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pI_{Cln} and Cytosolic F-Actin Constitute a Heteromeric Complex with a Constant Molecular Mass in Rat Skeletal Muscles[1]

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To elucidate the function of pI_{Cln}, its localization in subcellular organellae was investigated. A specific polyclonal anti-pI_{Cln} antibody detected the soluble 38-kDa pI_{Cln} exclusively in the cytosols of rat heart, lung, liver, spleen, skeletal muscle, testis, and brain, but not rat kidney. pI_{Cln}-associated proteins in skeletal muscle were also analyzed. Native-gradient PAGE showed a single 340-kDa protein band reactive to anti-pI_{Cln} antibody. This band also stained with anti-actin antibody. Two-dimensional PAGE and immunoprecipitation analysis indicated that all of the pI_{Cln} was present in association with actin of a constant length: the molecular ratio of pI_{Cln} to actin was roughly 1:7. In addition, all actin in the cytosol fractions was found in association with pI_{Cln}. These results suggest the possibility that skeletal muscle pI_{Cln} controls the length of cytosolic F-actin.

Key words: F-actin, native PAGE, pI_{Cln}, skeletal muscle.

Swelling-activated or volume-sensitive Cl^- currents are found in numerous cell types and play a variety of roles in their function (*1-3*). pI_{Cln}, a 235-amino-acid protein whose cDNA was first cloned from MDCK cells by Paulmichl *et al.* (*4*), was initially proposed to be a Cl^- channel-forming protein. Chen *et al.* knocked down the pI_{Cln} protein in nonpigmented ciliary epithelial cells using an antisense oligonucleotide complementary to human pI_{Cln} mRNA (*5*). The pI_{Cln}-knocked-down cells showed delayed activation of volume-activated Cl^- current and reduced ability to regulate volume. Recently, we reported that pI_{Cln} predominantly localizes at the luminal surface membranes of distal tubules and Henle's ascending limbs (*6*) and proximal tubules (*7*). pI_{Cln} seems to be involved in the activation pathway of the volume-activated Cl^- current. Except for the kidney, however, pI_{Cln} is a soluble protein localized predominantly in the cytoplasm (*8-10*). pI_{Cln} was observed on nuclei and their surroundings in pig renal proximal tubular cells, LLC-PK1, but not on the cell surface membranes, although it was present in soluble and insoluble forms (*11*). In this report, the exposure of LLC-PK1 cells to hypotonic media elevated the ratio of soluble to insoluble pI_{Cln} within 5 min. This intracellular translocation of pI_{Cln} was inhibited by extracellular ATP, which blocked the Cl^- current induced by the expression of pI_{Cln} in oocytes. When expressed in *Xenopus* oocytes, pI_{Cln} induced an outwardly rectifying anion conductance that was blocked by extracellular nucleotides (*4*). *Xenopus* oocyte swelling was shown to activate an endogenous $I_{Cln,swell}$ that superficially resembled the current induced by heterologous expression of pI_{Cln} (*12*).

Voets *et al.* characterized the current induced by expression of human pI_{Cln} in *Xenopus* oocytes in comparison with the endogenous $I_{Cln,swell}$ and showed clear differences between them in the rectification ratio of currents measured at +55 and −95 mV, the anion permeability sequence, the sensitivity to extracellular cAMP, and the effect of cell swelling (*13*). These observations cast significant doubt on the hypothesis that pI_{Cln} is an $I_{Cln,swell}$ channel regulator (*14*).

Suppression of cell-volume-induced activation of Cl^- channels by the antisense oligonucleotides for pI_{Cln} strongly suggests that pI_{Cln} relates to cell volume control, by either a direct or indirect mechanism (*15*). Krapivinsky *et al.* demonstrated that pI_{Cln} forms oligomeric complexes with other cytoplasmic proteins including actin in MDCK cells (*8*). The association of pI_{Cln} with actin was observed for red blood cells (*16*) and heart muscles (*17*). Emma *et al.* identified a non-muscle isoform of the alkali myosin light chain that binds selectively to pI_{Cln}, using affinity assays and immunoprecipitation with C6 glioma cells (*18*).

Filamentous actin (F-actin) has been suggested to play a role in regulatory volume decrease (*19-22*). The plasma membrane is supported by the F-actin-based network. Disruption of the cytoskeleton reduced $I_{Cl,swell}$ (*21*), and the absolute amount of intracellular F-actin is not critical for the volume regulation of cells (*23*). An F-actin disrupting drug, dihydrocytochalasin B, completely inhibited the translocation of pI_{Cln} from the insoluble to soluble fraction induced by the 50% hypotonic treatment of LLC-PK1 cells

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(11). Okada discussed the volume-sensitive sensors in connection with pI_{Cln} in detail, based on his finding that cytochalasin D increased current density per relative surface area of KB cells (3).

pI_{Cln} is present at high densities in organs not normally exposed to low osmotic stress, such as brain, testis, and adrenal gland (24). Native heart cytosolic pI_{Cln} forms several oligomeric complexes with apparent molecular masses of 73, 90, 300 kDa, while sf9 cells do not (8). In LLC-PK1 cells, the apparent molecular masses of the pI_{Cln}-complexes were 115 and 480 kDa (11). The differences in the apparent molecular masses of the oligomeric complexes with pI_{Cln} among the tissues and two types of cells suggest that the physiological roles of pI_{Cln} may differ depending on the tissues or cells. Questions have been raised regarding which of the several pI_{Cln}-including complexes contains actin, whether the actin that binds pI_{Cln} is globulous actin (G-actin) or F-actin, and whether the pI_{Cln}-actin complex also includes other proteins. The answers to these questions should help to clarify the functions of pI_{Cln}. In the present study, we investigated the intracellular localization of pI_{Cln} in several organs and pI_{Cln}-actin complexes in skeletal muscle cytosols.

