin-radixin-moesin (ERM) family of membrane/cytoskeletal linkage proteins, is known to be threonine-phosphorylated at Thr558 in activated platelets within its conserved putative actin-binding domain. The pathway leading to this phosphorylation step and its control have not been previously elucidated. We have detected and characterized reactions leading to moesin phosphorylation in human leukocyte extracts. In vitro phosphorylation of endogenous moesin, which was identified by peptide microsequencing, was dependent on phosphatidylglycerol (PG) or to a lesser extent, phosphatidylinositol (Pi), but not phosphatidylserine (PS) and diacylglycerol (DAG). Analysis of charge shifts, phosphoamino acid analysis, and stoichiometry was consistent with a single phosphorylation site. By using mass spectroscopy and direct microsequencing of CNBr fragments of phospho-moesin, the phosphorylation site was identified as KYK*LRQIR (where * indicates the phosphorylation site) (Thr558), which is conserved in the ERM family. Recombinant moesin demonstrated similar in vitro phospholipid-dependent phosphorylation compared with the endogenous protein. The phosphorylation site sequence of moesin displays a high degree of conservation with the pseudosubstrate sequences of the protein kinase C (PKC) family. We identified the kinase activity as PKC-θ on the basis of immunodepletion of the moesin kinase activity and copurification of PKC-θ with the enzymic activity. We further demonstrate that PKC-θ displays a preference for PG vesicles over PI or PS/DAG, with minimal activation by DAG, as well as specificity for moesin compared with myelin basic protein, histone H1, or other cellular proteins. Expression of a human His-tagged PKC-θ in Jurkat cells and purification by Ni2+ chelate chromatography yield an active enzyme that phosphorylates moesin. PG vesicle binding experiments with expressed PKC-θ and moesin demonstrate that both bind to vesicles independently of one another. Thus, PKC-θ is identified as a major kinase within cells with specificity for moesin and with activation under non-classical PKC conditions. It appears likely that this activity corresponds to a specific intracellular pathway controlling the function of moesin as well as other ERM proteins.

MATERIALS AND METHODS

All electrophoresis materials were supplied by Bio-Rad, and all other chemicals, as well as the catalytic subunit of PKA, were supplied by

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1 The abbreviations used are: ERM, ezrin-radixin-moesin; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; AML, acute myelogenous leukemia; CM, carboxymethyl; MBP, myelin basic protein; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BisTris, 2-h[[(2-hydroxyethyl)aminol-2-(2-hydroxyethyl)-propane-1,3-diol; HPLC, high pressure liquid chromatography; DAG, diacylglycerol; AKAP, protein kinase A anchoring protein; HIV, human immunodeficiency virus.
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Sigma unless otherwise noted. Protein kinase C (PKC), purified from rat brain, was obtained from Lipides (Westfield, NJ) and is a mixture of isozymes. Isozyme-specific anti-PKC sera were used for immunodepletion experiments were obtained from R & D Diagnostics (Berkeley, CA). These antibodies, with the exception of anti-PKC-γ, are capable of immunoprecipitation (32). Anti-PKC-γ (V3) and anti-PKC-θ sera were used as described previously (7); anti-PKC-θ (F-13) specific for the C-terminal residues 691–703, anti-PKC-α (C-20) specific for C-terminal residues 651–672, and anti-His₆ probe (H-15) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies and reagents for ECL detection were obtained from Amersham.

Phosphorylation—Phosphorylation reactions were carried out as described previously (6), except that the phospholipid was sonicated in water for 30 min to produce a suspension of vesicles. After the reactions, proteins were precipitated by adding 1/10 volume of 0.15% deoxycholic acid, mixing, and then quickly adding 1/10 volume of 100% (w/v) trichloroacetic acid, mixing, and centrifuging in a microcentrifuge for 5 min. This method improves protein yield and results in a stable precipitate (8). The precipitate was rinsed once with 20 μl of water, dissolved in 10 μl of 1× Laemmli sample buffer at 56 °C for 10 min, and subjected to 10% SDS-PAGE as described (9). The gels were stained with Coomassie Blue and dried before cellphane sheets, and autoradiograms were made. Protein determinations were done as described (10).

Purification of the pS7-Purification of the 73-kDa substrate was carried out as described previously, including ammonium sulfate fractionation and chromatography of proteins on CM-cellulose (6), which was based on earlier work (11). Fragments from a gradient elution of the CM-cellulose column were assayed for PG-stimulable phosphorylation of a 73-kDa substrate, and the peak fractions (about 0.1–0.15 μM NaCl) were combined and concentrated using a PM-30 membrane (Amicon, Desalting ammonium sulfate precipitate was collected by centrifugation, and the recombinant moesin was used in the moesin kinase assay described above. Purification of Moesin-specific Protein Kinase Activity from AML Cells—Each step in the purification of protein kinase activity utilized His₆-moesin (10 μg/ml) as a substrate in the phosphorylation reaction described above. Cells from patients with AML were obtained by leukapheresis. Cells were pelleted at 500 × g, rinsed with PBS, and stored in BSB (10 mM Na₂HPO₄, pH 6.5, 2 mM EDTA, 1 mM EGTA) at −70 °C. Cells were thawed, suspended in BSB (10 mM NaF, 1 mM Na₃VO₄, and glycerol was added to 7%, and the preparation was stored in an ultrafiltration cell. The sample was applied to a Millipore DEAE 8HR chelate affinity column (see below). The thawed suspension was sonicated with 40 bursts from a microtip at 70 °C for 20 s. Sonication was followed by centrifugation at 10,000 × g for 10 min. The supernatant was removed by centrifugation at 12,000 × g for 20 min, and the 3.2 ml of supernatant was combined with 3.2 ml of “bind” buffer and applied to a 2-ml Ni²⁺ chelate affinity column (Invitrogen). The column was rinsed according to the supplier's instructions, and the recombinant moesin was eluted with an imidazole gradient from 0 to 400 mM imidazole.

