Serine proteases are involved in many processes in the nervous system and specific inhibitors tightly control their proteolytic activity. Thrombin is thought to play a role in tissue development and homeostasis. To date, protease nexin-1 is the only known endogenous protease inhibitor that specifically interferes with thrombic activity and is expressed in the brain. In this study, we report the detection of a novel thrombin inhibitory activity in the brain of protease nexin-1−/− mice. Purification and subsequent analysis by tandem mass spectrometry identified this protein as the phosphatidylethanolamine-binding protein (PEBP). We demonstrate that PEBP exerts inhibitory activity against several serine proteases including thrombin, neuropsin, and chymotrypsin, whereas trypsin, tissue type plasminogen activator, and elastase are not affected. Since PEBP does not share significant homology with other serine protease inhibitors, our results define it as the prototype of a novel class of serine protease inhibitors. PEBP immunoreactivity is found on the surface of Rat-1 fibroblast cells and although its sequence contains no secretion signal, PEBP-H6 can be purified from the conditioned medium upon recombinant expression.

Serine proteases are involved in many processes during development and tissue homeostasis. Among other functions, they degrade components of the extracellular matrix to allow outgrowth of neuronal processes (1) or cell migration (2); they promote cell death (3) and act as mitogenic or survival factors (4). The activity of the proteases is regulated by their cognate inhibitors, which must act in an accurately balanced fashion to ensure a normal development and homeostasis. Disturbances of this balance in the nervous system have been proposed to be involved in pathological disorders such as Alzheimer’s disease (5).

To date, several serine proteases have been detected in the central nervous system, including tissue-type plasminogen activator (t-PA), 1 chymotrypsin, neuropsin, elastase, and thrombin (6). Many serine protease inhibitors regulating the activity of these proteases are found in the brain. However, protease nexin-1 (7) is the only known endogenous thrombin inhibitor present in the central nervous system. In vitro, the interplay of thrombin and PN-1 has been shown to modulate neurite outgrowth of neuronal cells (8, 9) and the stellation of astrocytes (10). Furthermore, PN-1 is highly expressed in response to injury of the nervous system (11). Despite these observations, mice lacking PN-1 show only a subtle phenotype in the nervous system (12), a result that can be explained by the existence of other unknown serine protease inhibitors that compensate for the lack of PN-1 function in these animals. To address this question, we searched for thrombin inhibitory activities in the brain of PN-1−/− mice.

In this report, we present the characterization and purification of a novel serine protease inhibitor that could be identified as the mouse phosphatidylethanolamine-binding protein, PEBP. This 187-amino acid-containing protein belongs to a family of phospholipid-binding proteins found in a wide range of species from flowering plants to mammals (13). In most cases the physiological role of these proteins in vivo is not yet understood. The demonstration of inhibitory activity against serine proteases indicates a new biological function for mouse PEBP that it might have in common with other members of the PEBP family.

**EXPERIMENTAL PROCEDURES**

**Preparation of Brain Homogenates**—The brains were prepared from C57bl/6 mice (RCC Ltd. Switzerland) of different ages. The deeply anesthetized animals were pericardially perfused with PBS without Ca2+ or Mg2+ for 5–10 min to obtain blood-free brains. These were homogenized either in parts (cerebellum, cortex, remaining parts of the brain) or as complete brains for 40 s using a Polytron homogenizer (Kinematica GmbH) in 10 mM Hepes, pH 7.5, 0.2% Tween 20, 320 mM sucrose, 1 mM EDTA. The homogenates were cleared by centrifugation (12,000 × g, 30 min, 4 °C) and the supernatants filtrated through a Millex-HA 0.45-μm filter unit (Amicon).

**Protease Inhibition Assays**—The human α-thrombin was previously prepared and characterized (14); all other proteases were commercially available products (Sigma, Fluka). The proteases were diluted in enzyme buffer (67 mM Tris, pH 8.0, 133 mM NaCl, 0.13% polyethylene glycol 6000; in case of chymotrypsin: 50 mM Tris, pH 8.0, 380 mM NaCl, 3 mM CaCl2) and used in the assays with the following final amounts: thrombin, trypsin, and chymotrypsin, 0.005 pmol; t-PA and neuropsin, 0.5 pmol; pancreatic elastase, 4 pmol. 80 μl of the samples were mixed with 10 μl of protease in a 96-well plate and incubated for 30 min at 37 °C. After addition of 10 μl of chromogenic substrate (H-n-Ile-Pro-Arg-p-nitroanilide, Chromogenix, 1.25 mg/ml in H2O; for chymotrypsin inhibition assay: N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, Sigma, 2.3 mg/ml in H2O), the remaining amidolytic activity was determined by measuring the rate of the hydrolysis of the chromogenic substrate at 405 nm over 30 min using a Thermomax microplate reader (Molecular Devices).