MATERIALS AND METHODS

Materials—Sprague-Dawley rats (male, 6 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu) and used in experiments that adhered to the Guidelines for Animal Experimentation of the Akita University School of Medicine. Goat anti-rabbit horseradish-peroxidase conjugated immunoglobulin (IgG) was from BioRad (Richmond, USA). Rabbit anti-actin antibody was from Sigma (IgG fraction, Catalogue No. A5060). This antibody was developed using the N-terminal peptide (amino acid residues 20-33) attached to a multiple antigen peptide backbone as an immunogen. The antibody recognizes the 42-kDa actin band in human or animal tissues extracts (according to the manufacturer's instructions). Other chemicals were purchased from Sigma Co. Japan. Rabbit anti-pI_{Cln} antibody developed using the N-terminal peptide was the same preparation as that described in our previous report (6). This antibody against the synthesized C-terminal 18 amino acid peptide sequence (NH_2-VDTTPTVAGQFEDADVDH-COOH) of pI_{Cln} strongly reacted with human recombinant pI_{Cln}.

Preparation of Rat Organs into Soluble and Insoluble Fractions—All operations were carried out at 4°C. Rats were sacrificed by cervical dislocation, and the organs were immediately removed, chopped, and homogenized with 3 volumes of Buffer A (20 mM Tris-HCl, 0.5 mM EDTA, 0.25 M sucrose, 2 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, pH 7.4). After centrifugation at $800\times g$ for 10 min, the supernatant was centrifuged at $100,000\times g$ for 90 min. The resulting supernatant was taken as the soluble fraction. The precipitate was washed twice by centrifugation in Buffer A and suspended in the same buffer to give the insoluble fraction.

Subcellular Fractionation—Rat brains, skeletal muscles, and kidneys were subcellularly fractionated into nuclear, mitochondrial, microsomal, and cytosol fractions as follows. Each organs was homogenized separately with 10 volumes of buffer A using a Polytron homogenizer (Kinematia Amb H, Switzerland) at half-maximal speed for approximately 2 min until all large pieces of tissue had disintegrated. The homogenate was centrifuged at $500\times g$ for 10 min. The precipitate (P1) and supernatant (S1) were further fractionated by centrifugation. P1 was suspended in Buffer B (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.32 M sucrose, 2 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, pH 7.4) and centrifuged at $1,000\times g$ for 10 min. This step was repeated once, and the resulting precipitate was suspended in Buffer B containing 0.1% Triton X-100. The suspension was overlayed on 2.2 M sucrose and centrifuged at $18,000\times g$ for 60 min. The precipitate was washed with Buffer A to yield the nuclear fraction. S1 was centrifuged at $22,000\times g$ for 15 min, and the resulting supernatant was centrifuged further at $105,000\times g$ for 60 min. The supernatant obtained was used as the cytosol fraction. The $22,000\times g$ precipitate was composed of two distinguishable layers: a fluffy white layer and a hard red layer. The fluffy layer was suspended in Buffer A and centrifuged at $10,500\times g$ for 8 min. The resulting supernatant was centrifuged at $38,500\times g$ for 30 min. The precipitate was washed once with Buffer A to give the microsomal fraction. The hard layer was suspended in Buffer C (1.0 mM Tris-HCl, 0.1 mM EDTA, 0.28 M sucrose, 2 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, pH 7.2) and centrifuged at $500\times g$ for 10 min. The supernatants was centrifuged at $10,700\times g$ for 12 min. The $10,700\times g$ precipitate was suspended in Buffer C and centrifuged at $500\times g$ for 10 min. The resulting supernatant was centrifuged at $1,900\times g$ for 8 min. The precipitate obtained was suspended in Buffer D (20 mM phosphate-KOH buffer, 0.02% BSA, 2 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, pH 7.2) and centrifuged at $500\times g$ for 10 min. The supernatant was centrifuged at $1,900\times g$ for 8 min. The resulting precipitate was washed once with Buffer A to yield the mitochondrial fraction.

Enzyme Assay—Succinate-cytochrome *c* reductase [EC 1.3.99.1] activity as a mitochondrial marker was measured by the method of Tisdale (25). Na^+-K^+-ATPase [EC 3.6.1.37] activity as a microsomal marker and fructose 1,6-bisphosphatase [EC 3.1.3.11] activity as a cytosolic marker were measured as described previously (Refs. 26 and 27, respectively). DNA as a nuclear marker was determined with bisbenzimidazole (Hoechst 33258) by the method of Labarca and Paigen (28). Protein was determined by Lowry's method (29).