Immunodepletion of Moesin Kinase Activity—Isozyme-specific PKC antibody rabbit antibodies were purified from crude antiserum by performing a 40% ammonium sulfate precipitation. This step effectively removed phosphatase activity present in serum. The precipitated IgG fraction was dialyzed into TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and the total protein was estimated by SDS-PAGE using different amounts of molecular weight standards. An aliquot of the CM-cellulose-purified moesin kinase was incubated with 1 μg each of the isozyme-specific and -specific IgG fraction was dialyzed into TBS at 4 °C for 60 min, followed by protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz) for an additional 30 min. The beads were collected by centrifugation, and the supernatant was used in the moesin kinase assay described above.

Purification of Moesin-specific Protein Kinase Activity from AML Cells—Each step in the purification of protein kinase activity utilized His₆-moesin (10 μg/ml) as a substrate in the phosphorylation reaction described above. Cells from patients with AML were obtained by leukapheresis. Cells were pelleted at 500 × g, rinsed with PBS, and stored in BSB (10 mM Na₂HPO₄, pH 6.5, 2 mM EDTA, 1 mM EGTA) at −70 °C. Cells were thawed, suspended in BSB (10 mM NaF, 1 mM Na₃VO₄, and glycerol was added to 7%, and the preparation was stored in an ultrafiltration cell. The sample was applied to a Millipore DEAE 8HR chelate affinity column (see below). The thawed suspension was sonicated with 40 bursts from a microtip at 70 °C for 20 s. Sonication was followed by centrifugation at 10,000 × g for 10 min. The supernatant was removed by centrifugation at 12,000 × g for 20 min, and the 3.2 ml of supernatant was combined with 3.2 ml of “bind” buffer and applied to a 2-ml Ni²⁺ chelate affinity column (Invitrogen). The column was rinsed according to the supplier's instructions, and the recombinant moesin was eluted with an imidazole gradient from 0 to 400 mM imidazole.

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NaCl, 10 μg/ml each of leupeptin, aprotinin, pepstatin, and 1 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C. Lysates were centrifuged at 15,000 × g for 15 min, and the supernatants were used for purification using 0.1 M of Ni²⁺ affinity beads. The beads were mixed with supernatants for 60 min at 4 °C and then rinsed twice with 1 ml of binding buffer and then three times with 1 ml of binding buffer containing 50 mM imidazole, pH 6.5. The beads were then rinsed once with TBSS to remove detergent, and bound proteins were eluted by incubation of the beads in 0.1 M of 500 mM imidazole, pH 6.5, in TBS containing 10 μg/ml bovine serum albumin and 50% glycerol for 15 min at 4 °C. Aliquots of the eluates from the Ni²⁺ affinity beads were subjected to SDS-PAGE and immunoblotting with anti-PKC-θ (V3-specific) and anti-His₆ antibodies. Both antibodies detected a single immunoreactive band at 80 kDa in the eluate from the pEF-PKC-θ-transfected cells but not pEF neo-transfected cells.

**Immunoblotting—**Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes in 62 mM Tris, 190 mM glycine containing 20% methanol at 280 mA for 45 min. The membranes were stained with Ponceau S in 1% acetic acid to identify the molecular weight standards and destained in PBST (PBS containing 0.01% Tween 20). The blots were incubated with 5% non-fat dry milk in PBST for 15 min and then with primary antibodies for 60 min. After several rinses in PBST, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or rabbit IgG for 30 min and then rinsed with PBST before staining with ECL reagent (Amersham). The blots were dried with a stream of N₂, and the dried lipid was suspended in water at a concentration of 1 mg/ml with vigorous vortexing. The solution was sonicated in a 20 °C water bath for 15 min to give phospholipid vesicles, and buffer was added to give a stock concentration of 0.5 mg/ml phospholipid, 0.5 M Tris-HCl, pH 7.5, 0.5 M NaCl. Phospholipid vesicles (100 μg/ml final concentration) were mixed with or without His₆-moesin (0.5 μg) and/or expressed PKC-θ-His₆ in a buffer containing 3 mM Mn²⁺, 20 μM ATP, and 10 μg/ml leupeptin, 100 mM Tris-HCl, pH 7.5, and 100 mM NaCl in 0.5-mL microcentrifuge tubes. The samples were incubated at 37 °C for 10 min and then for an additional 10 min on ice. The tubes were centrifuged at 12,000 × g for 15 min at 4 °C to obtain supernatant and pellet fractions, which were subjected to immunoblot analysis. Prior to the addition of PKC-θ-His₆ and His₆-moesin to vesicles, the solutions were centrifuged at 12,000 × g for 10 min to remove aggregated protein.

**RESULTS**

**Properties of the Partially Purified Kinase System—**The partial purification of pp73 from 40 ml of human leukemic cells utilized the same ammonium sulfate precipitation and CM-cellulose chromatography as were used previously for identifying pp47, which is phosphorylated under similar conditions (see “Materials and Methods”). The peak CM-cellulose fractions for pp73 purification were obtained at 0.1–0.15 M NaCl and contained both enzyme and substrate but very little phosphatase. We estimated phosphorylation at this step at about 100-fold, based upon a 50% yield estimate, which was inexact due to phosphatase activity in the starting material.

