Occludin phosphorylation: identification of an occludin kinase in brain and cell extracts as CK2

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Abstract In epithelial and endothelial cells, tight junctions limit paracellular flux of ions, proteins and other macromolecules. However, mechanisms regulating tight junction function are not clear. Occludin, a tight junction protein, undergoes phosphorylation changes in several situations but little is known about occludin kinases. A recombinant C-terminal fragment of occludin is a substrate for a kinase in crude extracts of brain. This activity was purified about 10 000-fold and identified as CK2 (casein kinase 2) by peptide mass fingerprinting, immunoblotting and mutation of CK2 sites within the occludin sequence. CK2 is therefore a candidate kinase for regulation of occludin phosphorylation in vivo.

Key words: Tight junction; Inflammation; Oedema; Signal transduction

1. Introduction

Endothelial and epithelial cells are coupled via intercellular junctions [1]. Tight junctions limit the flux of ions, proteins and other macromolecules via the paracellular pathway. They also polarize cells into apical and basolateral domains [2]. The protein composition and molecular architecture of tight junctions are being discovered. Peripheral cytoplasmic proteins, such as ZO-1 [3], interact with membrane-spanning proteins, such as occludin and claudins [2,4–6]. Mechanisms underlying the variability and regulation of the permeability properties of epithelial and endothelial cells remain to be established. These may play a role in tight junction breakdown occurring in inflammatory and hypoxic disorders.

In terms of regulation, phosphorylation of tight junction components, especially occludin, has been reported but the identity of the regulatory kinases and signalling pathways is still under investigation. In Madin–Darby canine kidney (MDCK) cells, calcium stimulates tight junction formation and, concomitantly, occludin becomes phosphorylated on serine and threonine residues [7,8]. In retinal endothelial cells, vascular endothelial growth factor stimulates occludin phosphorylation, perhaps playing a role in diabetic retinopathy [9]. In early development, occludin appears to undergo dephosphorylation and various pre-purified kinases, including casein kinase 2 (CK2) and cdc2, were proposed to phosphorylate occludin [10,11]. Protein kinase C (PKC) has also been suggested as an occludin kinase [12] or to regulate occludin phosphorylation in other ways [13]. Other studies link occludin phosphorylation with shear stress [14] and tight junction reassembly following cellular adenosine triphosphate (ATP) depletion [15]. Tyrosine kinases [16] and G-protein-coupled receptor-activated pathways involving rho-regulated signalling [17] have also been implicated in regulation of occludin phosphorylation.

Clearly, occludin phosphorylation is a real phenomenon and various kinases have been proposed as occludin kinases. Some approaches involved incubation of recombinant fragments of occludin with known kinases, and their physiological relevance must be questioned. We adopted the unbiased approach of incubating occludin fragments with tissue or cell extracts where kinase activity may be expected to be low and physiologically significant. Occludin phosphorylating activity was detected. This was purified and identified. The results are presented below.

2. Materials and methods

2.1. Materials

Suppliers of materials were: Amersham: [γ-^{33}P]ATP, Amplify, Rainbow markers, HiTrap columns, fast protein liquid chromatography (FPLC) systems, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody; Calbiochem: Brij-35, 1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA); Invitrogen: colloidal blue and silver quest staining kits; Pierce: bicinchoninic acid (BCA) protein assay reagents; BD Transduction Laboratories: CK2β and CK2α/α′ mouse monoclonal antibodies. Porcine brains were fresh from an abattoir, immediately snap frozen on dry ice for transport and then stored at −80°C. ECV304 cells were cultured as described [17].

2.2. Buffers

Buffer A: 20 mM HEPES/NaOH, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.03% (v/v) Brij-35, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 1 μg/ml aprotinin and 1 μg/ml leupeptin. Buffer B: Buffer A containing 1 M NaCl. Buffer C: Buffer A containing 150 mM NaCl. The final pH of the buffers was 8.0 at 4°C.