Electrophoresis and Immunoblot—Samples (20 μg of protein per lane) were subjected to SDS-PAGE (9% slab gel) by the method of Laemmli (30). Coomassie Brilliant Blue R-250 (CBB) was used for the staining of the proteins. Western blot analysis was performed by the method of Towbin *et al.* (31). The antibodies were used with 1,000-fold dilution. Native gradient polyacrylamide gel electrophoresis (Native-gradient PAGE) was carried out on a slab gel with linear gradient of polyacrylamide from 6 to 18% by a slight modification of the method described by Schägger *et al.* (32). After running, the gel was equilibrated with 1% SDS in the immunoblot buffer for 30 min, followed by immunoblotting. Two-dimensional gel electrophoresis was performed as follows: the first electrophoresis was the native-gradient PAGE on a slab gel, and the second electrophoresis was SDS-PAGE using the gel stripe of one lane cut from the first gel. The immunoblot was carried out

by the above-mentioned method of Towbin *et al.* (*31*). The densities of the protein bands or spots stained with CBB were measured with a scanning densitometer using a Scion Image program (Scion, USA). For determining protein molecular weights, High Molecular Weight Calibration Kit (Pharmacia, Uppsala, Sweden) was used. Phosphorylase *b* (94,000), albumin (67,000) and ovalbumin (43,000) were used for SDS-PAGE; and thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and albumin (67,000) were used for native gradient PAGE.

Immunoprecipitation—Anti-pI_{Cln} serum or preimmune serum, 10 μl, was mixed with 1 ml of the skeletal muscle cytosol fractions containing 4 mg of proteins and incubated for 2 h at 4°C. Protein A-Sepharose (0.5 g wet weight) was added, and the mixture was shaken gently for 2 h at 4°C. The resin was then washed five times with ice-cold 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM EGTA, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 0.1% Triton X-100. The washed resins were suspended in the buffer for SDS-PAGE, boiled for 4 min, and analyzed by SDS-PAGE followed by immunoblotting.

Phosphorylation and Dephosphorylation—The skeletal muscle cytosol fractions were incubated with protein kinase A catalytic subunit in a kinase buffer (10 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, 20 μM ATP, pH 7.4) at 30°C for 15 min, or alkaline phosphatase in a phosphatase buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.5) at 30°C for 30 min. The incubated samples were immediately analyzed by native-gradient PAGE and immunoblot assay with anti-pI_{Cln} or anti-actin antibody. The sample was also incubated in 10 mM EDTA or 10 mM $CaCl_2$ in 10 mM Tris buffer (pH 7.4) at 30°C for 5 min and used for native PAGE and immunoblot assay.

RESULTS AND DISCUSSION

Distribution of pI_{Cln} in Rat Organs—Northern blot analysis has revealed that pI_{Cln} is ubiquitously present in mammalian organs (*24, 33, 34*). The protein amount of pI_{Cln} in cells might not be proportional to the amount of mRNA for pI_{Cln}. Reports on its organ distribution by immunoblot are rather few. Using the anti-pI_{Cln} antibody, the distribution of pI_{Cln} was observed for rat organs: heart, lung, liver, spleen, kidney, skeletal muscle, testis, and brain. The tissue homogenates were divided into soluble and insoluble fractions as described under "MATERIALS AND METHODS."

As shown in Fig. 1, 38-kDa bands were recognized in the soluble fractions from all organs except the kidney. The renal extract showed no immunoreactive band. It is interesting to observe that pI_{Cln} was detected in the microsomal fractions of pig kidneys and on the luminal surface membranes of the distal tubules and Henle's loop ascending limbs (*6*). Rat and pig kidneys thus differ the localization of this protein. In the case of pig kidneys, pI_{Cln} was detected in both soluble and insoluble fractions.

Although the predicted molecular mass of pI_{Cln} from cDNA of MDCK cells is 27 kDa, the apparent molecular mass of antibody-recognizing endogenous pI_{Cln} was shown to be 37–43 kDa on SDS-PAGE (*6, 8, 32, 34*). This large apparent value on SDS-PAGE is a striking feature of pI_{Cln}. Our previous results (*35*) showed that a recombinant pI_{Cln} conjugated to 15.6-kDa thioredoxin (tag-peptide) showed