**Complex Formation Assay**—8 and 24 μl of brain homogenate (3.2 μg of protein/μl) of a perfused PN-1−/− mouse were incubated with 40 ng of human α-thrombin in a total volume of 40 μl in enzyme buffer for 30 min at 37 °C. After preincubation, 5 μl of nonreducing sample buffer...
were added, and the proteins resolved by SDS-PAGE without previous boiling. The resolved proteins were electroblotted on Protran nitrocellulose transfer membrane (Schleicher & Schuell) in a Transblot SD semidyry transfer cell (Bio-Rad) at 3 mA/m² for 40 min. The immunodetection of thrombin was performed using a polyclonal rabbit anti-human antibody (American Diagnostics). The membranes were extensively washed with 50 mM enzyme buffer (Amersham Pharmacia Biotech) as described elsewhere (15). EST-6 is a monoclonal antibody that recognizes free thrombin and also thrombin complexed with inhibitors (e.g. antithrombin III) (16). 50 μl of the EST-6 coated beads were added to the samples and the mixtures incubated at 4 °C overnight with gentle shaking. The beads were then washed three times with 1 ml of enzyme buffer each, and then resuspended in 50 μl of sample buffer, denatured at 95 °C for 5 min, and the supernatants loaded on a 12.5% SDS-PAGE. The gel was then silverstained following the manufacturer’s protocol (Bio-Rad Silver Stain).

**Characterization of Inhibitory Properties—** The inhibitory activity of purified PEBP-H₆ was tested against several serine proteases. To determine the rates for the hydrolysis of the chromogenic substrates, the obtained values were fitted with the software GraFit 4.0 (Erithacus Software) to the equation for a competitive inhibitor.

$$v = rac{V_{\text{max}} [S]}{K_m + [S]} + \frac{[I]}{K_I}$$

$[S]$ is the substrate concentration, $[I]$ the total inhibitor concentration, $K_m$ the Michaelis-Menten constant, and $K_I$ the inhibition constant.

**Immunocytochemistry—** To raise a polyclonal antiserum against PEBP, two COOH-terminal of the mouse PEBP (amino acids 144–159 and 174–187) were cross-linked to ovalbumin and injected into rabbits following standard protocols. The specificity of the immune serum was assessed by the detection of a single 21-kDa band on a immunoblot of mouse brain homogenate. Rat 1 fibroblasts were washed three times with PBS, fixed for 20 min with 4% paraformaldehyde in PBS at room temperature, and again washed with PBS. For permeabilization, the cells were fixed in 4% paraformaldehyde with 15% picric acid in PBS (20 min, room temperature), and washed with PBS with 0.2% Triton X-100. After blocking for 30 min with 3% BSA in PBS, the cells were incubated for 1 h with antiserum (1:1500 in blocking solution) and then washed with PBS. Immunofluorescence detection was performed using the Alexa 488 goat anti-rabbit IgG conjugate (Molecular Probes) as a secondary antibody.

**RESULTS**

**Detection of Thrombin Inhibitory Activity in PN-1⁻/⁻ Brains—** Perfused brains of PN-1⁻/⁻ and wild type mice were divided into cerebellum, cortex, and the remaining parts of the brain. Homogenates of these samples were checked for the presence of thrombin inhibitory activity. Stepwise dilutions of aliquots of the homogenates containing equal amounts of proteins revealed the presence of a thrombin inhibitory activity in all investigated brain compartments (Fig. 1). The level of this inhibitory activity was equivalent in the different parts of the brain tested. As expected, the thrombin inhibition was significantly higher in the wild type brains than in the PN-1⁻/⁻ brains. However, the fact that thrombin inhibition was also detected in the PN-1⁻/⁻ brains indicated indeed the presence of an additional thrombin inhibitor present in the mouse brain.

