Molecular Cloning and Characterization of a Novel Chloride Intracellular Channel-related Protein, Parchorin, Expressed in Water-secreting Cells

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We previously reported a 120-kDa phosphoprotein that translocated from cytosol to the apical membrane of gastric parietal cells in association with stimulation of HCl secretion. To determine the molecular identity of the protein, we performed molecular cloning and expression of the protein. Immunoblot analysis showed that this protein was highly enriched in tissues that secrete water, such as parietal cell, choroid plexus, salivary duct, lacrimal gland, kidney, airway epithelia, and choroid plexus. We obtained cDNA for parchorin from rabbit choroid plexus coding a protein consisting of 637 amino acids with a predicted molecular mass of 65 kDa. The discrepancy in size on 6% SDS-polyacrylamide gel electrophoresis is considered to be due to its highly acidic nature (pI = 4.18), because COS-7 cells transfected with parchorin cDNA produced a protein with apparent molecular mass of 120 kDa on 6% SDS-polyacrylamide gel electrophoresis. Parchorin is a novel protein that has significant homology to the family of chloride intracellular channels (CLIC), especially the chloride channel from bovine kidney, p64, in the C-terminal 235 amino acids. When expressed as a fusion protein with green fluorescent protein (GFP) in the LLC-PK1 kidney cell line, GFP-parchorin, unlike other CLIC family members, existed mainly in the cytosol. Furthermore, when Cl⁻ efflux from the cell was elicited, GFP-parchorin translocated to the plasma membrane. These results suggest that parchorin generally plays a critical role in water-secreting cells, possibly through the regulation of chloride ion transport.

When gastric acid secretion is stimulated, a marked redistribution of H⁺,K⁺-ATPase, the gastric proton pump, occurs in the parietal cell. In the resting state, H⁺,K⁺-ATPase exists mainly in the microsomal fraction (tubulovesicles) and is functionally silent because there is very low permeability to K⁺ and Cl⁻ ions. Upon stimulation, tubulovesicles containing H⁺,K⁺-ATPase are incorporated into the apical membrane, the latter acquiring K⁺ and Cl⁻ permeability, and thus H⁺,K⁺-ATPase can exchange K⁺ for protons, creating HCl together with water secretion (1). Recent studies on mechanisms of membrane fusion in parietal cells have made rapid progress, and several proteins involved in the recruitment/recycling process have been identified (2–4). In contrast, little is known of how the apical membrane acquires K⁺ and Cl⁻ ion permeability, which is thought to be the direct trigger for activation of the H⁺,K⁺-ATPase. The export of water then follows the osmotic gradient created by net ion transport into the extracellular solution. A similar mode of water transport, coupled to the export of KCl, is accomplished in many water-secreting cells.

In an earlier study, 32P-loaded rabbit gastric glands were screened for proteins that were phosphorylated, especially on the apical membrane, when acid secretion was activated (5). An interesting phosphoprotein was identified which migrated with apparent molecular mass of 120 kDa by 6–7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This so-called pp120 protein existed mainly in the cytosol and translocated to the apical membrane-rich fraction when acid secretion was activated. Translocation of pp120 was correlated with stimulation-associated redistribution of H⁺,K⁺-ATPase from tubulovesicles to the apical membrane. Interestingly, pp120 co-purified with a new type of protein kinase; thus, pp120 itself was suggested to be a kinase (6).

The aim of the present study was to identify molecular properties of pp120. Using antibodies to examine the tissue distribution, we found that pp120 was highly enriched in tissues associated with water transport, including parietal cells and choroid plexus. Thus, we named this protein “parchorin” based on the enrichment in the parietal and choroid cellular locations. We then cloned the cDNA encoding parchorin and revealed that this protein has significant homology with the chloride intracellular channel (CLIC) family (7). We now suggest that the expression of cellular parchorin is specified for water movement, possibly through the regulation of chloride ion transport.

