

Epithelial sodium channel regulatory proteins identified by functional expression cloning

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Epithelial sodium channel regulatory proteins identified by functional expression cloning. We describe here our current strategy for identifying and cloning proteins involved in the regulation of the epithelial sodium channel (ENaC). We have set up a complementation functional assay in the *Xenopus laevis* oocyte expression system. Using this assay, we have been able to identify a channel-activating protease (CAP-1) that can increase ENaC activity threefold. We propose a novel extracellular signal transduction pathway controlling ionic channels of the ENaC gene family that include genes involved in mechanotransduction (degenerins), in peptide-gated channels involved in neurotransmission (FaNaCh), in proton-gated channels involved in pH sensing (ASIC) or pain sensation (DRASIC).

The highly selective, amiloride-sensitive, epithelial Na channel (ENaC) is the rate-limiting step for Na reabsorption in the distal renal tubule, the distal colon, the distal ducts of several exocrine glands, and the airways. The activity of ENaC in kidney and colon is tightly controlled by aldosterone and is thus involved in the maintenance of Na balance and blood pressure [1, 2]. Aldosterone is a potent regulator of transepithelial Na transport in all vertebrates from amphibia to mammals. Aldosterone binds to an intracellular receptor, and the active hormone-receptor complex interacts with hormone-responsive elements in promoters of specific genes, hence modulating their transcription and leading to the expression and/or repression of specific proteins (AIP or ARP) [3, 4]. Candidate targets for the action of these proteins are the apical Na channel, the apical K channel, the Na,K pump, the basolateral K channel, and the tight junction proteins. As a general working hypothesis, we postulate that the gene network, which is under the specific control of aldosterone in renal epithelia, represents a set of candidate genes involved in the control of Na homeostasis and blood pressure through the aldosterone-dependent electrogenic Na transport process. Candidate genes belong to two categories: proteins

that transport Na, that is, the Na pump and the Na channel, and regulatory proteins controlling the activity of these proteins. Over the past 15 years, our laboratory has been involved in identifying and characterizing the Na channel and the Na pump. Major efforts were directed first to the molecular characterization of Na,K-adenosine triphosphatase from tight epithelia and its control by aldosterone [3]. More recently, we became interested in identifying the apical Na channel, technically a more difficult endeavor. Using functional expression cloning in *Xenopus* oocytes, we first identified tissues expressing the highest Na channel density (kidney and colon) using either a ^{22}Na -uptake assay [5] or a measurement of an amiloride-inhibitable Na current by a two-electrode voltage-clamp method [6]. With the latter method, we isolated from rat distal colon epithelial cells a cDNA identified by functional expression of an amiloride-sensitive Na current in *Xenopus* oocytes [7]. The Na current observed after a rENaC cRNA injection into the oocytes was smaller than that observed after injection of size-fractionated poly A⁺ RNA isolated from the tissue of origin, suggesting the existence of other subunits modulating the expression of the channel in the plasma membrane. At the same time, α rENaC had been characterized by Lingueglia et al [8]. Second, we designed a novel complementation functional assay to clone silent components of the channel that alone may have little Na channel activity but are of critical importance for the reconstitution of a fully active channel. Using our functional complementation assay, we were able to identify two other subunits (β and γ) [9]. The ion-selective permeability, the gating properties, and the pharmacological profile of the channel formed by coexpressing the three subunits in the oocyte were similar to those of the native channel. The α , β , and γ subunits from different species and from different organs have now also been reported by different groups, as reviewed in [10].

Recently, we have cloned the channel protein from A6 kidney cell [11], a cell line that expresses both the highly Na-selective channel (type 1) and the moderately selective channel (type 2). The biophysical characteristics are well conserved between rat and frog, suggesting molecular

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conservation as well. Three homologous subunits have been identified and coexpressed in the *Xenopus* oocyte system [11]. The reconstituted channel showed all the characteristics of the native type 1 ENaC described in A6 cells: high Na selectivity ($\text{Li} > \text{Na} \gg \text{K}$), low single-channel conductance (4 to 6 pS in presence of Na), slow gating kinetics (seconds), and high affinity for amiloride (submicromolar range). Transcript α , β , and γ subunits of *Xenopus* ENaC have been detected in A6 kidney cells and in *Xenopus* kidney, lung, and, to a lesser extent, stomach and skin. Each *Xenopus* ENaC subunit shares about 60% overall identity with the corresponding rat homologue (α , β , and γ rENaC), whereas subunits share only 35% identity. Our data suggest that the triplication of the ENaC subunits occurred before the divergence between mammalian and amphibian lineages.