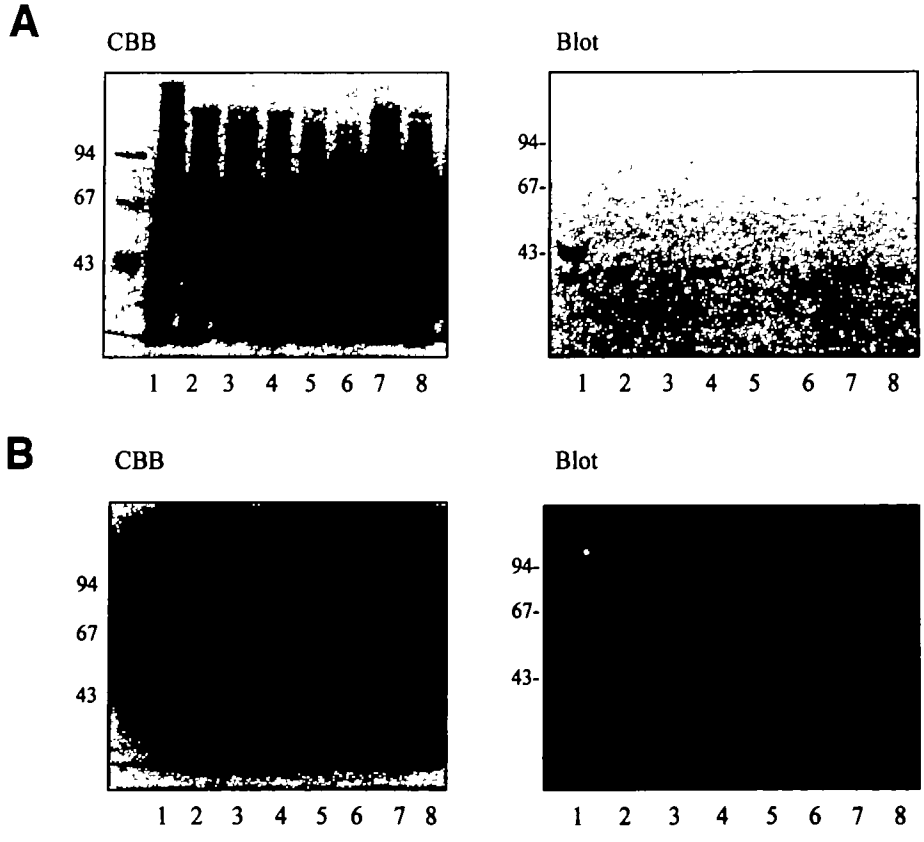

Fig. 1. **Distribution of pI_{Cln} in soluble and insoluble fractions from eight rat organs.** The organs were separated into soluble and insoluble fractions by centrifugation, as described in "MATERIALS AND METHODS." Each sample (20 μg protein/lane) was applied to SDS-PAGE (9%), followed by staining with Coomassie Brilliant Blue (CBB) or electrically transferred onto PVDF membranes and immunostained with anti-pI_{Cln} antibody (Blot). (A) Soluble fractions. (B) Insoluble fractions. Lane 1, heart; lane 2, lung; lane 3, liver; lane 4, spleen; lane 5, kidney; lane 6, skeletal muscle; lane 7, testis; lane 8, brain. The positions of molecular mass standards are shown with their values in kDa.

water-soluble characteristics, and mobility on SDS-PAGE corresponding to a molecular mass of 50 kDa. Assuming that the tag-portion has a molecular mass of 15.5-kDa on the SDS-PAGE, the portion of pI_{Cln} is calculated to have a molecular mass of 34.5 kDa (50–15.5 kDa). Therefore, the 38-kDa protein band detectable with the anti-pI_{Cln} antibody can be concluded to be pI_{Cln}. This calculated value is very near that of the present soluble pI_{Cln}, 38 kDa. This recombinant pI_{Cln} in *Escherichia coli* resulted in significant tolerance to a hyposmotic environment. The soluble 38-kDa protein in the present experiments is estimated to be pI_{Cln}, responsible for the tolerance to the hyposmotic environment.

The protein from skeletal muscle in Fig. 1 (lane 6) seems to show a slightly faster mobility, but the difference was not significant as shown in Fig. 2: the mobilities of the bands for skeletal muscle and brain were the same. Interestingly, the soluble fraction from the hearts showed another band with a larger molecular mass, 43 kDa. Krapivinsky and his coworkers showed only a single band using dog hearts (*8*). This discrepancy may come from the difference in animal species, like the localizations of pI_{Cln} in the kidneys of rat and pig.

No 38-kDa protein bands were observed in any insoluble fractions, as shown in Fig. 1B. A 47-kDa band was observed in all insoluble fractions except the lung and the spleen.

Another protein band with a molecular mass of 110-kDa was observed in the insoluble fractions from heart, liver, and skeletal muscle (Figs. 1B and 2). At the present time, there are no experimental results that allow discussion of the larger protein bands.

Intracellular Localization of pI_{Cln}—Subcellular localization of pI_{Cln} in three rat organs, skeletal muscle, brain, and kidney, was observed to determine the localization of the 38-, 47-, and 110-kDa proteins. As shown in Table I, the rat skeletal muscle, brain, and kidney were well-fractionated into the nuclei, mitochondria, microsomes, and cytosol, judging by the distribution of each marker enzyme.

The 38-kDa protein band on SDS-PAGE was observed in the cytosol fractions from skeletal muscle and brain, but not in renal cytosol, confirming the results in Fig. 1.

On the subcellular cell-fractionation, the activity of Na-K-ATPase, a highly specific marker enzyme of cell surface plasma membranes, was roughly 2.5–4 times higher in the microsomal fractions than in the mitochondrial fractions. On the other hand, the densities of the 47-kDa protein bands showed no significant differences between the mitochondrial and microsomal fractions from any organs tested. The 47-kDa protein seem to be associated at least with intracellular membranes other than the cell surface plasma membranes.