EXPERIMENTAL PROCEDURES

Isolation of Glands and Immunostaining—Isolated gastric glands were prepared from Japanese white rabbits (Shiraishi Co., Tokyo, Japan).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CLIC, chloride intracellular channel; FVD, polyvinylidene difluoride; 5'-RACE, 5'-rapid amplification of cDNA end; PCR, polymerase chain reaction; SPQ, 6-methoxy-N-[3-sulfopropyl] quinolinium; GFP, green fluorescent protein; MALDI, matrix-assisted laser desorption; PIPES, 1,4-piperazinediethanesulfonic acid.

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pan) by a combination of high pressure perfusion and collagenase digestion in a buffer containing 137 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.0 mM CaCl₂, 1.0 mM NaH₂PO₄, 10 mM Hepes, 5.5 mM d-glucose, and 1 mg/ml bovine serum albumin, pH 7.4, as described (8). For the isolation of salivary glands and lacrimal glands, an approximately 1 x 1 cm piece of gland was minced into a small fragment with anti-parchorn monoclonal antibody (Sigma) (6) and Cy3-anti-mouse IgG (1:50), and examined by microscopy (Nikon Eclipse TE300) with a x 60 water immersion objective (MIB Plan APO 60XWI) using a confocal laser scanning system (µRadiance; Bio-Rad). The sample was excited at 543 nm (green HeNe laser) and detected with E570LP. In some cases, confocal sections (0.5 µm each) were collected and composed along the z axis using LaserSharp software (Bio-Rad).

**Immunoblot Analysis**—Various tissues from rabbits or cows were collected and homogenized in 10 volumes of medium containing 113 mM mannitol, 37 mM sucrose, 5 mM PIPES, pH 6.7, 0.4 mM EDTA-Tris. For collecting airway epithelial cells, the rabbit trachea was isolated and opened along the main axis, and the inner surface was scraped with a spatula. For collecting the chorioepithelium, the rabbit eyeball was opened along the main axis, and the inner surface was rinsed. The epithelium was scraped off from the sclera with a spatula. This specimen was considered to include ciliary epithelium, retina, and choroid. For the renal specimen, a slice approximately 2 mm thick was taken from the middle portion of the rabbit kidney. Choroid plexus was harvested from the lateral ventricle of the rabbit or bovine cerebrum. The homogenates were spun down at 800 x g for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at 100,000 x g (tissues) or at 250,000 x g (cultured cells) for 1 h. The supernatant was used as the cytosolic fraction and the pellet as the membrane fraction for electrophoresis. SDS-PAGE was performed according to Laemmli (9). For immunoblotting, the proteins were electrochemically transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a semidyssary apparatus (1 nA/cm², for 40 min). The antigen was probed with antibodies and visualized using appropriate second antibodies linked with peroxidase.

**Partial Purification of Parchorin and Kinase Assay**—Parchorin was partially purified from the cytosol of rabbit gastric mucosa by ammonium sulfate precipitation, gel filtration, and DEAE-SEpharose chromatography (Amersham Pharmacia Biotech), as described (6). This material was used as a control purarchorin for this work. The DEAE-purified fraction was brought to 1.5 M ammonium sulfate, and the solubilized material was applied to a phenyl-SEpharose column (Amersham Pharmacia Biotech) and eluted with a gradient of ammonium sulfate (1.5 to 0 M) in 10 mM sodium phosphate, pH 7.0. The parchorin-containing fractions were collected, dialyzed against 10 mM sodium phosphate, pH 7.0, applied to a hydroxypapatite column (HA-Ultrogel, Sigma), and eluted with the gradient of 10-300 mM sodium phosphate, pH 7.0.

Kinase assays were performed on parchorin-containing fractions as described (6), with slight modifications. Briefly, samples were brought to 50 mM PIPES, pH 6.8, 10 mM MgCl₂, 0.1 mM dithiothreitol, about 5 µg of myelin basic protein, 0.2 mM ATP, and incubated at 25 °C for 15 min. The reaction was terminated by adding SDS-containing 4X buffer, and the sample was analyzed by SDS-PAGE and autoradiography.