As shown in Fig. 1, the partially purified system exhibits characteristics similar to our previous observations in whole cell extracts, with lane 3 demonstrating optimal phosphorylation of pp73 with Mn²⁺ and PG (3). The order of phospholipid preference was PG > PI > PS > no lipid, shown in lanes 2, 5, and 1, respectively. We also wished to determine whether the phosphorylation was due to cyclic AMP-dependent protein kinase (PKA) or to classical PKC. In lane 4, addition of rat brain PKC, under optimal conditions for phosphorylation of pp73, i.e. Mn²⁺ and PG, resulted in a decrease in pp73 phosphorylation compared to CM-cellulose fraction alone in lane 3. In lane 7, addition of rat brain PKC in the presence of PS/DAG or Ca²⁺ resulted in the phosphorylation of a 75-kDa protein, not pp73. In contrast to the lack of phosphorylation of pp73, the talin fragment was heavily phosphorylated in lane 7 compared with lane 6, merely by the addition of rat brain PKC. In lane 9, addition of the catalytic subunit of PKA under optimal PKA conditions gave almost no phosphorylation of pp73, whereas a band of about 55 kDa was heavily phosphorylated. Thus the CM-cellulose fraction exhibited the Mn²⁺ dependence and PG preference in phosphorylation noted previously, and the phosphorylation was not due to PKA or to classical PKC which is stimulated by PS/DAG/Ca²⁺.

**Purification and Identification of pp73—**Since two-dimensional gels showed that there were no significant amounts of other proteins at this pl (~6.5, data not shown), a preparative purification was conducted using adherence of proteins to PG vesicles under assay conditions as above. This was followed by isoelectric focusing in a sucrose gradient (12), and fractions containing pp73 were identified by SDS-PAGE and autoradiography. These fractions were digested with trypsin, and the resulting peptides were separated by HPLC. Three peptide sequences were obtained and were found to be identical to the ERK protein moesin (Table 1). The peptide sequences are inconsistent with those of the closely related proteins ezrin (17), radixin (18), merlin/schwannomin (19, 20), or talin (21).

**Phosphorylation of Bacterially Expressed Moesin—**To confirm the peptide sequencing identification, we tested recombinant His₆-moesin for its ability to be phosphorylated by endogenous kinase in the CM-cellulose fraction from leukemic cells. Fig. 2 shows that the phosphorylation of recombinant moesin is PG-stimulable (lane 4 versus lane 3) and that the amount of phosphorylation is roughly comparable to the phosphorylation of endogenous pp73 on a per weight basis (lane 4 versus lane 2). As a control, there is no kinase activity in the purified recombinant moesin fraction alone (lane 5). The cyanogen bromide fragmentation patterns of pp73 and recombinant moesin are identical upon SDS-PAGE (data not shown).

**In Vitro Phosphorylation of Moesin Is on Threonine 558—**Since ezrin has been reported to be multiply phosphorylated, we determined the extent of our in vitro reaction in two ways. First, the maximum phosphate we were able to incorporate was 0.8 mol of phosphate per mol of moesin, which suggests only one phosphorylation. Second, two-dimensional gel electrophoresis was performed on moesin (22), which showed two spots (Fig. 3, top) labeled 1 and 2. Carbamylation of His₆-moesin (23) generated a charge train of spots which established that the distance between 1 and 2 corresponded to 1 charge unit (data not shown). It is probable that spot 2 is the deamidation product of spot 1, since moesin contains an asparagine-
TABLE I
Sequences of three tryptic peptides from pp73 are identical to moesin

Purification of pp73 by ion exchange, adherence to PG vesicles, and isoelectric focusing were performed as described under "Materials and Methods." The resulting protein was >90% pure by two-dimensional electrophoresis and was submitted to the W. M. Keck Biotechnology Resource Laboratory for trypsin digestion, separation of the resulting peptides by HPLC, and microsequencing of three selected peptides. The sequences are compared to homologous sequences of human Moesin, Ezrin, Radixin, and Merlin with identical residues indicated by the dots.

| Peptide 1 | Y | G | D | F | N | K | E | V | H | K |
|-----------|---|---|---|---|---|---|---|---|---|---|
| Moesin 134 |   |   |   |   |   |   |   |   |   |   |
| Ezrin 134 | F | Y | Y | I | I | I | I | I | I | I |
| Radixin 134 | Y | Y | Y | I | I | I | I | I | I | I |
| Merlin 150 | Y | D | P | S | S | S | S | S | S | S |
| Peptide 2 | G | M | L | R | E | D | A | V | L | E | Y | L | K |
| Ezrin 181 | K | D | N | M | M | M | M | M | M | M |
| Radixin 181 | K | D | N | M | M | M | M | M | M | M |
| Merlin 197 | R | A | D | E | E | M | M | M | M | M |
| Peptide 3 | A | P | D | F | V | F | Y | A | P | R |
| Ezrin 264 |   |   |   |   |   |   |   |   |   |   |
| Radixin 264 |   |   |   |   |   |   |   |   |   |   |
| Merlin 280 | I | D | V | K | N | S | K | S | S | S |

FIG. 2. Recombinant moesin is phosphorylated by moesin kinase. Recombinant moesin containing a six-histidine tag was purified by nickel chelate affinity chromatography and CM-cellulose chromatography. CM-cellulose fraction proteins from AML cells were phosphorylated without and with recombinant moesin (r-moesin), and the recombinant moesin was phosphorylated alone. The products were resolved by SDS-PAGE; proteins were visualized with Coomassie Blue, and an autoradiogram was obtained. The additions for the phosphorylation reactions are given in the legend above the figures. A, autoradiogram. B, Coomassie Blue stain.

glycine sequence, which is most permissive for deamidation at mild pH and temperature (24), and we observed that more of spot 2 was produced by longer storage. Phosphorylation to approximately 20% gave two more spots (Fig. 3, middle) labeled 3 and 4; these spots were radioactive (Fig. 3, bottom). We interpret the data to infer that a single phosphorylation of native moesin, spot 1, produces spot 3 and that a single phosphorylation of deamidated moesin, spot 2, gives spot 4. Moesin phosphorylation did not result in additional spots. A similar pattern of spots using recombinant phospho- and dephospho-moesin was observed upon two-dimensional gel analysis (data not shown).