These results suggest the heterogeneity of mRNA for pI_{Cln}. Abe and his coworkers reported only one mRNA (1.8 kb) in all rat organs, including skeletal muscle, investigated by Northern blot analysis (*24*). We confirmed these results by similar experiments (data not shown). However, no further evidence is available regarding these speculations.

The 110-kDa band on SDS-PAGE was observed only in the nuclei fraction from skeletal muscle, as shown in Fig. 2. Although this exclusive localization of the 110-kDa protein is interesting, its relationship to pI_{Cln} is unknown and must await sequencing of the amino acid or the cDNA of this protein.

While preparing this manuscript, we succeeded in producing an antibody against pI_{Cln} using recombinant full-length pI_{Cln}. This antibody recognized only the 38-kDa protein band in SDS-PAGE, and not 47- and 110-kDa bands in the insoluble fractions of several organs tested, including skeletal muscle.

Native Form of Soluble pI_{Cln}—The pI_{Cln} with the molecular mass of 38 kDa on SDS-PAGE has been regarded as a regulatory protein of an osmo-sensitive chloride channel (*8*), and some proteins associated with pI_{Cln} have been reported: *e.g.*, actin (*8, 16*) and a protein kinase (*36*). The native pI_{Cln} was observed by a native-gradient PAGE, followed by immunoblotting with the anti-pI_{Cln} antibody. As shown in Fig. 3, only one band with a molecular mass of 340 kDa was detected. Our previous report described three

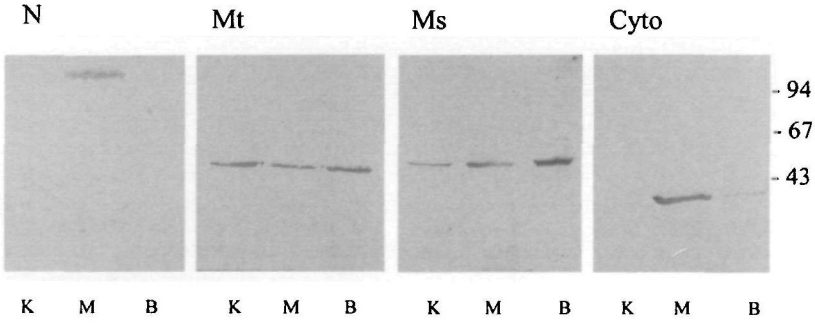

Fig. 2. **Subcellular distribution of pI_{Cln} in rat brain, skeletal muscle, and kidney.** The three organs were fractionated into nuclei (N), mitochondrial (Mt), microsomal (Ms), and cytosol (Cyto) fractions as described under "MATERIALS AND METHODS." In each lane, the sample (20 μg protein/lane) was applied to SDS-PAGE (9%), followed by electrical blotting onto PVDF membranes and immunostaining with anti-pI_{Cln} antibody. B, brain; M, muscle; K, kidney. The positions of molecular mass standards are shown with their values in kDa. Experiments were repeated three times, and the results were reproducible.

TABLE I. **Subcellular fractionation of rat brain, skeletal muscle and kidney.** Rat brain (B), skeletal muscle (M), and kidney (K) were fractionated, and their marker enzyme activities and DNA were measured. The values shown are the percentage of the maximal value in each fraction. S-CCRase, succinate-cytochrome *c* reductase; FBPase, fructose-1,6-bisphosphatase.

	DNA			S-CCRase			Na^+-K^+-ATPase			FBPase
	B	M	K	B	M	K	B	M	K	K
					(%)					
Nuclei	100	100	100	35	45	22	5	8	5	0
Mitochondria	0.5	0	0	100	100	100	35	30	25	1
Microsome	0	0	0	27	40	27	100	100	100	5
Cytosol	0	0	0	11	7	8	10	4	10	100

pI_{Cln}-containing complexes with molecular masses of 38, 115, and 480 kDa in LLC-PK1 cells using the same native-gradient PAGE (*11*). In MDCK cells, pI_{Cln} was observed to associate stably with actin (*8*). Based on these previous reports, the immunoblotting was carried out with the anti-actin antibody. Surprisingly, the results clearly showed only one band at the same position as that of pI_{Cln} and no other band, which suggested the absence of monomers and random oligomers of actin. The electrophoretogram stained with CBB after the native-gradient PAGE showed a dense and sharp protein band at the position of 340 kDa. By SDS-PAGE followed by immunoblotting for actin, the soluble fraction showed only one immunoreactive band, of approximately 43 kDa (data not shown).