**Protein Sequencing**—The DEAE-purified fraction containing about 100 µg of parchorin was separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane. As parchorin was known to migrate with an apparent molecular mass of 120 kDa by 6% SDS-PAGE, the Ponceau S-stained band of 120 kDa was excised and digested with endoproteinase Lys-C (Roche Molecular Biochemicals), Staphylococcus aureus V-8 protease (Sigma), or cyanogen bromide (Wako Pure Chemical Industries) (10). Released peptides were separated by reverse phase high pressure liquid chromatography using a C18 column (Shimadzu ODS-80TS) and were subjected to automated Edman degradation using PPSQ-10, Shimadzu amino acid sequencer (Shimadzu Co., Tokyo, Japan).

**In-gel Digestion and Matrix-assisted Laser Desorption Mass Spectrometry (MALDI-MS) of Parchorin Peptides**—A slightly modified method of Rosenfeld et al. (11) was used for in-gel digestion. Protein bands (~5 µg of protein) were minced and destained with three 10-min washes of 50% acetonitrile/25 mM NH₄HCO₃. Destained gel pieces were dried, rehydrated in 50 ml of 25 mM NH₄HCO₃ (pH 8.0), and incubated with 0.01 mg/ml trypsin for 15 h at 37 °C. Tryptic peptides were recov-
cell was monitored for 10 min of perfusion with normal Cl\textsuperscript{−} solution. The perfusate was then switched to Cl\textsuperscript{−}-free solution (101 mM sodium gluconate, 5 mM potassium gluconate, 2 mM calcium acetate, 2 mM MgSO\textsubscript{4}, 50 mM mannitol, 5 mM Hepes/Tris, pH 7.4), whereupon the efflux of Cl\textsuperscript{−} (reduction of intracellular [Cl\textsuperscript{−}]) was observed as an increase in fluorescence. As the passive loss of dye from the cell was quite fast (t\textsubscript{1/2} ~ 10 min) during perfusion at 37 °C, each record of time course was processed as follows. For the first 10 min in normal Cl\textsuperscript{−} solution, an exponential curve was constructed by regression analysis to estimate the diffusional loss of SFPQ from the cell. The projection of this regression curve was used to correct the relative fluorescence intensity for all subsequent time points in each experiment. These corrected time course data were used to calculate the Cl\textsuperscript{−} efflux rate. As about 2 min were required for replacement of the perfusate, the efflux rate was calculated by a linear fitting of the fluorescence values (slope) between 2 and 7 min after solution change.

Cell Culture and Transient Transfection—COS-7 or LLC-PK1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with gentamycin and 10% fetal bovine serum in a CO\textsubscript{2} incubator (5% CO\textsubscript{2}, 95% air) at 37 °C. COS-7 cells were transiently transfected by the DEAE-dextran method (14). LLC-PK1 cells were transiently transfected by use of Superfect reagent (Qiagen).

RESULTS

Tissue Distribution of Parchorin—We previously observed that parchorin was highly enriched in rabbit parietal cells (6). To determine whether parchorin was expressed in other rabbit tissues, we performed immunoblot analysis using anti-parchorin monoclonal antibody for the 800 kDa signal. Cryosection of fixed rabbit brain was stained with monoclonal anti-parchorin antibody (1/1000) and horseradish peroxidase-anti-mouse IgG (1/2000) and visualized with diaminobenzidine. Molecular masses (in kDa) of prestained standards are shown on the right.

In order to determine the cellular location of parchorin, gastric, lacrimal, and salivary glands were isolated from rabbits.
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The apical surface of ductal cells resulting in the appearance of a contiguous ductal lumen. This is analogous to gastric glands, where parchorin is present in the cells secreting water but not in the cells containing zymogen granules. These observations clearly indicate that parchorin is preferentially expressed in cells that secrete or transport water, such as saliva, lacrime, aqueous humor, cerebrospinal fluid, urine, periciliary tracheal fluid, and gastric juice.