2.3. Glutathione-S-transferase (GST)-occludin and mutagenesis

GST was fused to residues 264–521 of mouse occludin [4], and this resulted in a linker sequence (LVPRGSPEF). Mutagenesis (QuickChange kit, Stratagene) created either amino acid substitutions or, to truncate protein, stop codons. Plasmids from XL1-blue supercompetent cultures were purified (Qiagen midiprep system) and mutagenesis was confirmed by DNA sequence analysis. BL21 cells were trans-

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formed and cultures were induced with 1 mM isopropyl thiogalactose (IPTG) for 2 h at 37°C. Cells were solubilized in phosphate-buffered saline (PBS) containing 1.5% sarcosyl [18], 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 0.1 U/ml α_2-macroglobulin. After sonication on ice, insoluble material was removed by centrifugation. The supernatant was passed through a glutathione Sepharose column. After washing with PBS, fusion proteins were eluted in 50 mM Tris/HCl containing 10 mM reduced glutathione (pH 8.0). Fractions were analyzed for protein and dialyzed before use.

2.4. Kinase purification

Porcine brain was fragmented in N_2 (l) using a pestle and mortar. A Waring blender was used to extract the tissue into ice-cold buffer (1 g tissue: 2.5 ml buffer) containing 20 mM HEPES/10 mM NaOH, 5 mM EDTA, 0.1 mM vanadate, 1% Triton X-100, 0.1 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After centrifugation for 1 h at 100 000 × *g*, the supernatant was filtered through 60 μm and 0.2 μm filters and used immediately. The extract was applied at 5 ml/min onto a 5 ml SP-Sepharose HP column equilibrated in Buffer A. After washing in Buffer A until A_{280} had reached background, kinase activity was eluted using a step gradient of 50% Buffer B, collecting 5 ml fractions. Active fractions were pooled, diluted 4-fold in Buffer A and applied to a 5 ml Q-Sepharose HP column equilibrated in Buffer A. The column was developed with a 100 ml linear gradient of NaCl to 1 M at a flow rate of 5 ml/min, collecting 5 ml fractions. Active fractions were pooled, diluted 4-fold in Buffer A and applied to a 1 ml Q-Sepharose HP column equilibrated in Buffer A. A step gradient to 1 M NaCl was applied at a flow rate of 1 ml/min and 0.5 ml fractions were collected. Active fractions were further concentrated to 50 μl in a Microcon 10 spin filter unit (Anachem) and loaded onto a Superdex-200 PC 3.2/30 column at 40 μl/min in Buffer C, collecting 150 μl fractions. Active fractions were pooled, diluted to 1 ml in Buffer A and chromatographed on a 1 ml heparin HP column equilibrated in 50% Buffer B. The column was developed with a 10 ml linear gradient to 1 M NaCl at a flow rate of 1 ml/min, collecting 1 ml fractions. Proteins in the fractions were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% gels) and visualized by silver staining or, after transfer to nitrocellulose, by immunoblotting (see [17,19]). For mass spectrometry, protein in the entire fraction was precipitated [20], resolved by SDS–PAGE and stained with colloidal blue.

2.5. Kinase assay

Aliquots (5 μl) of extracts or fractions were assayed in a volume of 50 μl in the absence or presence of 1 μg GST-occludin. Buffer contained 50 mM β-glycerophosphate, 25 mM HEPES, 12.5 mM NaOH, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM BAPTA, 0.1 mM ATP and 0.37 MBq [γ-^{33}P]ATP, pH was 7.4 at 37°C. Reactions were at 37°C for 20 min and quenched by addition of 12.5 μl boiling hot 10% SDS in 50% glycerol/0.01% bromophenol blue. Samples were resolved by SDS–PAGE [21], fixed, stained with Coomassie blue (R250), impregnated with Amplify, dried and exposed to film at −80°C. Quantitation was by phosphorimaging using [^{33}P]phosphoprotein standards. Phosphoamino acid analysis was according to Boyle et al. [22].