We next determined the site of phosphorylation in moesin. Phosphoamino acid analysis showed that greater than 95% of the label was on threonine. Cyanogen bromide fragmentation of phosphorylated moesin showed one major radioactive fragment by SDS-PAGE (25) of about 3 kDa. About 80% of the radioactivity was in a single peak of approximately 3 kDa. Analysis of this peptide by mass spectrometry showed a peak at 3,517 Da, which is exactly the mass expected of a phosphorylated moesin CNBr fragment (550RLGRDKYKTLRQIRQGNTKQRIDEFESM577). Manual Edman degradation of this peptide coupled to an arylamine membrane (13) revealed significant release of radioactivity at cycle 9, corresponding to threonine 558 (Thr558). Ezrin also contains this sequence, and human placental ezrin (kindly provided by A. Bretscher, Cornell University) was phosphorylated by our preparations of kinase at about the same rate as moesin was phosphorylated (data not shown).

Moesin Kinase Activity Is Not a Classical PKC—Moesin kinase was purified from phosphatases using DEAE chromatography (see below), and its activity upon moesin and the purified 47-kDa talin fragment were tested under conditions optimal for moesin phosphorylation and optimal for most forms of PKC. In Fig. 4, lanes 1 and 3, negligible levels of phosphorylation of both moesin and talin fragment are seen in the absence of added lipids. In lane 2, enzyme added under conditions optimal for moesin kinase activity shows heavy labeling of both moesin and talin fragment. In lane 4, enzyme added under conditions optimal for most forms of PKC leads to heavy labeling of talin fragment and a negligible increase in labeling of moesin. Addition of a purified rat brain PKC preparation under optimal PKC conditions showed the same heavy phosphorylation of
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Table I

Comparison of the moesin phosphorylation site sequence with the pseudosubstrate sequences of PKC isozymes.

| Homology | % |
|----------|---|
| Moesin   | KYKTRLRQIR 100 |
| PKC-θ    | KGARIKQAK 75     |
| PKC-δ    | KGARIKQAK 75     |
| PKC-ε    | KGAVRRKRV 50     |
| PKC-η    | KGAMRRKRV 50     |
| PKC-ζ    | KGGRWRRK 38      |
| PKC-α    | KGALRRQKN 50     |
| PKC-β    | KGALRRQKN 50     |
| PKC-γ    | KGALRRQKV 50     |

Percent homology was calculated based on the number of identical and conserved amino acids indicated in bold by using eight amino acid residues surrounding the phosphorylated threonine in Moesin and alanine in the pseudosubstrate sequences of the PKC isozymes.

The Phosphorylation Site of Moesin Is Homologous to a PKC Pseudosubstrate Sequence—As an aid to identifying potential protein kinases that phosphorylate moesin, we examined the amino acid sequence of the phosphorylation site to determine whether it had sequence similarity with consensus protein kinase recognition sequences in other proteins (20). The phosphorylation site, KYKTRLRQ (where * indicates the phosphorylation site), was found to resemble the pseudosubstrate sequence found in the PKC family of enzymes. The sequence contains the requisite basic residues surrounding the phosphorylation site threonine with the phosphorylated residue, Thr558, at the alanine position in the pseudosubstrate sequences. As shown in Table II, homology is maximal with the novel PKC isozymes θ and δ (75%) compared with the conventional isozymes α, β, and γ (50%) and the isozymes ε, η, and ζ (38–50%). Although there is a mismatch between PKC-θ or PKC-δ and moesin of the two residues immediately N-terminal of Thr558, one basic residue within this span is common to all the sequences. This finding suggested that a novel PKC isozyme may be the moesin kinase we detected.

Immunological Identification of Moesin Kinase Activity as PKC-θ—Due to the high degree of sequence similarity of the moesin phosphorylation sequence with PKC-θ and δ, we analyzed the CM-cellulose fraction for the presence of PKC isozymes by immunoblotting. As shown in Fig. 5A, the CM-cellulose fraction contains three PKC isozymes, θ, α, and δ. The apparent relative abundance of the θ and δ isozymes is consistent with their known expression in hemopoietic cells. We next examined whether antibodies to specific PKC isozymes could immunodeplete moesin kinase activity from the CM fraction. As shown in Fig. 5B, antibodies raised against the V3 region of PKC-θ (7) removed 90% of the activity, whereas antibodies to the other PKC isozymes did not remove or inhibit moesin kinase activity. IgG specific for the C terminus of PKC-θ (Fig. 5B, anti-PKC-θ, C-Term) is less efficient at immunodepletion of kinase but was also correspondingly less efficient at immunoprecipitation of PKC-θ protein as assessed by immunoblotting. In Fig. 5C, analysis of the immunoprecipitate formed with anti-PKC-θ (V3-specific) by immunoblotting with anti-PKC-θ and α indicated that only PKC-θ is in the immunoprecipitate and that PKC-α is not. Anti-PKC-θ IgG does not cross-react with PKC-α, the next most abundant PKC isozyme in the CM fraction (Fig. 5, compare A and C). However, we were unable to demonstrate activity in the immunoprecipitates, possibly due to the inactivation of the enzyme bound to IgG and protein A/G beads or an inability of the bound enzyme to be optimally activated with phosphatidylglycerol vesicles. Taken together, these data suggest that the moesin kinase activity in the CM-cellulose purified cell extracts is attributable to PKC-θ.