cDNA Cloning of Parchorin—To acquire further information on the properties of parchorin, cDNA cloning was performed. As the N terminus of purified rabbit gastric parchorin was found to be blocked, the protein was digested and its fragments were sequenced. We obtained partial protein sequences from eight peptide fragments (Fig. 4a). BLAST homology analysis showed that seven of eight sequenced fragments had high degrees of homology with the predicted amino acid sequences of previously cloned bovine chloride channel protein, p64 (15). Based upon the sequences of obtained peptide fragments and that of p64, we synthesized degenerate primers and carried out reverse transcription-PCR at low stringency. Because material from the gastric tissue abundantly contains mRNA of pepsinogen, total RNA from choroid plexus was used as a template for reverse transcription-PCR. Using cDNAs generated from reverse transcription-PCR as a probe, we screened a cDNA library from rabbit brain and obtained one positive clone coding a cDNA of 2.4 kilobase pairs containing a 3’-coding region with an in-frame stop codon preceded by 1.8 kilobase pairs of a 3’-untranslated region but lacking the 5’-end of the full-length cDNA. To extend the partial length cDNAs toward the 5’-end of the gene, 5’-RACE was performed. Because the computer analysis predicted that the 5’-end of the known part of mRNA is highly complicated in its secondary structure, the temperature at the annealing step of PCR was set on high, and “touchdown PCR” (17) was done. Through three rounds of 5’-RACE, an obtained PCR clone had a presumed initiating methionine codon in a consensus region favorable for Kozak’s rules (18), and there was an in-frame stop codon in the upstream region of the methionine.

The full-length cDNA, constructed from the clone of cDNA library and 5’-RACE, encoded a polypeptide of 637 amino acids with a predicted Mr of 64,918. The nucleotide and deduced amino acid sequences of parchorin are shown in Fig. 4a. The full-length amino acid sequence contained all of the peptides obtained from peptide sequence analysis, as indicated by the wavy underline. When MALDI-MS was performed on the tryptic fragments from SDS-PAGE-purified parchorin, peaks were obtained that corresponded to the masses of 23 theoretical tryptic peptides within less than 0.2 mass units. These peptide regions, as indicated by the overline in Fig. 4a, covered 39.1% of the total predicted mass of parchorin. The encoded sequence of parchorin is markedly rich in acidic residues and has a predicted pI of 4.18, which is close to what we previously estimated by isoelectric focusing (4.5 or less) for native parchorin (6). However, the predicted molecular mass of 65 kDa is only half of the apparent molecular mass of native parchorin by 6% SDS-PAGE.

We then subcloned the full-length cDNA into pcDNA3 and transfected COS7 cells. Fig. 5 shows Western blotting of the recombinant parchorin expressed in COS7 cells. The expressed parchorin clearly migrated around 120 kDa, as did native parchorin. It is also obvious from Fig. 5 that most of the expressed parchorin is cytosolic (supernatant of 250,000 x g for 1 h). Thus, the actual molecular mass of pp120/parchorin is 65 kDa, and its apparent large size as measured by SDS-PAGE is considered to be due to its highly acidic nature, reducing the SDS/protein ratio. This property also explains its anomalous

Fig. 3. Cellular localization of parchorin. Isolated glands were fixed, permeabilized, and probed with anti-parchorin monoclonal antibody (1/1000). The first antibody was visualized by Cy3-anti-mouse antibody (1/50). a, resting rabbit gastric gland. Isolated glands were incubated with 100 μM cimetidine for 30 min at 37°C and processed as described above. Note that parchorin exists exclusively in the parietal cell (P), whereas the position where the chief cell exists (C) is dark; also note that most of parchorin in the parietal cell appears to be distributed throughout the cytosol. b, stimulated gastric gland. Isolated glands were maximally stimulated with 100 μM histamine plus 50 μM isobutylmethylxanthine for 30 min at 37°C and processed as above. Note that membranous structure is seen in contrast to the resting gland. c, isolated lacrimal gland stained with anti-parchorin antibody. Confocal sections (0.5 μm each) were composed along with z axis. d, transmission image of c. Note that all the cells in the acinus are parchorin-positive. e, isolated submandibular gland stained with anti-parchorin antibody. Note that only the ductal cells (D), not the acinar cells, were parchorin-positive. Not only the cytosol but also the apical surface of the duct is heavily stained (inset). f, transmission image of e. Bars: a–d, 10 μm; e and f, 50 μm; inset to e, 5 μm.
mobility in SDS-PAGE with different concentrations of acrylamide (6).

