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# A corticosteroid-induced gene expressing an "IsK-like" $K^+$ channel activity in *Xenopus* oocytes

**(ion channels/phospholemman/$Na^+$,$K^+$-ATPase/epithelial transport/aldosterone)**

BERNARD ATTALI*, HEDVA LATTER†, NURIT RACHAMIM†, AND HAIM GARTY†

Departments of †Membrane Research and Biophysics and *Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

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**ABSTRACT** **Screening a rat colon cDNA library for aldosterone-induced genes resulted in the molecular cloning of a cDNA whose corresponding mRNA is strongly induced in the colon by dexamethasone, aldosterone, and a low NaCl diet. A similar mRNA was detected in kidney papilla but not in brain, heart, or skeletal muscle. *Xenopus laevis* oocytes injected with cRNA synthesized from this clone, designated CHIF (channel-inducing factor), express a $K^+$-specific channel activity. The biophysical, pharmacological, and regulatory characteristics of this channel are very similar to those reported before for IsK (minK). These include: slow ($\tau$ > 20 s) activation by membrane depolarization with a threshold potential above −50 mV, blockade by clofilium, inhibition by phorbol ester, and activation by 8-bromoadenosine 3′,5′-cyclic monophosphate and high cytoplasmic $Ca^{2+}$. The primary structure of this clone, however, shows no homology to IsK. Instead, CHIF exhibits >50% similarity to two other short bitopic membrane proteins, phospholemman and the $\gamma$ subunit of $Na^+$,$K^+$-ATPase. The data are consistent with the possibility that CHIF is a member of a family of transmembrane regulators capable of activating endogenous oocyte transport proteins.**

Aldosterone is the major steroid regulating electrolyte transport in the renal and intestinal tracks, acting by altering gene expression (1–4). In particular, the hormone enhances vectorial $Na^+$ reabsorption and $K^+$ secretion across tight epithelia in the distal segments of the nephron and colon. These processes take place via apical $Na^+$ and $K^+$ channels and basolateral $Na^+/K^+$ pumps. Stimulation of ion transport can be induced *in vivo* by perfusing animals with various corticosteroids or by a low salt diet, which enhances adrenal secretion of aldosterone (5, 6). The mechanisms underlying these responses are poorly understood. Accumulating data indicate that at least part of the increase in $Na^+$ transport is mediated by the induction of a factor(s) that activates preexisting apical $Na^+$ channels (7, 8). More recently, it has been reported that a chronic hyperaldosteronism induces one $Na^+$ channel subunit, while the other two subunits are constitutively expressed (9). The hormonal effect on epithelial $K^+$ channels is even less understood and it is unclear whether aldosterone induces $K^+$ channels, elevates their open probability, or simply acts to increase the membrane potential that drives $K^+$ secretion (10, 11).

The current study was undertaken to identify aldosterone-induced genes that might be involved in the regulation of ion transport. One cDNA cloned in this way showed surprising structural or functional similarities to two bitopic channel-inducing proteins, IsK and phospholemman. IsK (minK) is a 130-amino acid protein that transverses the membrane once (12). Injecting oocytes with IsK cRNA evokes a unique, slowly activating depolarization-induced $K^+$-specific current. The current amplitude can be increased by activating protein kinase A and by elevating intracellular $Ca^{2+}$ (13, 14). It has a species-specific response to protein kinase C and is inhibited by the antiarrhythmic compound clofilium (15). Phospholemman is a 72-amino acid transmembrane polypeptide, isolated as a major sarcolemmal phosphoprotein (16). Oocytes injected with phospholemman cRNA express a $Cl^-$-specific current slowly activated by membrane hyperpolarization (17). It has been suggested that IsK and phospholemman do not form ion-conducting pores but are regulatory proteins capable of activating endogenous oocyte $K^+$ and $Cl^-$ channels (18). The data reported here are consistent with this view and suggest the existence of a family of short transmembrane polypeptides involved in mediating ion transport.‡