Partial Purification of PKC-θ from AML Cells—We next endeavored to purify the moesin kinase activity from human AML cells using His6-moesin as a substrate in our standard assay (see “Materials and Methods”). Immunoreactivity of individual fractions with anti-PKC-θ, as well as anti-PKC-α, was also monitored. A 35–65% ammonium sulfate fraction of the cell lysate was chromatographed over a CM-cellulose column, and aliquots of each fraction were assayed using PG vesicles (Fig. 6A, upper panel), and in addition PS/DAG without Ca2+ to assay for novel PKCs (Fig. 6A, middle panel). PG-stimulable moesin kinase activity elutes from the column at about 0.09–0.18 M NaCl; activity in the presence of PS/DAG/EGTA is about 10-fold lower than with PG. Immunoblot analysis of the CM-cellulose fractions indicates that the fractions displaying moesin kinase activity contain PKC-θ (Fig. 6A, lower panel), as well as PKC-α (data not shown). No PKC-θ immunoreactivity was found in side fractions. A pool of the active CM-cellulose fractions eluting at 0.09–0.18 M NaCl was fractionated on a DEAE 8HR column. As shown in Fig. 6B, two pools of moesin kinase activity using PG vesicles are found. A major peak of activity eluting at a lower [NaCl] in fractions 4–6 contain PKC-θ, whereas fractions 8–12, which display lower PG-stimulable moesin kinase activity, contain PKC-α. The peak of moesin kinase activity and PKC-θ immunoreactivity is found in fraction 5, which has no detectable PKC-α. This chromatographic...
with the V3-specific anti-PKC antibody from each immunoprecipitation was used to assay moesin kinase activity and detection of PKC-α. An identical amount of the supernatant fraction from each immunoprecipitation was also assayed for moesin kinase activity using His6-moesin as a substrate and PG vesicles (upper panel), for PS/DAG/EGTA-stimulated PKC activity using His6-moesin as a substrate (middle panel), and for PKC-θ immunoreactivity (V3 region-specific). B, Millipore DEAE 8HR column; the fractions from the CM-cellulose column eluting at 0.09–0.14 M NaCl were pooled and chromatographed on a Millipore DEAE 8HR column using a linear 0–0.5 M NaCl gradient and assayed for moesin kinase activity with PG vesicles (arrow, His6-moesin), and for immunoreactivity with antibodies specific for PKC-θ and PKC-α (arrows). C, heparin-Sepharose chromatography: DEAE fraction 5 was subjected to heparin-Sepharose chromatography using a 0–0.5 M NaCl gradient. Fractions were assayed for moesin kinase activity and for PKC-θ immunoreactivity. The positions of phosphorylated His6-moesin and immunoreactive PKC-θ and PKC-α are indicated by arrows.

Fig. 6. Partial purification of PKC-θ from AML cells. A, CM-cellulose chromatography. The column was eluted with a 0–0.3 M NaCl gradient. Aliquots of the fractions were assayed for moesin kinase activity using His6-moesin as a substrate and PG vesicles (upper panel), for PS/DAG/EGTA-stimulated PKC activity using His6-moesin as a substrate (middle panel), and for PKC-θ immunoreactivity (V3 region-specific). B, Millipore DEAE 8HR column; the fractions from the CM-cellulose column eluting at 0.09–0.14 M NaCl were pooled and chromatographed on a Millipore DEAE 8HR column using a linear 0–0.5 M NaCl gradient and assayed for moesin kinase activity with PG vesicles (arrow, His6-moesin), and for immunoreactivity with antibodies specific for PKC-θ and PKC-α (arrows). C, heparin-Sepharose chromatography: DEAE fraction 5 was subjected to heparin-Sepharose chromatography using a 0–0.5 M NaCl gradient. Fractions were assayed for moesin kinase activity and for PKC-θ immunoreactivity. The positions of phosphorylated His6-moesin and immunoreactive PKC-θ and PKC-α are indicated by arrows.

FIG. 5. Immunological identification of moesin kinase activity as PKC-θ. An aliquot of the CM fraction containing moesin kinase activity was subjected to immunoblotting with PKC isozyme-specific monoclonal antibodies (A) as indicated in the figure. The sizes of the full-length PKC-α, -δ, and -θ are 82, 78, and 80 kDa, respectively. The additional bands of lower molecular mass detected with α- and θ-specific antibodies are degradation products. B, immunodepletion of moesin kinase activity with 1 µg of each isozyme-specific anti-PKC IgG as indicated or with control rabbit IgG or no IgG (–IgG). An aliquot of the CM-cellulose fraction was incubated with IgG fractions from antisera specific for each isozyme, followed by protein A/G agarose to remove IgG and immune complexes. An identical amount of the supernatant fraction from each immunoprecipitation was used to assay moesin kinase activity. The kinase activity was assayed using His6-moesin (arrow). The minus (–) enzyme lane contains no added enzyme. The immunodepletion and moesin kinase assays were carried out as described under “Materials and Methods.” C, immunoprecipitation (IP) of moesin kinase activity and detection of PKC-θ in the immunoprecipitate. An aliquot of the CM-cellulose fraction was subjected to immunodepletion with the V3-specific anti-PKC-θ (labeled θ) or non-immune IgG (N) as described in B, and the immunoprecipitates were analyzed by immunoblotting with the monoclonal anti-PKC-θ and anti-PKC-α. The position of PKC-θ at 80 kDa is indicated by the arrow.