The BLAST homology analysis revealed the coding sequence of parchorin had a significant homology to the CLIC family in the COOH-terminal region of the molecule (7), especially to the bovine kidney chloride channel, p64 (16). The deduced amino acid sequence derived from p64 is aligned with parchorin in Fig. 4b. The COOH-terminal 235 amino acids of parchorin show 75.3% homology to the bovine kidney chloride channel, p64 (16).

Fig. 4. Nucleotide and predicted amino acid sequence of parchorin. a, amino acid sequences obtained from protease digests of parchorin are shown with a wavy underline. Presumed mass regions (within 0.2 mass units) from MALDI-MS and electrospray-MS are indicated by an overline. The characteristic sequence repeats in the N-terminal region are shaded. The in-frame stop codon in the upstream region of the predicted first methionine is shown with a boldface underline. b, encoded sequences of parchorin and CLIC family members, i.e. CLIC2 (7), p64 (16), p64H1 human (25), NCC27 (21), and CLIC3 (22) were aligned by CLUSTALW multiple sequence alignment software. Dark shading indicates identical residues, and light shading indicates conserved substitutions.
identity with that of p64. This COOH-terminal region is highly conserved throughout the family and has one or two potential membrane spanning regions predicted by the hydropathy profile (16) and protease digestion (19). However, the entire parchorin molecule is predicted to be cytosolic because of its highly charged nature in the N-terminal region, and this is consistent with its observed intracellular distribution.

Although no homology to any known protein was found in the N-terminal 2/3 of the sequence, there is a characteristic structure. In the region from amino acid 155–244, a sequence of 6 amino acids, GGSVDA or some very similar sequence, is repeated 15 times (Fig. 4a). Interestingly, p64 has four repeats of ASDPEEPQ in the N-terminal region (118–149), although there is no obvious homology between the two proteins in the N-terminal region.

Based on further analyses with PROSITE (Swiss Institute of Bioinformatics), there are a number of consensus sites for phosphorylation by several kinases. Parchorin contains 5 potential phosphorylation sites for protein kinase C (Thr-220, Ser-356, Ser-423, Thr-517, and Ser-559) and 10 potential casein kinase II phosphorylation sites (Ser-83, Ser-122, Ser-284, Ser-288, Thr-336, Ser-338, Thr-440, Thr-517, Ser-552, and Thr-570), but no predicted protein kinase A sites, which exist in p64 or several other members of the CLIC family.

As a result of Northern blot analysis on total RNA from several rabbit tissues, a single transcript of 4.2 kilobases was expressed. This size is consistent with the total length of cloned parchorin described above. The message of parchorin was found prominently in choroid plexus and gastric mucosa, slightly in kidney, and not at all in brain (omitting choroid plexus), heart, or lung (Fig. 6). These results are consistent with the immunoblot data described above.

**Parchorin Is a Novel Protein of CLIC Family**—It is possible that parchorin is a rabbit homologue of the bovine CLIC family member p64, despite the facts that the similarity is found only in the COOH-terminal region and that the tissue distribution is completely different for the two proteins (16). To examine this possibility, we performed immunoblot analysis of choroid plexus from bovine brain. As shown in Fig. 7, a single band of 130 kDa, not 64 kDa, reacted with the anti-parchorin monoclonal antibody, indicating that a homologue of parchorin other than p64 was also expressed in the bovine tissue.
result was obtained using anti-parchorin polyclonal antibody against the COOH-terminal 50 amino acids (amino acids 588–637) of parchorin fused to glutathione S-transferase (data not shown). Thus, we conclude that parchorin is a novel protein and a new member of CLIC family.

We also made recombinant peptide fragments of parchorin fused to glutathione S-transferase (amino acids 409–613), corresponding to the C-terminal part that is common within the CLIC family, and found that the monoclonal antibody did not react with either fragment 588–637 or 409–613 (data not shown). It was concluded that the recognition epitope existed in the N-terminal region (1–408) that is unique to parchorin, assuring that the antibody staining specifically indicated parchorin but not other CLIC family members.