## METHODS

**Animal Treatment and Nucleic Acid Isolation.** Male 8- to 10-week-old Wistar rats were used. Enhancement of epithelial ion transport was induced by one of two ways: (*i*) subcutaneous injections of dexamethasone§ (0.6 mg/100 g of body weight, suspended in corn oil) or aldosterone (15 $\mu$g/100 g of body weight, dissolved in phosphate-buffered saline) at ≈66, 42, and 18 hr before the experiment and (*ii*) feeding rats a sodium-free diet (no. 902902; ICN) for 10–14 days. Plasma levels of aldosterone were determined in rats fed a normal diet and low NaCl diet using a radioimmunoassay kit (Coat-A-Count aldosterone DPC, Los Angeles). Animals were killed by cervical dislocation, various organs were excised, and total RNA was isolated by the procedure of Chomczynski and Sacchi (21). Poly(A)$^+$ RNA was selected by two passes through an oligo(dT)-cellulose column.

**cDNA Library Screening and Sequence Analysis.** An oligo(dT)-primed cDNA library was constructed in λZAP II vector, using 10 $\mu$g of dexamethasone-stimulated colonic poly(A)$^+$ RNA. The library was screened for differentially expressed transcripts as described (22). In brief, total [$^{32}$P]cDNA probes were reverse transcribed from matched preparations of poly(A)$^+$ RNA extracted from dexamethasone-stimulated and untreated rats. Fifty thousand phages were plated at a density of 5000 plaque-forming units per 132-mm plate and blotted onto duplicate nitrocellulose filters. The two sets of filters were hybridized with the matched probes and plaques that hybridized only with dexamethasone-induced cDNA were cored out and rescreened. The pBluescript plasmids were excised from λZAP phages using ExAssist helper phage (Stratagene) and differential expression was confirmed by Northern hybridization of the insert DNA with

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Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L41254).

§This synthetic glucocorticoid was favored because of the possibility that part of the response to aldosterone requires occupancy of the glucocorticoid receptor (19, 20).

RNA from induced and noninduced rats. Three clones corresponding to the same ≈1380-bp cDNA, designated CHIF, were isolated in this way. Both strands of the insert cDNA were sequenced using an automatic sequencer and Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Data base searches and sequence analysis were done using the GCG sequence analysis software package (Genetic Computer Group, Madison, WI).

**Expression in *Xenopus laevis* Oocytes.** For expression, CHIF cDNA was digested with *Bgl* II, and the ≈800-bp fragment (containing the coding region and ≈450-bp 5′ untranslated sequence) was purified. It was subcloned into the *Bgl* II site of a pSP64T-derived vector containing 3′ and 5′ untranslated regions of *Xenopus* β-globin (23). Proper insertion and orientation were confirmed by sequencing. Plasmid DNA was linearized by digestion with *Sma* I. Sense cRNA was synthesized from the T3 promoter and capped, using an RNA transcription kit (Stratagene). Oocytes were treated and injected, and channel activity was recorded as described (24, 25).

## RESULTS AND DISCUSSION

Screening a rat colon cDNA library for steroid-induced transcripts that might be involved in the regulation of ion transport resulted in the molecular cloning of a polyadenylylated 1383-nucleotide cDNA. Colonic mRNA corresponding to this clone was strongly enhanced by dexamethasone (Fig. 1*A*), aldosterone (data not shown), or a low NaCl diet (Fig. 1*B*). In all cases, a similar 1.1- to 1.4-kb transcript was detected. In addition, cDNA probes synthesized from the most 5′ 350 nucleotides and the most 3′ 590 nucleotides hybridized to the same mRNA species. For reasons that are discussed below, we have termed this steroid-induced mRNA CHIF (channel-inducing factor). Induction of CHIF by salt deprivation was accompanied by an increase in plasma aldosterone from 1.0 ± 0.1 to 12.8 ± 1.4 nM (mean ± SE, $n = 3$). Thus, the observed induction (or decreased degradation) of colonic CHIF is likely to be mediated by occupancy of the mineralocorticoid receptor ($K_d$ = 4.1 nM) and not the glucocorticoid receptor ($K_d$ = 25–60 nM) (26, 27). Resalinating the $Na^+$-deprived rats for 24 hr lowered plasma aldosterone from 12.8 ± 1.4 to 1.1 ± 0.3 nM ($n = 3$) and fully reversed the steady-state increase in CHIF message (Fig. 1*B*). Northern hybridization with total RNA from various organs of dexamethasone-stimulated and untreated rats indicates that in addition to the colon, CHIF's mRNA is abundant in kidney papilla, but not in brain, heart, and skeletal muscle (Fig. 1 *C* and *D*).