step effectively separates PKC-θ and PKC-α and enriches the moesin kinase activity contained in fractions containing PKC-θ. Fraction 5 was used for heparin-Sepharose chromatography because it contained maximal moesin kinase activity and PKC-θ. The moesin kinase activity again co-fractionates with intact PKC-θ as detected by immunoblotting (Fig. 6C). Furthermore, the activity obtained cannot be ascribed to the catalytic fragment of PKC-θ in the preparation because no immunoreactive fragment was detected with a PKC-θ antibody directed to the C terminus. The peak of moesin kinase activity and PKC-θ immunoreactivity also coeluted from a Superdex-200 FPLC column at 85 kDa, which is consistent with the molecular mass of PKC-θ (data not shown). Therefore, the purification used here results in the enrichment of moesin kinase activity corresponding to intact PKC-θ and removes the immunoreactive fragment present at the CM-cellulose step (Fig. 5A). However, attempts to purify the moesin kinase activity further resulted in a drastic loss of phosphorylating activity, which precluded direct sequencing of purified kinase. A similar loss of activity with purification has been observed for some PKC isozymes, for example PKC-δ purified from K562 hematopoietic cells (27) and PKC-γ from murine brain (28). All further experiments using PKC-θ from AML cells were done with a kinase preparation containing the full-length enzyme as determined by SDS-PAGE/immunoblotting and Superdex-200 chromatography.

Preferential Activation of Moesin Kinase (PKC-θ) by PG Vesicles—We next examined the activation of moesin kinase by PG and PS in combination with DAG. Previous studies using the [A25SS]PKC substrate peptide, which corresponds to the pseudosubstrate sequence of PKC-α with an alanine to serine substitution, showed that PS at optimal concentrations gave near maximal activation of PKC-θ in the absence of either phorbol 12-myristate 13-acetate or DAG (Ref. 29, Fig. 5). We examined the activation of moesin kinase with PG and PS
vesicles with added DAG constituting 10% (30) of the total lipid concentration of either 20 and 200 μg/ml. As shown in Fig. 7, addition of DAG to PG vesicles did not lead to increased moesin phosphorylation. By using PS and PS/DAG vesicles, less moesin phosphorylation was obtained compared with PG vesicles. Addition of 10% DAG to PS vesicles led to a minimal increase in moesin phosphorylation (Fig. 7, compare 100% PS and 90% PS + 10% DAG) and is more evident using 20 μg/ml total lipid. In addition, we investigated activation of PKC-θ using PC vesicles containing 20% acidic lipids (PG, PS, or PI), a typical concentration of the inner leaflet of the plasma membrane (31). PC vesicles alone did not activate PKC-θ. PG, in mixed vesicles with PC, maximally activated PKC-θ, and DAG again had no effect (data not shown).

Moesin Kinase (PKC-θ) Activity toward Different Substrates—We next tested whether the moesin kinase activity has particular substrate and cofactor requirements. By using moesin, MBP, and histone H1, the kinase activity toward each was tested in the presence of PG, PI, or PS/DAG. As shown in Fig. 8, maximal kinase activity was obtained using PG and His6-moesin, whereas the kinase activity toward MBP was lipid-independent, and histone H1 phosphorylation was minimal. These results are consistent with previous findings of PKC-θ using an MBP-derived peptide and histone H1 (29).

Recombinant His6-PKC-θ Phosphorylates Moesin—To verify that PKC-θ phosphorylates moesin, we transfected eukaryotic cells with a plasmid bearing PKC-θ with a His6-tag at its C terminus (16, 29). Jurkat (T-lymphoblastic leukemia) cells were transfected with the His6-PKC-θ plasmid and with the neo vector alone. Cell lysates from each transfection were subjected to immunoblot analysis with anti-PKC-θ, followed by microsequencing and confirm by phosphorylation of the His6-moesin. Furthermore, about 50% of the His6-moesin was pelleted in association with PG vesicles, and binding was independent of His6-moesin.

Fig. 8. Substrate and lipid dependence of moesin kinase activity. Aliquots of fraction 8 from the heparin-Sepharose column were assayed using His6-moesin (H₆M), myelin basic protein (MBP), and histone H1 in the presence of PG, PI, and PS/DAG phospholipid vesicles. The lanes indicated by the lines are reactions in buffer alone without added lipid. H₆M, MBP, and Histone H1 are 73, 22, and 34 kDa, respectively.

Fig. 9. Expressed PKC-θ-His₆ phosphorylates moesin. Jurkat T₆ cells were electroporated with pEF plasmids containing PKC-θ-His₆ and empty vector (pEF neo), and cell lysates were purified on Ni²⁺ affinity beads. A, The eluates and unbound (UNBD) fractions were subjected to immunoblot analysis with anti-PKC-θ (V3-specific). B, the Ni²⁺ column eluates from neo and PKC-θ-His₆-transfected cells were assayed using PG and PI phospholipid vesicles and His₆-moesin.

Fig. 10. PKC-θ was found in the pellet fractions of reactions containing PG vesicles, and binding was independent of His₆-moesin. Furthermore, about 50% of the His₆-moesin was pelleted in association with PG vesicles.

DISCUSSION

In this report we present reaction conditions under which PKC-θ threonine phosphorylates moesin at its previously identified in vivo site within its actin-binding domain (1). PKC-θ is a recently discovered novel PKC isozyme, which has not previously been shown to phosphorylate moesin or to require the unusual conditions defined here nor has it been linked to any other substrate of likely physiological significance. Although phosphorylation of ERM proteins has been previously described, the work we present is the first evidence linking proteins of this class to a specific kinase and suggests a novel and specific signaling pathway regulating membrane-cytoskeleton linkages.