2.6. Peptide mass fingerprinting

In-gel digestion of proteins was performed by established methods [23]. Briefly, gel slices containing bands were cut into 1 mm^3 pieces, washed with 25 mM NH_4HCO_3 in 50% acetonitrile, reduced with dithiothreitol (10 mM, 56°C, 30 min) and S-alkylated with 55 mM iodoacetamide (45 min in the dark). The gel pieces were washed twice with 50 mM NH_4HCO_3 in 50% acetonitrile and, to dehydrate, once in 100% acetonitrile. Sequencing grade modified trypsin (Promega, 20 μg) was reconstituted in 200 μl of 1 mM HCl then diluted 8-fold in 50 mM NH_4HCO_3. Gel pieces were rehydrated in trypsin solution for 45 min on ice. Excess solution was removed and replaced with buffer. Samples were then incubated overnight at 37°C and then extracted two or three times in 5% trifluoroacetic acid/50% acetonitrile and once in 100% acetonitrile. Supernatants were pooled, dried in a Speed Vac and then resuspended in 10–30 μl of 0.1% trifluoroacetic acid (TFA) containing 1–10% acetonitrile.

The masses of tryptic peptides were determined by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) on a Bruker REFLEX instrument. A small portion of the digest (~0.5 μl) was spotted onto a thin layer of α-cyano-4-hydroxycinnamic acid (twice-recrystallized; Sigma)/nitrocellulose previously deposited from solutions in acetone (three parts 20 mg/ml matrix: one part 10 mg/ml nitrocellulose; 0.5 μl applied). Spectra were obtained by summing at least 150 individual laser shots. The masses obtained were then entered into the MASCOT search engine (www.matrixscience.com, [24]; parameters: error ±0.35 Da; one missed cleavage allowed; mammalian database searched).

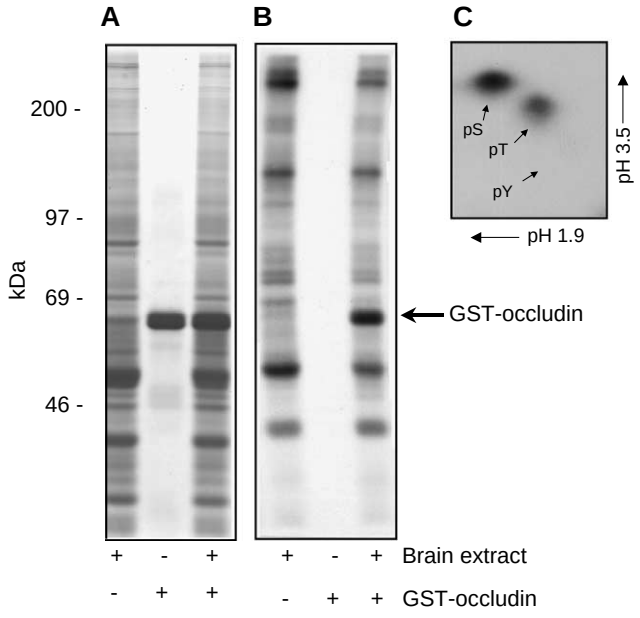

Fig. 1. An occludin kinase activity is detectable in brain extracts. A: Brain extracts and purified GST-occludin$^{264-521}$ were incubated either alone or together in the presence of kinase assay buffer. Proteins were analyzed by SDS–PAGE and stained with Coomassie blue. The migration of GST-occludin is indicated. B: The corresponding autoradiogram of A. C: Phosphoamino acid analysis of phosphorylated GST-occludin.

3. Results and discussion

3.1. An occludin phosphorylating activity is detected in brain extracts

Occludin spans the plasma membrane four times [4,5]. This

Table 1
Purification of an occlusion kinase from porcine brain

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
1. Extract	23	395	33.4	0.084	–	–
2. SP-Sepharose	25	39.3	241	6.132	1	100
3. Q-Sepharose	10	5.4	464	85.9	14	193
4. Superdex-200	0.45	0.78	66.6	85.3	13	27.6
5. Heparin-Sepharose	3	0.001	54.0	54 000	8806	22.4

The amount of brain used in this preparation was 20 g. A unit is defined as pmol of phosphorylated GST-occludin produced in a 20 min reaction. Protein for Steps 1–3 was determined by the BCA method. A_{280} was used to estimate the protein in Steps 4 and 5.