The longest open reading frame in CHIF's nucleotide sequence predicts an 87-amino acid protein (upper sequence in Fig. 2*A*). A computer search of GenBank and EMBL data bases revealed significant homology to two other short polypeptides. One is phospholemman, a major sarcolemmal phosphoprotein that evokes $Cl^-$ channel activity in oocytes (16, 17). The other is the γ subunit of $Na^+$,$K^+$-ATPase, a 58-amino acid polypeptide associated with the pump, whose function is yet unknown (29). In both cases, ≈34% identity and ≈57% similarity were observed. Statistical significance of these homologies was estimated by aligning phospholemman and the γ subunit with 10 randomized CHIF sequences and found to be very high ($P < 0.001$). On the other hand, no significant homology between CHIF and IsK was noted.

The topological similarity between CHIF and phospholemman is even higher, and hydropathy plots of the two proteins practically overlap (Fig. 2*B*). Based on data obtained for phospholemman (16), we propose that the first ≈20 hydrophobic amino acids in CHIF are a cleavable signal sequence. The mature protein will therefore have an extracellular $NH_2$ terminus of ≈18 amino acids, a hydrophobic transmembrane domain of ≈20 residues, and an intracellular COOH terminus of 27 residues. Similarities between CHIF and the other two proteins are particularly noticeable in a stretch of 38 amino acids around the transmembrane domain (50% identity and 74% similarity). On the other hand, the COOH termini are quite different and CHIF lacks the phosphorylation sites typical to phospholemman.

Injecting CHIF cRNA into *Xenopus* oocytes was found to induce a slowly activating current, not seen in water-injected oocytes (Fig. 3*A*). This current is strongly voltage-dependent and seen only above a potential of −50 mV (Fig. 3*B*). It was identified as a $K^+$-specific current according to two criteria: (*i*) changing external $K^+$ from 2 mM to 20 mM shifted the reversal potential of the tail current from −101 ± 4 mV to −41 ± 3 mV ($n = 4$), a value expected according to the Nernst equation, for a $K^+$-selective conductance; and (*ii*) substituting external $Na^+$ and $Cl^-$ by *N*-methyl-D-glucamine and aspartate, respectively, had no effect on the reversal potential (data not shown). The depolarization-induced outward $K^+$ current develops very slowly and fails to reach steady value even after several