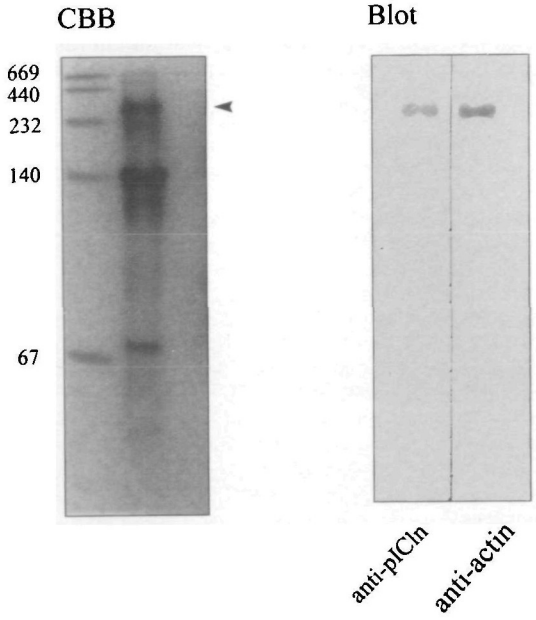

Fig. 3. **Native-gradient PAGE of cytosolic pI_{Cln} of rat muscle.** Rat skeletal muscle cytosol (40 μg protein/lane) was analyzed by native-gradient PAGE (6-18%). The obtained gels were stained with Coomassie Brilliant Blue (CBB) or equilibrated with 10% SDS solution, then blotted onto PVDF membranes and immunostained with anti-pI_{Cln} or anti-actin antibody. The positions of molecular mass standards are shown with their values in kDa. The arrowhead indicates the position of the immunostained band.

The association of pI_{Cln} with actin in the soluble fraction was analyzed by two-dimensional gel electrophoresis. In general, isoelectrofocusing is used for the first dimension of two-dimensional PAGE. This conventional method did not work for pI_{Cln}. The native-gradient PAGE was applied for the first dimension, and SDS-PAGE for the second as described under "MATERIALS AND METHODS." As shown in Fig. 4, this new type of two-dimensional PAGE showed well-separated results. The gels stained with the anti-pI_{Cln} antibody or the anti-actin antibody each showed only one immunoreactive spot. The CBB-staining of the native-gradient PAGE showed a dense spot at the position of actin of approximately 43 kDa and a faint spot at the position of pI_{Cln} of approximately 38 kDa. These electrophoretic patterns indicate that no other protein associates with the pI_{Cln}-actin complex and, consequently, that actin in the soluble fractions of rat skeletal muscles is present as a 340-kDa heteropolymer in association with pI_{Cln}. The density ratio of the actin spot to the pI_{Cln} spot stained with CBB was 6.79 ± 0.98 ($n=5$). One pI_{Cln} and seven actin monomers can form a 338-kDa ($37+43\times7$) pI_{Cln}-actin complex. This value is consistent with the apparent value obtained from the native-gradient PAGE. The density of staining by CBB is known to vary depending on the protein species. Therefore, the ratio of 1 pI_{Cln} to 7 actins might be different from the real ratio. However, this value indicates the binding of one pI_{Cln} to one F-actin with a constant length.

The specific association of pI_{Cln} with actin was confirmed by immunoprecipitation of the pI_{Cln}-actin complex from the muscle-soluble fractions using anti-pI_{Cln} antibody. The SDS-PAGE followed by immunoblot of the immunoprecipitates showed a 38-kDa band stained with the anti-pI_{Cln} antibody and a 43-kDa band stained with the anti-actin antibody, indicating co-immunoprecipitation of the pI_{Cln}-actin complex (data not shown).

This association of pI_{Cln} with actin might be controlled by phosphorylation-dephosphorylation (*36*) or divalent cations such as Ca^{2+}. The soluble fractions of rat skeletal muscles were treated with the catalytic subunit of protein kinase A or with alkaline phosphatase as described under

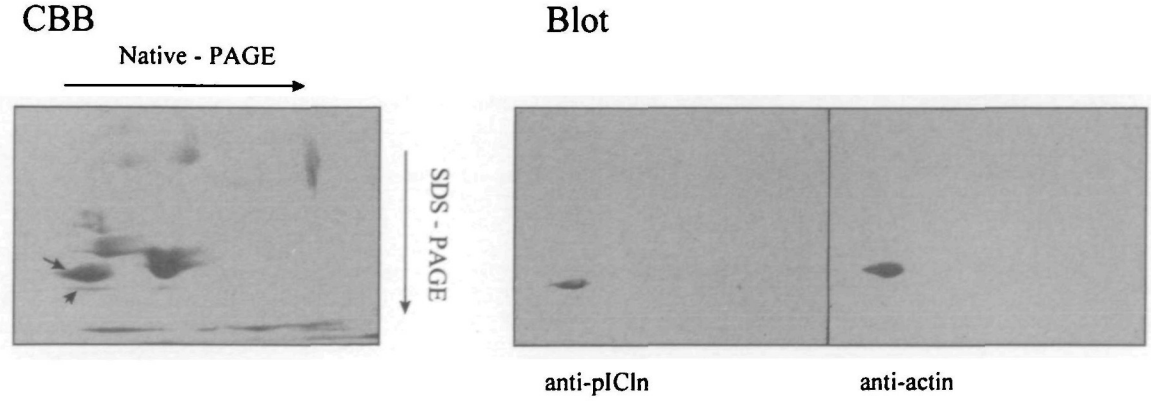

Fig. 4. **Two-dimensional PAGE of cytosolic pI_{Cln} in rat skeletal muscle.** The first dimension was native-gradient PAGE (6-18%), and the second dimension, SDS-PAGE (9%). The cytosolic sample (80 μg protein) was used for one analysis. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) or analyzed by immunoblot assay using anti-pI_{Cln} or anti-actin antibodies. The upper and lower arrows show the positions of spots detected by immunostaining with anti-pI_{Cln} and anti-actin antibodies, respectively.