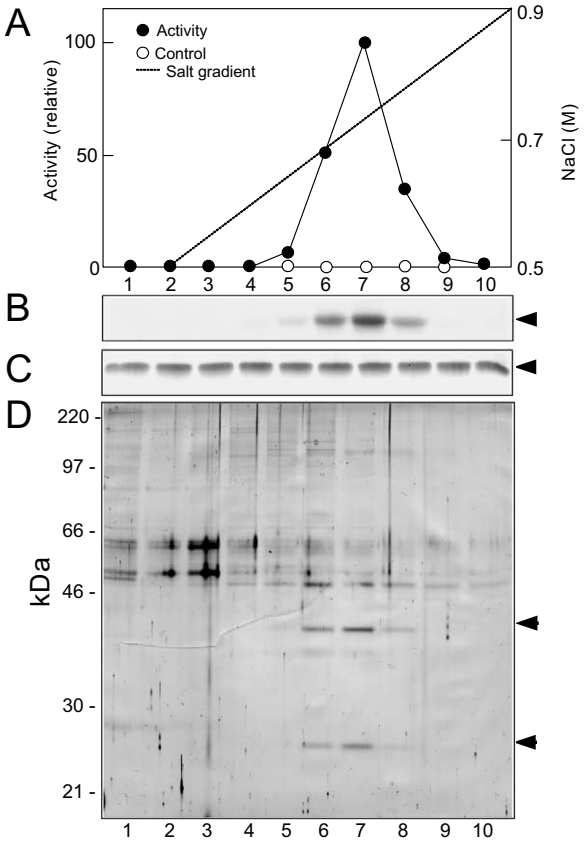

Fig. 2. Fractionation of kinase activity using heparin-Sepharose. Activity phosphorylating occludin was purified up to Step 4 as described in Table 1. Protein was then fractionated using heparin-Sepharose, employing a 0.5–1.0 M salt gradient. The gradient is based on conductivity measurements. A: Elution profile of GST-occludin phosphorylating activity (filled circles) with salt gradient. In the absence of GST-occludin (open circles), phosphorylating activity was not detected. B: Phosphorylation of GST-occludin. C: Coomassie stain of GST-occludin. D: Fractions were analyzed by SDS–PAGE and silver staining. Arrows indicate bands of 41 and 26 kDa.

results in three regions of the protein having access to the cytoplasm, and which therefore could be accessible to kinases for phosphorylation. The N-terminal region (murine sequence) is composed of about 66 residues. Transmembrane regions two and three are linked by residues 159–168. The C-terminal region extends from residues 264–521. GST fused to N- or C-terminal regions was prepared and a peptide corresponding to the linker region was synthesized. These were then tested as potential kinase substrates in extracts of brain tissue. Reactions were set up and analyzed. The staining of proteins from brain extracts shows many stained bands, as expected (Fig. 1A). GST-occludin$^{264-521}$ migrates as an obvious 64 kDa protein, even when incubated with brain extracts (Fig. 1A). The autoradiogram (Fig. 1B) shows many phosphoproteins produced by incubation of brain extracts with ATP. GST-occludin is phosphorylated in the presence but not absence of brain extract. Therefore, kinase activity is present in brain extract that is capable of phosphorylating residues within GST-occludin$^{264-521}$. Control experiments (see later) show that this phosphorylation is within the occludin residues. Other experiments (results not shown) using either GST-occludin$^{1-66}$ or peptide containing residues 159–168 as substrates did not show phosphorylation in incubations with brain extract. Phosphoamino acid analysis (Fig. 1C) of phosphorylated GST-occludin$^{264-521}$ revealed mainly phosphoserine and some phosphothreonine. Phosphotyrosine was not detectable.