Separation of Kinase Activity from Purified Parchorin—In a previous study, the highly purified parchorin fraction contained a kinase activity; therefore, we suggested that parchorin itself was a kinase (6). However, no consensus sequence for a kinase was found throughout the full length of the protein, suggesting that parchorin was not a kinase but a substrate for a co-purified kinase. To confirm this, we further purified parchorin from rabbit gastric mucosa. Cytosol of rabbit gastric mucosa possesses an activity to phosphorylate parchorin without exogenous kinase, and its activity decreases as parchorin is purified by ammonium sulfate precipitation, gel filtration, and DEAE-Sepharose chromatography. The loss of activity is not mainly due to the inactivation or removal of the kinase but is considered to be due to the removal of a putative activator.
because the activity is recovered by exogenously added substrate (6). We found in the present study that myelin basic protein was the most potent activator, and further purification became possible. The material after DEAE-Sepharose chromatography was brought to 1.5 M ammonium sulfate, applied to a phenyl-Sepharose column, and eluted with the reduction of ammonium sulfate concentration. Fig. 8 shows a typical experiment in which we separated myelin basic protein-activated kinase from parchorin using hydroxyapatite chromatography. With a gradient of sodium phosphate, parchorin eluted as a peak around fraction 6 (Fig. 8a). When each fraction was incubated in the presence of myelin basic protein and [γ-32P]ATP, both parchorin and myelin basic protein were phosphorylated. Fig. 8b shows that the peak of kinase activity, manifested by the phosphorylation of myelin basic protein, is located around fraction 16, clearly separating from the parchorin peak. The slight 32P labeling in the tail of the parchorin peak (Fig. 8b, fractions 10–14) is likely due to fractional overlap with the kinase activity. Redistribution of Parchorin Is Associated with Efflux of Cl−—We previously demonstrated in rabbit parietal cells that parchorin resides mainly in the cytosol and that some of this cytoplasmic parchorin is translocated to the apical membrane in the stimulated state (6), and this was confirmed in the present study (Fig. 3, a and b). As described above, parchorin is especially prominent in tissues that participate in the transport of aqueous secretions. Therefore, we used a cultured cell line to examine what kind of stimulus might cause this translocation. When GFP-tagged parchorin (GFP-parchorin) was transiently transfected into LLC-PK1 cells, most of the GFP-parchorin signal was diffusely distributed throughout the cytosol (Fig. 9c). In order to effect a change in ion transport, the extracellular solution was changed to Cl−-free solution. Removal of Cl− caused an obvious translocation of parchorin to the plasma membrane within 2 min (Fig. 9b) and became even more marked as time progressed (Fig. 9c). When the extracellular solution was returned to the normal Cl−-containing solution, GFP-parchorin gradually left the plasma membrane and became distributed more randomly throughout the cytosol (Fig. 9d).

Parchorin Activates the Export of Cl− Ion in LLC-PK1 Cells—Some CLIC family members, such as NCC27/CLIC1 (21), p64H1 (19), CLIC3 (22), and p64 (16, 23, 24), have been shown to play a role in intracellular chloride ion transport. Because encoded sequences of parchorin have significant homology with this family, we examined whether parchorin also activated Cl− transport. To detect the function of parchorin, we used the Cl−-sensitive fluorophore, SPQ, to monitor intracellular concentration of Cl−. Because the intensity of SPQ is inversely proportional to intracellular Cl− concentration, elevation of fluorescent intensity indicates a loss, or efflux, of Cl− from the cells. Fig. 10 shows a typical record of the time course for the change in SPQ fluorescence before and after removal of extracellular Cl−, corrected for the spontaneous decrease in fluorescence due to the loss of the dye. Because cells distant from each other responded differently to removal of extracellular Cl−, the untransfected control cells (lacking GFP-parchorin) were selected within the same field as transfected cells for the evaluation of SPQ fluorescence. The time course data demonstrate that cells transfected with GFP-parchorin showed some potentiation of SPQ signal when switched to Cl−-free medium, suggesting that Cl− efflux was enhanced compared with untransfected cells. The Cl− efflux rate was estimated by the slope of ∆fluorescence after Cl− removal (i.e. between 12 and 17 min, as shown in Fig. 10, considering the dead volume of the appara-
tus). The initial rate (Δfluorescence/min) of 0.027 ± 0.002 for untransfected cells was significantly increased to 0.044 ± 0.004 for GFP-parchorin-transfected cells (mean ± S.E. of 11 cells from three independent transfections; statistically significant by Student’s t test at p < 0.05). In control experiments done with cells transfected with GFP alone, no potentiation of Cl\textsuperscript{-} efflux was observed (initial rate, 0.026 ± 0.003; n = 7). These results suggest that parchorin potentiates chloride ion efflux across the plasma membrane of cells.