**Complex Formation Assay—** An electrophoretic mobility shift assay was performed to address the question whether a complex is formed between thrombin and one or more components of PN-1⁻/⁻ brains. Aliquots of brain homogenates from perfused PN-1⁻/⁻ mice were preincubated with human α-thrombin and applied to an SDS-PAGE under nonreducing, seminative (0.1% SDS) conditions. The resolved proteins were analyzed for high molecular weight complexes by subsequent immunoblot analysis with a polyclonal anti-thrombin antibody (Fig. 2A). Preincubation of the brain homogenates with thrombin resulted in the formation of complexes of ~60 kDa. This size suggested a complex between thrombin (37 kDa) and a second protein of ~23 kDa.

**Coimmunoprecipitation—** A coimmunoprecipitation was performed to confirm the formation of such a complex between thrombin and an inhibitory protein. Brain homogenates were incubated with human α-thrombin for 30 min at 37 °C. After
Mouse PEBP Inhibits Serine Proteases

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FIG. 1. Thrombin inhibitory activity in brains of wild type and PN-1\(^{-/-}\) mice. Different parts of wild type (○, cerebellum; □, cortex; ●, remaining parts) and PN-1\(^{-/-}\) (△, cerebellum; ■, cortex; ●, remaining parts) brains were homogenized and their protein content measured. Aliquots with equal protein contents were stepwise diluted and assayed for thrombin inhibition.

FIG. 2. Complex formation and coimmunoprecipitation of brain homogenates with human \(\alpha\)-thrombin. Panel A, the indicated protein amounts of cleared homogenates from PN-1\(^{-/-}\) brains were preincubated with 40 ng of thrombin for 30 min at 37 °C and then applied to an SDS-PAGE gel under non-denaturing conditions. Immunoblotting was performed with a polyclonal anti-human thrombin antibody. Panel B, brain homogenates were preincubated with buffer or with thrombin and then coimmunoprecipitated with protein A-Sepharose beads coated with a monoclonal antibody against thrombin. The proteins were resolved under denaturing conditions on an SDS-PAGE gel and visualized by silver staining. A protein of approximately 20 kDa (arrowhead) coimmunoprecipitated after preincubation with thrombin.

preincubation in an immunoprecipitation was performed with protein A-Sepharose beads coated with the anti-thrombin monoclonal antibody EST-6. Brain homogenate preincubated with buffer only was used as a negative control. The coimmunoprecipitated proteins were applied to an SDS-PAGE and visualized by silver staining (Fig. 2B). As the binding properties between thrombin and the putative inhibitor were not known, the coimmunoprecipitation was performed at very low stringency conditions, resulting in a high background. Nevertheless, a protein of \(\sim 20\) kDa was coimmunoprecipitated after preincubation with human \(\alpha\)-thrombin, again indicating the existence of an inhibitory thrombin binding protein of 20–23 kDa.

Gel Filtration—To partially purify the inhibitor, brain homogenates from PN-1\(^{-/-}\) mice of different ages were fractionated according to their molecular weight with a Superdex-200 gel filtration column. The fractions were assayed for the presence of thrombin inhibitory activity. As shown in Fig. 3, thrombin inhibitory activity eluted from the column in fractions 56–63, corresponding to an elution volume of 14–15.75 ml. Calibration of the gel filtration column with ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa) under the same conditions indicated a molecular mass for the putative inhibitor of 21–24 kDa. The amount of this inhibitory activity was detected at equal levels in brains of wild type and PN-1\(^{-/-}\) mice (data not shown). In addition the activity did not depend on the age (from 14 days to 2 years). The peak in hydrolysis rate detected in fractions 64–66 of brain homogenates obtained from older animals corresponds to a proteinase, not further characterized, that seems to be up-regulated with age.

Taken together, the results of the thrombin assays, the complex formation assay, the coimmunoprecipitation, and the gel filtration suggested an unidentified thrombin inhibitor in the mouse brain. The finding that heat treatment of the brain homogenates (95 °C for 5 min) completely abolished the thrombin inhibition (data not shown) supported the hypothesis that the inhibitory activity is indeed due to a protein.