3.2. Purification of the occludin phosphorylating activity

The occludin phosphorylating activity was purified (Table 1). Extract was applied to a cation exchange column and eluted using a step gradient. About 90% of bulk protein was lost at this point. Fractions containing activity were diluted and applied to an anion exchange column. Occludin phosphorylating activity eluted at about 0.4 M NaCl. This fraction was concentrated and applied to a gel filtration column (Superdex-200) and activity eluted between ferritin (440 kDa) and aldolase (158 kDa) standards. The heparin step gave significant purification. Most protein in the sample at this stage was eluted with <0.5 M NaCl. However, the occludin phosphorylating activity had high affinity for heparin and eluted at about 0.7 M NaCl. At this point, protein was just detectable by ultraviolet (UV) absorbance. Quantitation showed purification of about 10 000-fold with a yield of approximately 20%.

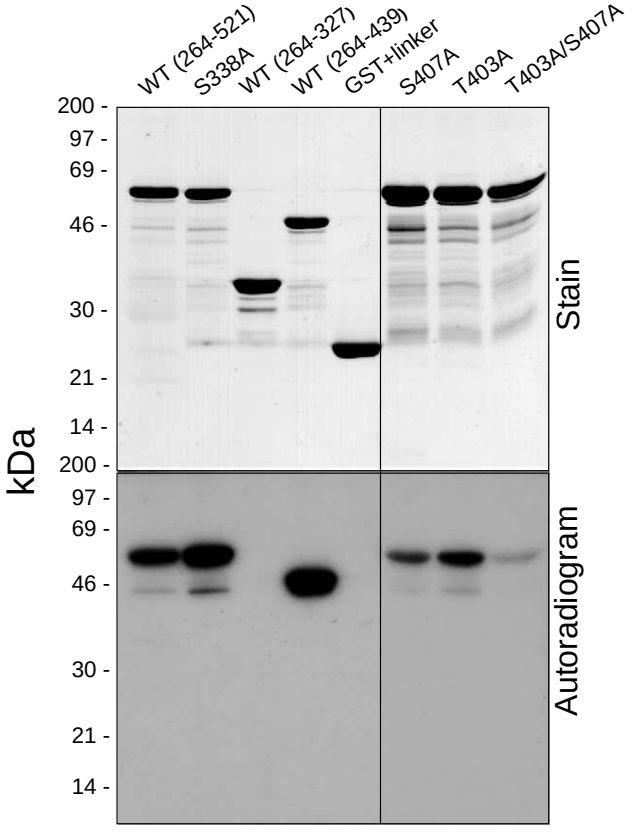

Fig. 3. Phosphorylation site analysis. Truncated and mutant forms of GST-occludin were analyzed for their ability to be phosphorylated. The kinase was purified as described in Table 1. Upper panels show Coomassie staining of substrates; lower panels, the corresponding autoradiograms. GST was fused to parts of occludin as follows: WT (264–521), residues 264–521; S338A, as WT (264–521) but serine 338 mutated to alanine; WT (264–327), residues 264–327; WT (264–439), residues 264–439; GST+linker, GST fused to linker sequence found in all GST-occludin variants; S407A, as WT (264–521) but serine 407 mutated to alanine; T403A, as WT (264–521) but threonine 403 mutated to alanine; T403A/S407A, a double mutant of WT (264–521).

Quantitation (Fig. 2A) of phosphorylating activity (Fig. 2B) eluting from the heparin column using equal amounts of GST-occludin (Fig. 2C) shows a peak in fraction 7. Fractions were analyzed by SDS–PAGE and silver staining to reveal two bands of 41 and 26 kDa that also peaked in fraction 7 (Fig. 2D). As the 41 and 26 kDa bands were the only ones co-eluting with the occludin kinase activity at a stage when purification was substantial, they were analyzed further.