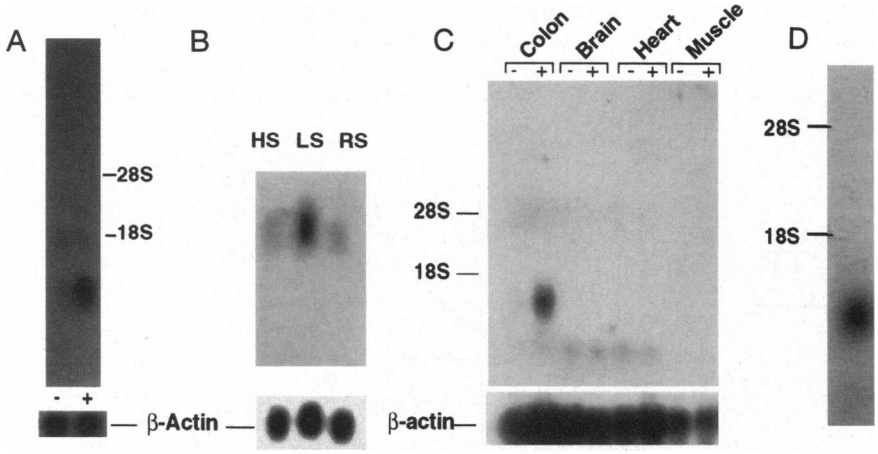


FIG. 1. Induction of CHIF by dexamethasone and $Na^+$ deprivation. (*A*) Colonic RNA was extracted from matched groups of rats that were injected with dexamethasone (+) or diluent (−). Aliquots of ≈2 μg of poly(A)$^+$ were resolved on agarose gel, transferred to nylon membrane, and hybridized sequentially with [$^{32}$P]cDNA probes corresponding to CHIF and β-actin. (*B*) Three groups of three rats each were treated for 12 days as follows: (*i*) fed a normal rat chow (HS), (*ii*) fed a $Na^+$-free ration (LS), and (*iii*) fed a $Na^+$-free ration and then resalinated for 24 hr (RS). Resalination was done by feeding the animals a normal rat chow and adding 110 mM NaCl to their drinking water. RNA was extracted and hybridized as above. Plasma levels of aldosterone were determined in blood samples taken from each rat, immediately after sacrifice. (*C*) Northern hybridization of CHIF and β-actin with aliquots of ≈10 μg of total RNA extracted from various organs of dexamethasone-injected (+) and untreated (−) rats. (*D*) Northern hybridization of CHIF with ≈10 μg of total RNA extracted from kidney papilla of dexamethasone-injected rats.