**DISCUSSION**

We have identified, cloned, and partially characterized a novel protein, parchorin, belonging to the CLIC family. Parchorin is distributed exclusively in tissues that secrete aqueous fluid, such as saliva, tears, aqueous humor, cerebrospinal fluid, urine, periciliary tracheal fluid, and gastric juice. These secretions must be strictly regulated to maintain physiological homeostasis; thus, the existence of specific proteins that function as mediators of regulated secretion is indispensable. Parchorin is the first candidate protein of this type, as far as we are aware. Considering the fact that all parchorin-containing tissues affect the active efflux of water, this protein may play a central role in regulated secretion. We hesitate to use the CLIC nomenclature, e.g. CLIC6, because this name is an abbreviation of “chloride intracellular channel” for the ubiquitous endosomes. As discussed below, parchorin could not be a conventional channel, and it does not work in endosomes but possibly in transcellular secretion. We have therefore used a name based on its characteristic tissue distribution.

Members of the CLIC family other than parchorin exist mainly in the membrane fraction; p64, in particular, exists mainly in the membrane fraction and has been proposed to have two membrane-spanning regions in its C-terminal sequence (15). Although there is good homology in the C-terminal region, previous experiments have shown that parchorin exists predominantly in the cytosolic fraction of parietal cells (6), as also observed for COS-7 cells transfected with parchorin or LLC-PK1 cells transfected with GFP-parchorin in the present study. Actually, parchorin contains an abundance of hydrophilic amino acids, and therefore, computer analysis by SOSUI topology system (Department of Biotechnology, Tokyo University of Agriculture and Technology) also predicts a cytosolic localization. Membrane-bound parchorin in the parietal cell was easily solubilized from the membrane-bound fraction by relatively low concentrations of n-octylglucoside that did not solubilize H\textsuperscript{+},K\textsuperscript{+}-ATPase, a typical integral membrane protein (6). These data indicate that parchorin exists in a soluble form in the resting state and then associates with the membrane in the secreting state by some mechanism presently unknown. A similar argument has been made by Edwards (25): there might be two forms for members of the p64 family, because a considerable amount of the soluble form as well as the membrane-bound form exists in certain tissue, or in overexpressed cell lines, suggesting membrane insertion of the molecule by some modification. Although stimulus-related translocation has not been reported for CLIC family members other than parchorin, it is conceivable that translocation might occur under an appropriate stimulus.

As for the machinery for the translocation of parchorin, we have no data at present. Possible mechanisms would be binding to membrane protein(s), lipid modification, and covering the acidic N-terminal region with other basic protein(s). It is reasonable to suppose that the C-terminal sequence common among CLIC family members is essential for their function, i.e. chloride conductance or its activation, whereas the N-terminal part affords its specific role. The repeat of GGSVDA motif in the N-terminal domain of parchorin is considered to be one of the most interesting candidates for the interaction with another molecule.

Exogenously transfected GFP-parchorin in LLC-PK1 cell was translocated when Cl\textsuperscript{-} was removed from the extracellular medium. This is the first example of a translocation event among the CLIC family members. Export of water is driven by osmotic forces constituted by the balance of ionic concentrations between intracellular and extracellular solutions. Thus, redistribution of parchorin may be a result of downstream signaling from a stimulus of osmotic pressure. It has been reported that osmotic pressure results in the activation of an unidentified kinase (26), protein kinase C (27, 28), or tyrosine kinase (29). Parchorin was originally found as a phosphoprotein (5), and we know that parchorin has several consensus sites for protein kinase C and casein kinase. Thus, it is possible that phosphorylation by a specific protein kinase promotes the redistribution of parchorin.