3.3. Identification of the occludin phosphorylating activity

Protein in the entire fractions was quantitatively precipitated and resolved by SDS–PAGE and stained with colloidal Coomassie to reveal the same 41 and 26 kDa bands. In addition, a band of 44 kDa was observed (result not shown) that also co-eluted with activity. The three proteins were subjected to tryptic peptide mass mapping. In all cases, good sequence coverage was obtained and MOWSE scores were significant (Table 2). The data identify the three proteins as subunits of CK2. CK2 is highly conserved across species; α/α′ subunits are catalytically active and the β subunits serve a regulatory role [25].

3.4. Occludin mutagenesis and CK2 sites

Analysis of GST-occludin phosphorylation was performed (Fig. 3). Purified kinase (Table 1) was incubated with a variety of forms of the protein. Mutation of serine 338, a site for phosphorylation by PKC [12], to alanine (S338A) did not have any effect on phosphorylation. Removal of the last 194 residues (wild-type (WT) (264–327)) or the entire occludin sequence (GST+linker) prevented phosphorylation, whereas removal of the last 82 residues (WT (264–439)) had no effect. Therefore, phosphorylatable residues lie between amino acids 328 and 439. The minimum consensus sequence for CK2 is [S/T-X-X-D/E], and further negatively charged residues at positions −1 to +7 enhance CK2 phosphorylation [11]. Residues serine 407 (−1 to +7: ESCEELEED) and threonine 403 (−1 to +7: TTGGESCEE), as suggested previously [11], are good candidates for CK2 sites within the region of interest within occludin. Mutagenesis of serine 407 to alanine resulted in reduced ability of the kinase to phosphorylate occludin. The threonine 403 to alanine mutant had a smaller effect but the double mutant (T403/S407A) was even less phosphorylated than either of the single mutants. These data are consistent with the claim that CK2 is the kinase in brain extracts responsible for phosphorylation of occludin.

3.5. Co-elution of CK2 with occludin phosphorylating activity in extracts of ECV304 cells

Brain tissue is a convenient starting point for kinase purification. However, the observations on occludin phosphorylation that were of interest to us were made using ECV304 cells [17]. The possibility that CK2 was responsible for occludin phosphorylation in extracts of these cells was investigated. As CK2 has a distinctive chromatographic profile on heparin columns, fractionation of ECV304 cell extracts was performed and showed activity eluting at about 0.7 M NaCl (Fig. 4A, B), as with extracts of brain. Equal use and loading of GST-occludin was verified (Fig. 4C). Immunoblot analysis of the fractions showed co-elution of activity with the β (27 kDa, Fig. 4D) and α/α′ subunits of CK2 (∼44 kDa, Fig. 4E).

Table 2
Peptide mass fingerprint identification of protein bands as CK2 subunits

Protein (i.d.)	Observed MH^+	Expected MH^+	Delta
26 kDa (CK2β)	2007.71	2007.82	−0.11
	1687.63	1687.85	−0.22
	1390.54	1390.58	−0.04
	1084.69	1084.59	0.10
41 kDa (CK2α)	1864.87	1864.85	0.02
	1798.93	1798.85	0.08
	1771.99	1771.88	0.11
	1524.92	1524.80	0.12
	1449.93	1449.79	0.14
	1331.78	1331.61	0.17
	1106.89	1106.55	0.34
	1050.83	1050.55	0.28
44 kDa (CK2α′)	1873.58	1873.85	−0.27
	1770.66	1770.85	−0.19
	1742.63	1742.83	−0.20
	1731.71	1731.86	−0.15
	1726.59	1726.84	−0.25
	1622.60	1622.68	−0.08
	1613.62	1613.76	−0.14
	1606.58	1606.69	−0.11
	1590.54	1590.69	−0.15
	1565.66	1565.66	0.00
	1549.66	1549.66	0.00
	1528.68	1528.76	−0.08
	1357.68	1357.69	−0.01
	1310.81	1310.76	0.05
	1122.70	1122.79	−0.09
	1106.71	1106.55	0.16
	1019.68	1019.51	0.17

The 26 kDa protein gave seven peaks of which four matched those expected of the CK2β subunit, giving 23% sequence coverage and a MOWSE score of 80. The 41 kDa protein gave eight matched peaks out of 10 accounting for 27% coverage of protein identified as CK2α subunit and a MOWSE score of 105. The 44 kDa protein gave 28 mass peaks of which 17 matched sequences in the CK2α′ subunit accounting for 38% coverage and giving a MOWSE score of 124.