"MATERIALS AND METHODS." Neither treatment produced changes in the mobility of pI_{Cln} (340 kDa) in the native-gradient PAGE, indicating no dissociation of the complex (data not shown). Similarly, the treatment of the soluble fractions with 10 mM EDTA or 10 mM Ca^{2+} showed no dissociation of the complex (data not shown). Our preliminary experiments could not show the construction of the complex with recombinant pI_{Cln} and purified F-/G-actin from rat skeletal muscles.

The actin-based cytoskeleton plays a role in the regulation of cell shape, cell mobility, and membrane function. Disruption of the cytoskeleton alters the volume sensitivity of $I_{Cl,swell}$ (*37*). Expression of pI_{Cln} with an apparent molecular mass of 38 kDa on SDS-PAGE in *Xenopus* oocytes produced an outwardly rectifying chloride current (*8*), and the display features were consistent with swelling-induced chloride current (*4*). Expression of pI_{Cln} in *E. coli* resulted in significant tolerance to a hyposmotic environment (*35*). The specific association of F-actin with pI_{Cln} may be concerned with swelling-induced cell volume control. Although it is unlikely that skeletal muscles are physiologically exposed to a hyposmotic environment, they may change their cell volumes by contraction and relaxation. The cytosolic pI_{Cln} of rat skeletal muscles may contribute to regulation of the mechanical changes in cell volume through maintaining and regulating cytoskeletal function.

In conclusion, 38-kDa pI_{Cln} was found exclusively in the cytosols of all rat organs tested except the kidney. The anti-pI_{Cln} antibody did not detect any proteins in renal cytosols. All 38-kDa pI_{Cln} in the cytosol fractions from rat skeletal muscle was present in association with actin, and all the cytosolic actin was found in association with pI_{Cln} at a 1:7 stoichiometry of pI_{Cln} binding to actin, forming a complex with molecular mass of 340 kDa.