The reaction conditions described here result in greatly increased phosphorylation of the prominent 73-kDa protein substrate we originally described in hemopoietic cells (3–5), which we here identify as moesin on the basis of protein purification followed by microsequencing and confirm by phosphorylation of...
related to a family of proteins involved in linking the membrane and cytokinesis. Moesin was first isolated by its ability to bind heparin (36); subsequent cDNA cloning and sequence analysis showed it to be a 68-kDa protein intensely stained band corresponds to bovine serum albumin present in the PKC-θ-His6 preparation.

recombinant moesin with partially purified kinase. Several further observations corroborate this identification. First, both pp73 and PKC-θ bind to PG vesicles, and moesin is known to bind to the plasma membrane (32, 33). Second, in Fig. 2 we observed four spots of pp73 by two-dimensional gel analysis with pI ranging from 6.6 to 6.45, which is similar to the four spots of moesin reported in a comprehensive protein data base (34). Our results indicate that the latter distribution results from deamidation and mono-phosphorylation.

By using recombinant moesin as a substrate for in vitro kinase assays, we have partially purified and identified PKC-θ as a protein kinase that phosphorylates moesin at Thr558 in the actin-binding domain, and we further conclude that it corresponds to the protein kinase previously described (3). This identification is based upon immunodepletion experiments and the demonstration of moesin phosphorylation by recombinant PKC-θ. The phosphorylation site of moesin, KYKTyr558RQ, containing Thr558, is phosphorylated by a partially purified PKC-θ preparation and is highly homologous with the pseudosubstrate sequence of PKC-θ. The lipid dependence of PKC-θ phosphorylation of moesin displays a preference for PG over PS. Furthermore, both moesin and PKC-θ associate with PG phospholipid vesicles, and PKC-θ displays a strict substrate preference for moesin, rather than MBP or histone H1. PKC-θ is thus the major moesin kinase directly identifiable in the cell extracts we studied. The single phosphorylation site identified corresponds exactly with that which is now well established to occur in cells (1, 33). We are currently extending this work to test further our putative PKC-θ/moesin link in cellular systems and to analyze its control and effects. We cannot, however, rule out the participation of other moesin kinases in other cell types or tissues or under other activating conditions. This is especially a consideration since ERM proteins are ubiquitous, whereas PKC-θ is not. PKC-α can phosphorylate moesin, albeit at an apparently lower rate, which may be adequate to elicit a physiologic response.

ERM proteins are involved in a wide variety of cellular functions, including cell-cell adhesion, cell-substrate adhesion, microvillar structure, and cytokinesis. Moesin was first isolated by its ability to bind heparin (36); subsequent cDNA cloning and sequence analysis showed it to be a 68-kDa protein related to a family of proteins involved in linking the membrane of a cell to its cytoskeleton (32). The ERM proteins identified to date have a common domain structure based upon amino acid sequence homology to the erythrocyte band 4.1 protein. They are comprised of an N-terminal domain which is involved in membrane binding, an α-helical central domain, and a C-terminal actin-binding domain (37). In vitro work has shown that native ERM proteins bind to beads displaying the cytoplasmatic tail of the transmembrane protein CD44 at physiological ionic strength in the presence of phosphatidylinositol-4,5-diphosphate (38). Ezrin has been shown to bind filamentous β-actin, not α-actin, at physiological ionic strength (39), whereas such an interaction between moesin and F-actin has not yet been demonstrated. The ezrin/β-actin interaction correlates with the fact that β-actin and ezrin are colocalized to diverse cellular extensions, whereas α-actins are largely restricted to sarcomeres and stress fibers (40, 41).

Does moesin phosphorylation affect actin binding? Moesin was originally immunolocalized to structurally and functionally diverse cellular extensions, and it was speculated that these “rapidly assembled cell surface structures may be akin to environmental sensors in a very general sense” and that phosphorylation of moesin might be related to these morphological changes (33). The Thr558 site in moesin was phosphorylated in platelets upon thrombin stimulation, which drastically alters platelet shape. Using a phosphorylation state-specific antibody to KYKpT558LR, only a fraction of total moesin molecules found in filopodia and retraction fibers are phosphorylated in RAW macrophages (35). In the presence of the phosphatase inhibitor calyculin A, phosphorylated moesin is increased to nearly 100% and is redistributed from filopodia and retraction fibers into an F-actin containing ring-like structure in the cytoplasm. Thus, this phosphorylation step accompanies actin-based morphological changes (1). We are currently investigating possible interactions of moesin and phosphorylated moesin with various actin isoforms.

ERM proteins have now been shown to interact with several molecules and signaling pathways in addition to those described here. Tsukita and co-workers (38) found that the affinity of the moesin and ezrin interaction with the cytoplasmic tail of CD44 was markedly enhanced by phosphoinositides. In A431 cells, the redistribution of ezrin into membrane ruffles after epidermal growth factor stimulation is concomitant with both serine and tyrosine phosphorylation (42). Studies using gastric mucosal cells indicate that ezrin associates with the regulatory (R1) subunit of PKA, suggesting that ezrin functions as an protein kinase A anchoring protein (AKAP) (43). The R1-binding site in ezrin, corresponding to residues 417–432, is highly conserved in moesin and corresponds to the R1-binding site in the C terminus of AKAP79 (44). Additionally, AKAP79 contains a binding sequence for PKC in the N terminus, and a peptide corresponding to this sequence inhibits PKC activity. A similar PKC-binding site has not been identified in either ezrin or moesin, but several sites in ezrin/moesin have significant homology with this sequence in AKAP79. We demonstrate that moesin is not phosphorylated by the catalytic subunit of PKA (Fig. 1) but is phosphorylated at the KYKTVLRQ site in the actin-binding domain by PKC-θ. ERM proteins may participate in a dynamic, macromolecular signaling complex, analogous to the model proposed by Faux and Scott (45) in which AKAP79 anchors PKC, PKA, and phosphatase 2B at neuronal synapses. ERM proteins may similarly function as anchoring proteins linking the actin cytoskeleton with phospholipid, phosphatidylinositol-4,5-diphosphate, CD44, PKA, and PKC. Our data demonstrating that moesin and PKC-θ bind to phospholipid vesicles and that the phosphorylation site sequence in moesin is homologous to the pseudosubstrate sequence of PKC-θ support this
hypothesis. Furthermore, preliminary immunofluorescence studies show significant colocalization of PKC-θ and moesin in punctate structures within protrusions at the cell periphery in RAW macrophages and HL-60 cells (data not shown). This pattern appears similar to that recently described for PKC-θ in resting T-cells (46). Thus, PKC-θ may regulate the interaction of ERM proteins with the actin cytoskeleton and may also interact with other components of such a complex.