Purification and Identification of the Inhibitor—The inhibitor did not bind to Q- and heparin-Sepharose at pH 9.0 and SP-Sepharose at pH 5.0 under the conditions we used. Nevertheless, we succeeded to purify the inhibitor by a combined anion exchange and heparin affinity chromatography followed by a cation exchange chromatography. The activity was always recovered from the flow-through of the column. The heparin column was included to bind and thereby remove PN-1 whose thrombin inhibitory activity is well established (19). The cleared homogenate of two wild type brains was applied to the combined Q and heparin columns; the flow-through containing the inhibitor was recovered and concentrated. The sample was then diluted with a buffer appropriate for the cation exchange chromatography and loaded on an equilibrated SP-Sepharose column. The concentrated flow-through of this purification step was applied to a 15% SDS-PAGE. Silver staining of the gel revealed a single band of the expected size of 20–25 kDa. The tryptic peptides derived from this SDS-PAGE separated inhibitor were analyzed by tandem mass spectrometry. Sequence tags from five peptides were obtained that all fitted to the amino acid sequence of the mouse phosphatidylethanolamine-binding protein (Fig. 4, bold).

Recombinant Expression of Mouse PEBP-H\(_6\)—For the cloning of the cDNA coding for mouse PEBP, we used IMAGE clone...
1921274 (Sugano mouse, kidney) as a template. Sequencing of this clone showed that the 3' end of this cDNA does not correspond to the published sequences for the mouse PEBP mRNA. This would result in an incorrect sequence for the last 10 amino acids of PEBP. As the NanoESI mass spectrometry had shown that the COOH terminus of the purified protein is identical with the published sequence for mouse PEBP (GenBank accession no. P70296), we decided to use an antisense primer in the PCR that codes for the correct last 10 amino acids. This cDNA served as a template for the generation of a cDNA coding for mouse PEBP fused to six histidine residues at the COOH terminus to obtain highly purified protein. In initial experiments we found that the recombinant PEBP-H6 is most easily purified from the conditioned medium of transfected cells. The second elution fraction of the purification using Ni2+NTA-agarose resulted in a single band after SDS-PAGE (Fig. 5A, lane 4). The identity of this protein with PEBP-H6 was confirmed by immunoblot analysis (data not shown). The inhibitory properties of PEBP against thrombin, chymotrypsin, trypsin, t-PA, elastase, and neuropsin were investigated using this highly purified PEBP-H6 preparation. Thrombin (Fig. 5B) and chymotrypsin (Fig. 5C) are competitively inhibited by PEBP-H6 with apparent K values in the nanomolar and low micromolar range, respectively (Table I). Neuropsin was inhibited as well (data not shown), whereas the amidolytic activities of trypsin, tissue plasminogen activator, and pancreatic elastase were not affected.

FIG. 3. Thrombin inhibitory activity in gel filtration fractions. Brains obtained from PN-1/2 mice of different ages (○, 14 days; ■, 6 months; ▲, 1 year; ●, 2 years) were homogenized and applied to a Superdex-200 gel filtration column (dashed line, molecular weight calibration) and the collected fractions assayed for thrombin inhibitory activity (×, brain homogenation buffer only).

FIG. 4. Identification of the inhibitor by NanoESI mass spectrometry. The tryptic peptides found to be identical to mouse PEBP are shown in bold. The serine at position 116 originally published (Swiss-Prot P70296) was found to be a glycine that is in fact conserved in all known proteins belonging to the phosphatidylethanolamine-binding protein family. This finding was confirmed by DNA sequencing of IMAGE clone 1921274 that has as the 116th codon a GGT instead of the previously published AGT (GenBank accession no. U43206).