REFERENCES

1. Nilius, B., Eggermont, J., Voets, T., and Droogmans, G. (1996) Volume-activated Cl^- channels. *Gen. Pharmacol.* **27**, 1131-1140
2. Strange, K., Emma, F., and Jackson, P.S. (1996) Cellular and molecular physiology of volume-sensitive anion channels. *Am. J. Physiol.* **270** (*Cell Physiol.* **39**), C711-C730
3. Okada, Y. (1997) Volume expansion-sensing outward-rectifier Cl^- channel: fresh start to the molecular identity and volume sensor. *Am. J. Physiol.* **273** (*Cell Physiol.* **42**): C755-C789
4. Paulmichl, M., Li, Y., Wickman, K., Ackerman, M., Peralta, E., and Clapham, D. (1992) New mammalian chloride channel identified by expression cloning. *Nature* **356**, 238-241
5. Chen, L., Wang, L., and Jacob, T.J.C. (1999) Association of intrinsic pI_{Cln} with volume-activated Cl^- current and volume regulation in a native epithelial cell. *Am. J. Physiol.* **276** (1 Pt 1), C182-192
6. Tao, G.Z., Komatsuda, A., Miura, A.B., Kobayashi, A., Itoh, H., and Tashima, Y. (1998) pI_{Cln} predominantly localizes at luminal surface membrane of distal tubules and Henle's ascending limbs. *Biochem. Biophys. Res. Commun.* **247**, 668-673
7. Laich, A., Gschwentner, M., Krick, W., Nagl, U.O., Fürst, J., Hofer, S., Susanna, A., Schmarda, A., Deetjen, P., Burckhardt, G., and Paulmichl, M. (1997) I_{Cln}, a chloride channel cloned from kidney cells, is activated during regulatory volume decrease. *Kidney Int.* **51**, 477-478
8. Krapivinsky, G.B., Ackerman, M.J., Gordon, E.A., Krapivinsky, L.D., and Clapham, D.E. (1994) Molecular characterization of a swelling-induced chloride conductance regulatory protein, pI_{Cln}. *Cell* **76**, 439-448
9. Emma, F., Breton, S., Morrison, R., Wright, S., and Strange, K. (1998) Effect of cell swelling on membrane and cytoplasmic distribution of pI_{Cln}. *Am. J. Physiol.* **274**, C1545-C1551
10. Mursh, M.W., Davis-Amaral, E.M., Vandenburgh, H.H., and Goldstein, L. (1998) Hypotonicity stimulates translocation of I_{Cln} in neonatal rat cardiac myocytes. *Pflügers Arch.-Eur. J. Physiol.* **436**, 415-422
11. Ma, Y., Tao, G., Kobayashi, A., Itoh, H., Tashima, Y., Kojima, N., and Senoo, H. (1999) Translocation of pI_{Cln} in a pig kidney cell line, LLC-PK1, by low osmotic stress. *Clin. Exp. Nephrol.* **3**, 163-168
12. Ackerman, M.J., Wickman, K.D., and Clapham, D.E. (1994) Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.* **103**, 153-157
13. Voets, T., Buyse, G., Tytgat, J., Droogmans, G., Eggermont, J., and Nilius, B. (1996) The chloride current induced by expression of pI_{Cln} in *Xenopus* oocytes differs from the endogenous volume-sensitive chloride current. *J. Physiol.* **495**, 441-447
14. Strange, K. (1998) Molecular identity of the outwardly rectifying, swelling-activate channel: Time to reevaluate pI_{Cln}. *J. Gen. Physiol.* **111**, 617-622
15. Gschwentner, M., Nagl, U.O., Woll, E., Schmarda, A., Ritter, M., and Paulmichl, M. (1995) Antisense oligonucleotide suppresses cell-volume-induced activation of chloride channels. *Pflügers Arch.* **430**, 464-470
16. Schwartz, R.S., Rybicki, A.C., and Nagel, R.L. (1997) Molecular cloning and expression of a chloride channel-associated protein pI_{Cln} in human young red blood cells: association with actin. *Biochem. J.* **327**, 609-616
17. Mursh, M.W., Luer, C.A., Davis-Amaral, E.M., and Goldstein, L. (1997) Hypotonic stress induces translocation of the osmolyte channel protein pI_{Cln} in embryonic skate (*Raja eglanteria*) heart. *J. Exp. Zool.* **277**, 460-463
18. Emma, F., Sanchez-Olea, R., and Strange, K. (1998) pI_{Cln} may function as a channel in intracellular membranes. *Biochim. Biophys. Acta* **1404**, 321-328
19. Mill, J.W. and Lubin, M. (1986) Effect of adenosine 3′5′-cyclic monophosphate on volume and cytoskeleton of MDCK cells. *Am. J. Physiol.* **250**, C319-C324
20. Hallows, K.R., Paackman, C.H., and Knauf, P.A. (1991) Acute cell volume changes in anisotonic media affect F-actin content of HL-60 cells. *Am. J. Physiol.* **261**, C1154-C1161
21. Schwiebert, E.M., Mills, J.W., and Stanton, B.A. (1994) Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. *J. Biol. Chem.* **269**, 7081-7089
22. Tilly, B.C., Edixhoven, M.J., Tertoolen, L.G.J., Morii, N., Saitoh, Y., Narumiya, S., and Jonge, H.R. (1996) Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. *Mol. Biol. Cell* **7**, 1419-1427
23. Szucs, G., Buyse, G., Eggermont, J., Droogmans, G., and Nilius, B. (1996) Characterization of volume-activated chloride currents in endothelial cells from bovine pulmonary artery. *J. Membr. Biol.* **149**, 189-197
24. Abe, T., Takeuchi, K., Ishii, K., and Abe, K. (1993) Molecular cloning and expression of a rat cDNA encoding MDCK-type chloride channel. *Biochim. Biophys. Acta* **1173**, 353-356
25. Tisdale, H.D. (1967) Preparation and properties of succinic-cytochrome *c* reductase (complex II-III). *Methods Enzymol.* **10**, 213-215
26. Tashima, Y., Hasegawa, M., Lane, L.K., and Schwartz, A. (1981) Specific effects of spermine on Na^+-K^+-adenosine triphosphatase. *J. Biochem.* **89**, 249-255
27. Tashima, Y., Mizunuma, H., Shima, H., Kobayashi, R., Tanka, S., Maeda, N., and Kumegama, M. (1984) Induction of fructose 1,6-bisphosphatase and glucose 6-phosphatase by dibutyryl cyclic adenosine monophosphate in fetal mouse liver. *J. Biochem.* **96**, 805-813
28. Labarca, C. and Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 344-352
29. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurements with the Folin phenol reagent. *J.*

Biol. Chem. **193**, 265-275
30. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellular sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
32. Schägger, H., Cramer, W.A., and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* **217**, 220-230
33. Okada, H., Ishii, K., Nunnoki, K., and Taira, N. (1995) Cloning of a swelling-induced chloride current related protein from rabbit heart. *Biochim. Biophys. Acta* **1234**, 145-148
34. Buyse, G., De Greef, C., Raeyamaekers, L., Droogmans, G., Nilius, B., and Eggermont, J. (1996) The ubiquitously expressed pI_{Cln} protein forms homomeric complexes in vitro. *Biochem. Biophys. Res. Commun.* **218**, 822-827
35. Tao, G.-Z., Kobayashi, A., Itoh, H., and Tashima, Y. (1998) Expression of pI_{Cln} in *Escherichia coli* gives a strong tolerance to hypotonic stress. *FEBS Lett.* **434**, 28-32
36. Sanchez-Olea, R., Emma, F., Coghlan, M., and Strange, K. (1998) Characterization of pI_{Cln} phosphorylation state and a pI_{Cln}-associated protein kinase. *Biochim. Biophys. Acta* **1318**, 49-60
37. Levitan, I., Almonte, C., Mollard, P., and Garber, S.S. (1995) Modulation of a volume-regulated chloride current by F-actin. *J. Membr. Biol.* **147**, 283-294