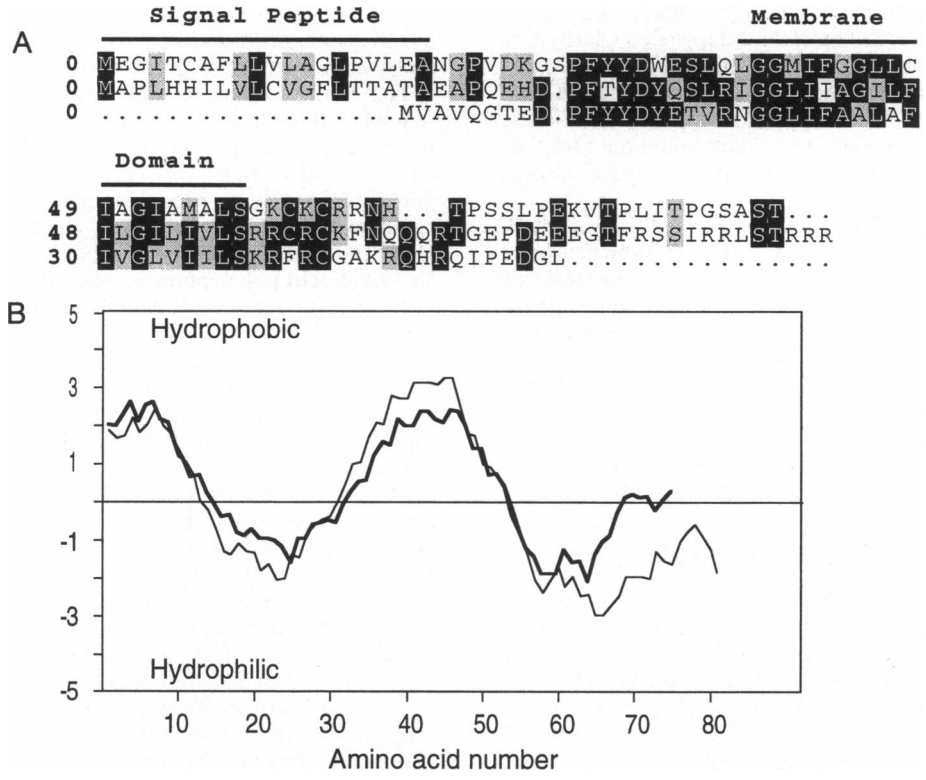


FIG. 2. Sequence and hydropathy analysis of CHIF. (*A*) Sequence alignment of CHIF (top line), canine phospholemman (middle line, GenBank accession no. M63934), and cattle $\gamma$ subunit of $Na^+,K^+$-ATPase (bottom line, GenBank accession no. X70059). The black and shaded boxes represent identical and similar amino acids, respectively. The horizontal bars indicate positions of the signal sequence and putative transmembrane domains. (*B*) Hydropathy plot analyses of CHIF (thick line) and phospholemman (thin line). Data were calculated according to Kyte and Doolittle (28), using a window of 12 amino acids.

hundreds of seconds. At −20 mV, the activation kinetics can be fitted to a single exponential with a time constant of ≈35 s. At +20 mV, the activation is best described as the sum of two exponentials with $\tau_1$ = 3.5 s and $\tau_2$ = 26.4 s ($r$ = 0.99). The relative amplitude of the fast component is 25%. The above kinetics and current–voltage relationships are remarkably similar to those previously reported for IsK (12). Like IsK (18, 30), the amplitude of CHIF-induced current saturates at <10 ng of cRNA (Fig. 3*C*). This concentration is well below the amount needed to saturate the oocyte's translational machinery and indicates limitation of channel activity by other factors—e.g., endogenous oocyte proteins. We have also noted considerable variations in the level of the CHIF-induced current expressed by different batches of oocytes, and two of seven batches expressed very little or practically no $K^+$ current. In spite of its homology to phospholemman, CHIF failed to induce a slowly activating $Cl^-$ current even at 100 ng of injected cRNA and at a test potential of −130 mV. Oocytes from the same frog injected with IsK or phospholemman cRNA exhibited at −130 mV a slowly activating $Cl^-$ current similar to the one reported in refs. 17 and 18.

The CHIF-induced current is sensitive to several blockers and second messengers. It is inhibited by the class III antiarrhythmic drug clofilium (≈60% inhibition at 100 $\mu$M, Fig. 4*A*), tetraethylammonium (50% at 30 mM), and $Ba^{2+}$ (80% at 5 mM). On the other hand, the following channel inhibitors were

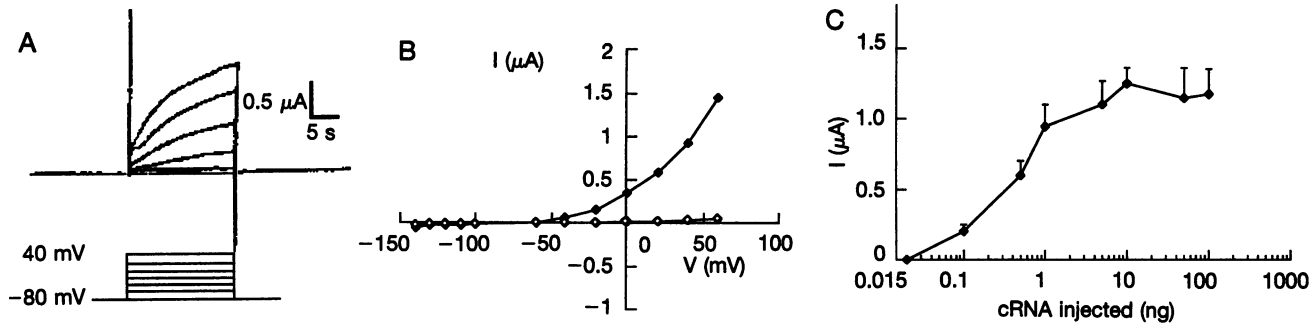


FIG. 3. Functional expression of CHIF in *Xenopus* oocytes. (*A*) Superimposed current traces elicited by 20-s depolarization steps from a holding potential of −80 mV to +40 mV, in 20-mV increments. Representative traces in an oocyte injected with 10 ng of cRNA are depicted. Similar traces were recorded in >30 oocytes from five different frogs, injected with three different cRNA preparations. (*B*) Current–voltage relationships in cRNA-injected (filled diamonds) and water-injected (open diamonds) oocytes. The current amplitudes were measured during 20-s depolarizing pulses from −80 mV to +60 mV. Leakage currents were subtracted. (*C*) cRNA dose–response relationships. Oocytes were injected with the indicated amounts of CHIF cRNA and currents were recorded 3 days later. A test potential of +30 mV for 20 s was used. Each point represents the mean ± SE of the currents recorded in 12–16 oocytes from three different frogs.