1921274 (Sugano mouse, kidney) as a template. Sequencing of this clone showed that the 3' end of this cDNA does not correspond to the published sequences for the mouse PEBP mRNA. This would result in an incorrect sequence for the last 10 amino acids of PEBP. As the NanoESI mass spectrometry had shown that the COOH terminus of the purified protein is identical with the published sequence for mouse PEBP (GenBank accession no. P70296), we decided to use an antisense primer in the PCR that codes for the correct last 10 amino acids. This cDNA served as a template for the generation of a cDNA coding for mouse PEBP fused to six histidine residues at the COOH terminus to obtain highly purified protein. In initial experiments we found that the recombinant PEBP-H6 is most easily purified from the conditioned medium of transfected cells. The second elution fraction of the purification using Ni2+NTA-agarose resulted in a single band after SDS-PAGE (Fig. 5A, lane 4). The identity of this protein with PEBP-H6 was confirmed by immunoblot analysis (data not shown). The inhibitory properties of PEBP against thrombin, chymotrypsin, trypsin, t-PA, elastase, and neuropsin were investigated using this highly purified PEBP-H6 preparation. Thrombin (Fig. 5B) and chymotrypsin (Fig. 5C) are competitively inhibited by PEBP-H6 with apparent K values in the nanomolar and low micromolar range, respectively (Table I). Neuropsin was inhibited as well (data not shown), whereas the amidolytic activities of trypsin, tissue plasminogen activator, and pancreatic elastase were not affected.

An interference of the COOH-terminal histidine tag could be excluded by the use of purified NH2-terminal His-tagged PEBP or partially purified PEBP with a NH2- or COOH-terminal hemagglutinin tag, or, furthermore, by the use of the protein encoded by IMAGE clone 1921274 with 10 different amino acids at the COOH terminus. All these different proteins show similar inhibitory properties.

FIG. 5. Inhibitory properties of PEBP-H6. Panel A, following expression in Rat-1 cells, PEBP-H6 was purified using Ni2+NTA-agarose. Samples of the purification step containing 100 ng of protein were subjected to electrophoresis on a 12.5% SDS-PAGE. The gel was silver-stained. Lane 1, flow-through of Ni2+NTA-agarose; lane 2, first washing; lane 3, first elution; lane 4, second elution. Panel B, 0.1 nM thrombin was incubated with increasing amounts of PEBP-H6 and the velocity of the hydrolysis of the chromogenic substrate was determined as described under “Experimental Procedures” (PEBP-H6 concentrations: ○, dashed and dotted line, 0 μM; ●, dotted line, 0.74 μM; ■, dashed line, 1.10 μM; □, straight line, 1.47 μM). Panel C, 0.1 nM chymotrypsin was incubated with increasing amounts of PEBP-H6 (PEBP-H6 concentrations: ○, dashed and dotted line, 0 μM; ●, dotted line, 1.48 μM; ■, dashed line, 2.22 μM; □, straight line, 2.95 μM). The data represent the mean of three separate determinations.
tracellular localization of PEBP, Rat-1 fibroblast cells were immunostained with a polyclonal antiserum directed against mouse PEBP. The detection of PEBP immunoreactivity at the surface of nonpermeabilized Rat-1 cells (Fig. 6A) showed that an extracellular localization of PEBP has to be considered as well.

**DISCUSSION**

In this study, we report the detection of a novel serine protease inhibitor in mouse brain. Purification and analysis by NanoESI mass spectrometry identified this protein as PEBP. We demonstrate that PEBP competitively inhibits the amido-lytic activities of thrombin, chymotrypsin, and neutropsin, whereas the activities of trypsin, t-PA, and pancreatic elastase were not affected.