Our previous suggestion that parchorin itself was a new type of kinase was based on the kinase activity of a highly purified fraction from rabbit gastric mucosa, which was activated by basic proteins and was resistant to inhibitors of known kinases, such as protein kinase A, protein kinase C, and calmodulin kinase II (6). However, no consensus sequence of any kinase was found in parchorin, suggesting that parchorin is not a kinase but a substrate for the unknown kinase. This was further confirmed by our observation that parchorin and the kinase activity can be separated by hydroxyapatite chromatography. This myelin basic protein-activated kinase might be a key molecule for regulating water secretion via the modulation of parchorin. We are now trying to identify this enzyme.

In order to clarify the physiological function of parchorin, GFP-parchorin was transfected into LLC-PK1 cells. We supposed that any cell type enriched in parchorin might not respond to parchorin-overexpression due to an adequate endogenous supply; moreover, a cell without parchorin might not respond either, because it might lack the putative target for parchorin. We chose the LLC-PK1 line because of its origin from kidney that expresses a moderate amount of the protein. Using this cell line, it was found that GFP-parchorin potentiated the efflux of Cl\textsuperscript{-} from cells when Cl\textsuperscript{-} was removed from the extracellular solution. The parchorin-accelerated Cl\textsuperscript{-} efflux was only about twice that of control, possibly reflecting the fact that endogenous parchorin or other proteins exhibiting parchorin-like functions might operate in the LLC-PK1 cells. More work is necessary, possibly including making parchorin-deficient cells, to clarify the physiological function of this protein.

Although it is still possible that parchorin itself is a chloride channel, the existence of a predominant soluble form of parchorin more likely suggests that it acts as an activator or regulator of a Cl\textsuperscript{-} channel. This reminded us of a soluble chloride channel, pICln, which was originally cloned as a swelling-activated chloride channel. When overexpressed in Xenopus oocytes, pICln gave rise to an anion conductance that was constitutively active and outwardly rectifying. In addition, antisense pICln oligonucleotides and a monoclonal anti-pICln antibody inhibited the endogenous outwardly rectifying swelling-activated anion currents in NIH3T3 cells (30) and Xenopus oocytes (31). However, like parchorin, this protein exists mainly in the cytoplasm and indirectly associates with a kinase (32). These data strongly suggest that pICln is not a channel itself, but a component or regulator of a Cl\textsuperscript{-} channel. We would suggest that parchorin may also act in the same manner. In rabbit parietal cells, the CIC-2G chloride channel was suggested to be a key molecule for HCl secretion (33). CIC-2G was activated in a voltage- and protein kinase A-dependent manner. It is possible that parchorin acts in concert with a Cl\textsuperscript{-} channel.
channel, such as CIC-2G, to regulate secretion in parietal cells and other cells. We consider that there is no direct connection between protein kinase A and parchorin, because parchorin lacks potential phosphorylation sites for protein kinase A and the translocation of parchorin was not potentiated by stimulating protein kinase A in cell lines. However, in parietal cell extracts, there is a tightly associated myelin basic protein-activated kinase, the specific regulatory function of which is unknown (it might be termed “parchorin kinase”). Further study is clearly necessary to elucidate the intracellular signal for the activation of parchorin.

In conclusion, we have identified a novel protein, parchorin, as the first translocating protein in the CLIC family. Distribution of the protein strongly suggests a physiological role in the regulation of water secretion. Parchorin translocates from cytosol to plasma membrane in association with Cl⁻ efflux, and it augments Cl⁻ transport. The search for specific proteins such as a putative kinase for parchorin and proteins associating with parchorin could greatly contribute to our understanding of the functional activities of water secreting cells. This reminds us of cystic fibrosis transmembrane conductance regulator protein; the secretory defect in cystic fibrosis has been attributed to a defect in the translocation ability of the cystic fibrosis transmembrane conductance regulator protein (34). Although its distribution overlaps with that of parchorin, e.g. kidney and airway epithelia, there are clear distinctions, e.g. pancreas and heart. It should be an important future project to search for whether a disease is associated with a dysfunction of parchorin.

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