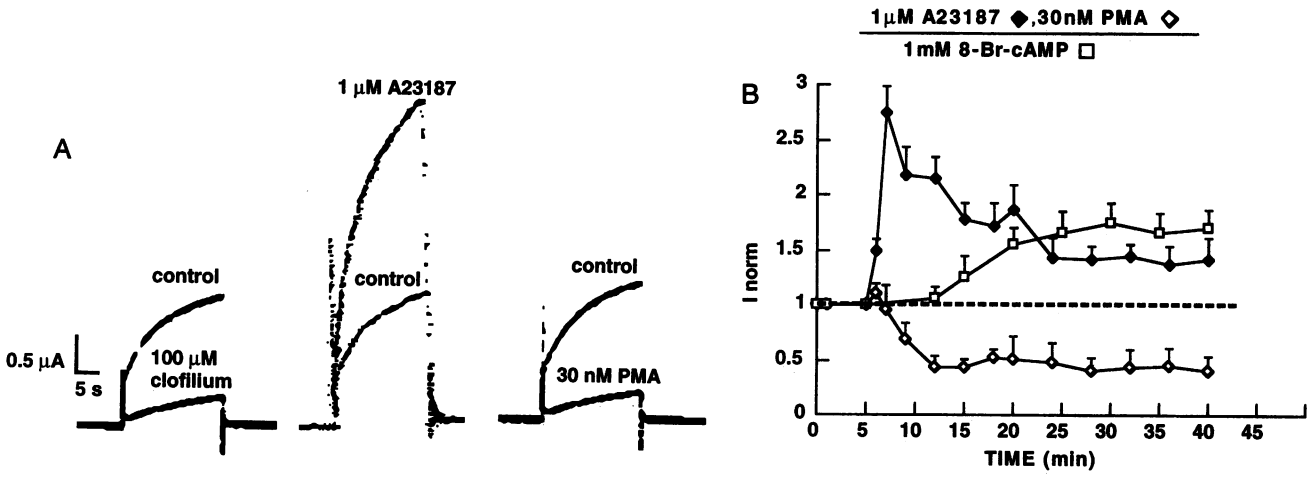


FIG. 4. Pharmacological and regulatory properties of the CHIF-induced current. (*A*) Current traces were recorded before and after superfusing oocytes with clofilium (100 μM), A23187 (1 μM) and niflumic acid (100 μM), and phorbol 12-myristate 13-acetate (PMA) (30 nM). Oocytes were injected with 10 ng of CHIF cRNA and currents were recorded during a 20-s test potential from −80 mV to +30 mV, applied 5–15 min after initiating superfusion with the above reagents. (*B*) Time course of the change in current amplitude induced by A23187, PMA, and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP). Superfusion was initiated at $t = 5$ min (upper horizontal bar) and currents were recorded, as above, in 3- to 6-min time intervals. Current amplitudes were normalized to the values measured at $t = 0$. Each data point represents a mean ± SE of the amplitudes measured in 12–16 oocytes from four different frogs. Currents measured 2 min after applying A23187, 15 min after adding 8-Br-cAMP, and 4 min after superfusing PMA were significantly different from the control values ($P < 0.01$).