The phosphatidylethanolamine-binding protein was originally purified from bovine brain and described as a soluble 23-kDa basic cytosolic protein (20). Binding studies revealed its affinity for phosphatidylethanolamine (21), nucleotides like GTP and GDP and small GTP-binding proteins and other hydrophobic ligands (22). Independently, PEBP was purified from human brain as neuropolypeptide h3 (23); sequencing of this protein (24) showed 95% amino acid sequence identity with the sequence for the bovine PEBP (25). The sequence of PEBP shares no significant homology with other proteins so that it is assumed to be the prototype member of the PEBP family. PEBP homologues have been identified in other mammals including rat (26), mouse (27), and monkey (28). Other members of the PEBP family include the putative odorant-binding protein in Drosophila (29), the putative PEBP of the malaria parasite Plasmodium falciparum (30), and the Ov-16 antigen of Onchocerca volvulus (31) and the toxocara excretory-secretory antigen 26 of Toxocara canis (32), two parasitic nematodes. A dosage-dependent suppressor of CDC25 mutations in Saccharomyces cerevisiae, TFS1 (33), and several proteins in flowering plants (Arabidopsis thaliana (Refs. 34–36) and Antirrhinum (Ref. 37)) also belong to this family. Despite this widespread expression and the resolution of the three-dimensional structures of bovine and human PEBP by x-ray crystallography (38, 39), only very little is known about the functions of this protein family. In rat, PEBP was described to be the precursor of the hippocampal neurostimulating peptide, an undecapeptide that is involved in the differentiation of neurons in the medial septal nucleus, where it enhances the synthesis of choline acetyltrans-ferase (40). However, being the precursor of hippocampal neurostimulating peptide cannot be the only biological function of PEBP, as it is not only expressed in the central nervous system but also in a wide variety of other tissues including spleen, testis, ovary, muscle, and stomach (41, 42). The members of the PEBP family are often highly expressed in growing or elongated cells such as oligodendrocytes, spermatides, and the inflorescence meristem of flowering plants (36). This expression pattern and the binding to phospholipids located mainly on the inner leaflet of the plasma membrane suggest a role of PEBP in the organization of the plasma membrane during cell growth and development. Serine proteases are known to inhibit or reverse morphological changes of neuronal cells in vitro; thrombin, for example, is characterized as a neurite retraction signal (9). PEBP could therefore be important in the outgrowth and maintenance of neuronal processes. PEBP was reported to be one of several cellular proteins present inside the human immunodeficiency virus type 1 virions (43), again indicating a possible role for PEBP in membrane organization.

On the other hand, the interaction between PEBP and small-GTP binding proteins leads to speculation that PEBP could be involved in the signaling machinery. Recently, the human PEBP was described as a Raf-1 kinase inhibitor protein that suppresses the mitogen-activated protein kinase signaling (44). The authors of this study showed that PEBP regulates the extracellular signal-regulated kinase pathway at the Raf/mito-gen-activated protein kinase/extracellular signal-regulated kinase kinase interface by binding to Raf-1 and thus leading to a competitive inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase. They further showed that the binding of PEBP/Raf kinase inhibitor protein to Raf-1 decreases during mitogenic stimulation and could characterize the binding sites (45). Interestingly, thrombin is a mitogenic signal and Raf-1 is phosphorylated upon binding of thrombin to its receptor, a G protein-coupled receptor (46). Therefore, PEBP could modulate the cellular response to stimulation of protease-activated receptors.

As PEBP shares no significant homology with other known classes of serine protease inhibitors such as the serpins, the Kunitz, the Kazal, or the Bowman-Birk family (47), the results of this study define it as the prototype for a novel family of serine protease inhibitors. Of special interest is the fact that

| Protease   | $K_i$  |
|------------|-------|
| Thrombin   | $3.8 \pm 1.3 \times 10^{-7}$ |
| Chymotrypsin| $1.8 \pm 1.0 \times 10^{-6}$ |
| Trypsin    | Not inhibited |
| t-PA       | Not inhibited |
| Elastase   | Not inhibited |

* The protease inhibition constants ($K_i$) were determined as described under “Experimental Procedures.” The data represent the mean ± S.D. of three separate determinations.
PEBP inhibits a serine protease with specificity for hydrophobic and aromatic amino acids (chymotrypsin) at the P1 position as well as one that cleaves only after basic amino acids (thrombin). The active site and the mechanism by which PEBP inhibits the serine proteases remain to be determined.

Our finding that mouse PEBP acts as an inhibitor of trypsin-like serine proteases is supported by a report that the yeast homologue of PEBP inhibits a serine protease with specificity for hydrophobic and aromatic amino acids (chymotrypsin) at the P1 position as well as one that cleaves only after basic amino acids (thrombin). The active site and the mechanism by which PEBP inhibits the serine proteases remain to be determined.

The amino acid sequence of PEBP contains no obvious secretion signal, and previous immunohistochemical studies attributed a cytoplasmic localization to this protein. The immunodegeneration of endogenous PEBP on the cell surface and the presence of active PEBP in the supernatant of transfected cells shown here demonstrate that, at least under cell culture conditions, the localization of PEBP is not restricted to the cytoplasm or the inner leaflet of the plasma membrane. Whether this is due to shedding of membranous material as is found for the nematodes' homologues of PEBP (32) or whether the binding to phosphatidyethanolamine plays a role in a putative transport across the plasma membrane remains to be investigated.

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