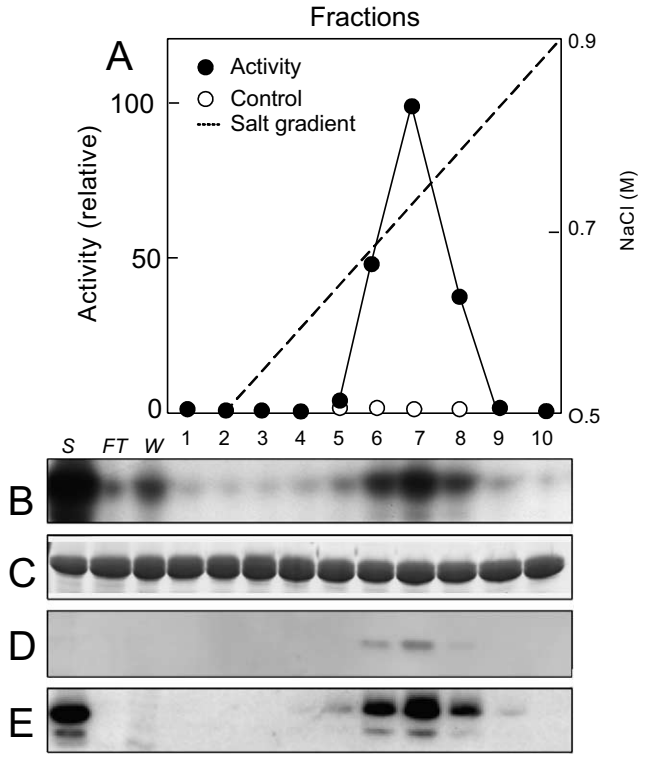

Fig. 4. Occludin phosphorylating activity in extracts of ECV304 cells. Extracts were loaded onto a heparin column. After washing the column in Buffer A containing 0.5 M NaCl, proteins were eluted using a gradient of 0.5–1.0 M NaCl. The gradient is based on conductivity measurements. A: Quantitation of GST-occludin phosphorylating activity (closed circles). Activity was not detected in the absence of GST-occludin (open circles). B: Autoradiogram showing GST-occludin phosphorylation. S, starting material; FT, flow-through; W, 0.5 M NaCl wash; fractions 1–10. C: Coomassie staining of GST-occludin. Immunoblots of fractions using antibody recognizing the β (D) and α subunits (E) of CK2.

These data strongly support the notion that CK2 is also an occludin kinase in ECV304 cells.

3.6. Conclusions

Much evidence indicates that occludin is a substrate for kinase(s) in a variety of model systems (see Section 1). Some studies describe occludin phosphorylation changes taking place in different situations. Others attempted to identify occludin kinases. One approach involves incubation of recombinant fragments of occludin with pre-purified and known kinases. Our approach was unbiased and was to examine if any occludin phosphorylating activity existed in extracts of tissue. In these experiments, the concentration of kinase would be expected to be much lower raising the possibility of physiological relevance. Activity was detected and its purification and characterization revealed it to be CK2. Pre-purified CK2 was also reported to phosphorylate *Xenopus laevis* occludin in vitro [10,11]. The in vivo relevance of CK2 as an occludin kinase should be now examined. Also, observations implicating other serine/threonine kinases, such as PKC [12], and the tyrosine kinase c-Yes [16] as occludin kinases need to be considered when examining the physiological and pathological relevance of occludin phosphorylation.

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