without effect: 4-aminopyridine (4 mM), quinine (0.5 mM), charybdotoxin (50 nM), amiodarone (300 μM), amiloride (50 μM), niflumic acid (100 μM), SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; 300 μM), and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; 1 mM). Elevating internal $Ca^{2+}$ by superfusing oocytes with 1 μM A23187 in the presence of 300 μM niflumic acid plus 300 μM DIDS (to block $Ca^{2+}$-activated $Cl^-$ current) increases the current 2- to 3-fold (Fig. 4*A*). This effect is transient; it peaks at ≈2 min and declines over the next 20-min period to a new steady value ≈50% above the control level (Fig. 4*B*). 8-Br-cAMP at 1 mM increases the current amplitude by ≈50% over a period of 30 min, while 30 nM PMA partially inhibits it (Fig. 4). Interestingly, CHIF's deduced amino acid sequence has no consensus sites for phosphorylation by protein kinase A and only one site for a protein kinase C-dependent phosphorylation (serine 58 located at the membrane cytoplasm interface). Again, the responses to clofilium, A23187, and kinases activators are very similar to the behavior reported earlier for IsK (13, 25, 31, 32).

The oocyte data summarized in Figs. 3 and 4 indicate that CHIF cRNA transcribes either a $K^+$-specific pore or a transmembrane regulator capable of activating endogenous oocyte $K^+$ channels. In the first case, the data would suggest the existence of an epithelial channel involved in the regulation of $K^+$ transport by aldosterone. Such interpretation makes the striking functional (but not sequence) similarity between CHIF and IsK very hard to explain. In principle, it is possible that two entirely different proteins will form channels with identical biophysical and regulatory properties. However, CHIF and IsK are very short polypeptides and, in both cases, ion conduction and voltage gating involve <70 amino acids (33). It is hard to imagine that channels formed by monomeric or oligomeric forms of such short polypeptides will exhibit functional identity with no significant sequence similarity. Therefore, we believe that the current data are more consistent with the possibility that CHIF is a regulatory protein that activates endogenous oocyte $K^+$ channels. The functional similarity between CHIF and IsK can thus be explained by assuming that IsK is another transmembrane regulator of the same oocyte channel.

The unique membrane topology of IsK and phospholemman and the fact that their channel activity is usually not seen in native cells have led several investigators to propose that these bitopic proteins are regulators of oocyte $K^+$ and $Cl^-$ channels and not membrane pores. Findings that argue against this view are the abilities to alter gating kinetics and ionic selectivity by point mutations (17, 33, 34). These findings indicate that the two proteins closely interact with the ion-conducting pore and are part of the channel complex. Evidence in favor of the regulatory hypothesis have been reported by Attali *et al.* (18). These authors have demonstrated that at a sufficiently high concentration, IsK can induce in addition to the $K^+$ current a "phospholemman-like" $Cl^-$ current and concluded that IsK is a dual regulator of $K^+$ and $Cl^-$ channels. The fact that we now find a phospholemman-like transcript with an "IsK-like" activity is in agreement with this view. If, indeed, CHIF functions to activate an oocyte channel rather than forming a $K^+$ pore, it may act in one of two ways: (*i*) activate channels that are already in the membrane by a direct, protein–protein interaction or (*ii*) function as a chaperon that assists the cellular translocation, membrane insertion, and folding of other proteins—i.e., its role may be analogous to that described for the β subunit of the $Na^+,K^+$-ATPase (35) and rBAT, a kidney cortex cDNA clone (36).

The considerable homology between CHIF, phospholemman, and the γ subunit of $Na^+,K^+$-ATPase appears to be of particular interest, irrespective of the question whether CHIF is a $K^+$ pore or a transmembrane regulator. This finding suggests that all three proteins are members of the same gene family involved in the regulation of ion transport. While this manuscript was in preparation, another protein that evokes a phospholemman-like $Cl^-$ channel activity in oocytes was reported (37). This protein, denoted Mat-8, is expressed in breast tumors as well as several epithelia. It exhibits >51% identity to CHIF, indicating that the two are closely related. An important, unresolved issue is which are the transporters affected by CHIF in native cells and what is its role in the physiological response to aldosterone.

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