The PKC family of serine/threonine kinases consists of multiple isozymes that are grouped into three categories based on their cofactor and lipid dependence for activation. The conventional PKCs (α, β1, β2, and γ) are activated by Ca2+, phospholipid, and DAG; novel PKCs (δ, η, and ζ) are insensitive to Ca2+, and the atypical PKCs (ζ and λ) are insensitive to both Ca2+ and DAG (47). Although some isozymes have widespread tissue and cell type distribution (α, δ, and ζ), other isozymes such as PKC-γ, -η, and -θ are more restricted. PKC-θ is the main novel isozyme expressed in skeletal muscle, testes, platelets, and their megakaryoblastic precursor cells, T-lymphocytes, and neoplastic hematopoietic cells (7, 48, 49). We have found moesin phosphorylation activity in HL-60 cells, spleen cells, and AML cells, which is consistent with the known distribution of PKC-θ (29) and in contrast to the tissue distribution of the μ (50) and the λ/α isofoms, which are not found in spleen (51, 52). We have previously described modulation of this activity under various conditions affecting cellular proliferation and differentiation (4, 5). It will be of interest to determine if PKC-θ immunoreactivity is modulated in parallel as expected. If so, this previous work could be the basis for physiologic models of PKC-θ activation and function.

The unusual lipid activation pattern we have observed in vitro in the presence of Mn2+ (PG > PI > PS/DAG) is reminiscent of the unusual lipid activation conditions described for other novel PKCs. Our data demonstrating a minimal contribution of DAG to the PS activation of PKC-θ phosphorylation of moesin is consistent with the findings of Baier et al. (29), who showed a similar minimal effect of phorbol 12-myristate 13-acetate to the PS activation of PKC-θ. A comparable pattern has been observed with PKC-δ phosphorylation of the γ chain of the IgE receptor, where the rate of threonine phosphorylation was increased 2.5-fold using PI compared with PS, whereas the effect of PG was not tested (53). PKC-ε displays PG-stimulable activity toward a PKC-ε synthetic peptide resembling the PKC-ε pseudosubstrate site, with a serine residue at the alanine position (54). PKC-ε was also found to phosphorylate histones poorly, similar to the present findings with PKC-θ.

The activation of classical and novel PKCs by PS and DAG is thought to reflect an allosteric enzymic effect: DAG generation causes increased PKC membrane affinity and accompanies release of the pseudosubstrate sequence from the active site. However, we suspect the mechanism of PKC-θ activation and moesin phosphorylation to be more complex. Our findings indicate first that both moesin and PKC-θ bind to PG vesicles independently of one another; the phospholipid activation observed may be based upon closely co-concentrating the two components on a suitable surface. Phospholipid binding could correlate with the activity of moesin in the presence of Mn2+, similar to the present findings with PKC-θ and Moesin Phosphorylation (29), who recently investigated such interactions of moesin and PKC-θ.

The in vitro function of PKC-θ has mainly been studied in cells of the hematopoietic system. The chromosomal location of the human PKC-θ gene was mapped to the short arm of chromosome 10 (10p15), a region that is frequently deleted or subject to translocations in T-cell leukemia, lymphoma, and T-cell immunodeficiency (59). In T-lymphocytes, PKC-θ in conjunction with 14-3-3 may be a constituent of the signaling cascade leading to T-cell activation by activating the AP-1 transcriptional complex (60). Recent evidence clearly shows that antigen stimulation results in the translocation of PKC-θ, along with talin, to the site of contact between T-cells and antigen-presenting cells, whereas in unstimulated cells PKC-θ is found in a punctate distribution (46). The HIV protein Nef, whose association with PKC-θ results in a loss of enzyme, is known to down-regulate CD4 in infected T-cells and blocks interleukin-2 production in Jurkat cells, possibly by inhibiting NFkB and AP-1 activation (61). The identification of moesin, ezrin, actin, and collagen inside HIV-1 virions provides evidence for a role of the cytoskeleton in virus budding from microvillar-like pseudopods of infected T-cells (62). Taken together, these findings raise the intriguing possibility that the pathway described here may play a role in virus production from infected T-cells as well as normal T-cell functions.

In summary, this laboratory first characterized the surprisingly specific phosphorylation of two proteins in crude extracts under somewhat unusual conditions: in the presence of Mn2+, the addition of PG or PI vesicles resulted in 10-fold stimulation of phosphorylation (3). We previously identified one of these substrates as the 47-kDa calpain fragment of talin (6), and we here identify the second substrate as moesin. These two proteins are structurally related and are proteins linking the plasma membrane to the actin cytoskeleton. We provide further evidence that the moesin kinase activity corresponds to PKC-θ, using both purified kinase from human leukemic cells and recombinant PKC-θ. The assay requirement for vesicles correlates with their cellular localization on the cytoplasmic surface of the plasma membrane. The in vitro phosphorylation site of moesin Thr558 corresponds to that occurring in activated platelet membranes and in macrophages (1, 35). We are currently investigating such interactions of moesin and PKC-θ with cells to address the physiologic significance of this system.

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