We have examined the effect of aldosterone on ENaC mRNA abundance and the rate of protein synthesis for each of the three ENaC subunits in the A6 *Xenopus* kidney cell line [12]. In cells grown on a porous substrate, amiloride-sensitive electrogenic Na transport is expressed and is up-regulated by aldosterone (300 nM) after one hour. α , β , and γ -mRNA abundance is not changed by aldosterone during the first three hours stimulation but increases four-fold over control after 24 hours. The rate of α -subunit protein synthesis is significantly increased above control after 60 minutes. β -Subunit synthesis increases only after six hours, and there is no significant change for the γ subunit. The half-lives of each subunit were short (40 to 50 min), as assessed by ^{35}S methionine pulse-chase experiments, and were not modified by aldosterone [12]. Taking into account the short half-life of ENaC protein and assuming that the synthesis of the α subunit is a limiting factor in the assembly and expression of new channels at the cell surface, we have proposed that the aldosterone regulation of Na transport might be, in part, mediated by *de novo* synthesis and assembly of the channel protein [12].

One of our current aims is to define regulatory proteins that could control Na channel activity. We would like to identify proteins that interact either directly or indirectly with the channel protein to control its activity. To address this question, three different strategies are currently available.

The first strategy is to identify the proteins interacting directly with the Na channel. Because the cytoplasmic domains of the protein (the N terminus and C terminus) are short (about 9 kDa), the number of interactions may be rather limited. Affinity columns can be designed, using either the N terminus or the C terminus of each subunit, to isolate cytoplasmic components that can bind selectively to these two domains. This approach has been used successfully to identify α spectrin as interacting directly with the C terminus of α rENaC [13] and, more recently, Nedd-4 [14] that interacts with the C termini of the α , β , and γ subunits. In this study, the double-hybrid cloning technique in yeast

was used to identify interacting proteins. This is a powerful method for identifying any protein(s) that interacts directly with each other.

The second strategy for identifying regulatory proteins under the control of aldosterone is to use either a cDNA differential subtractive approach, as used by Attali et al [15], or the now popular technique of differential display reverse transcription-polymerase chain reaction. The latter may be difficult due to large numbers of false positive and negative clones but nevertheless has been used successfully by François Verrey in Zurich [16]. For both approaches, the demonstration of the physiological relevance of the identified genes remains difficult.

The third strategy designed in our laboratory is the use of a complementation functional assay that allows selection of gene products that can regulate the activity of the Na channel expressed in the oocyte. This approach has allowed us to clone two silent subunits (β and γ) of the Na channel [9]. The same technique may also be used to identify regulatory proteins induced by aldosterone. Both activators and repressors of channel activity may be cloned by expression in the oocyte. In this report, we discuss briefly the results we have obtained recently using this approach.

CONSTRUCTION OF THE A6 CDNA LIBRARY AND CLONING STRATEGY

The A6 cell line is a *Xenopus* kidney cell line that expresses a high degree of epithelial differentiation and regulation [17]. A6 cells grown on filters exhibit transepithelial Na transport that can be stimulated by aldosterone. The time course of aldosterone action on Na transport and ENaC expression can be divided into three steps: a latent period (30 to 45 minutes), an early phase (45 minutes to hours) in which Na transport increases and transepithelial electrical resistance falls, but without significant changes in ENaC mRNA abundance, and a late phase (3 to 24 hours) in which Na transport increases further without change in transepithelial electrical resistance but with a significant increase in ENaC mRNA abundance [12]. Assuming that a regulatory protein(s) should be induced by aldosterone during the latent period, A6 cells were grown on filters and stimulated with aldosterone for 45 minutes before recovery of mRNA. After 45 minutes, the transepithelial Na current just began to increase (by about 1.4-fold: 5.9 ± 0.09 mA/cm⁻² with aldosterone vs. 4.2 ± 0.02 mA/cm⁻² in control). Total RNA was then extracted and polyA⁺ mRNA purified by oligo(dT) column affinity. To obtain a restricted cDNA library, we size fractionated the mRNA by sucrose gradient centrifugation, and six mRNA fractions (0.5 and 5 kb) were tested for their ability to increase ENaC activity. Each fraction (50 ng mRNA per oocyte) was injected into oocytes together with a near saturating amount (0.3 ng of each subunit per oocyte) of $\alpha\beta\gamma$ -ENaC cRNA. ENaC activity was then measured as an amiloride-sensitive Na current. A current of 224.2 ± 45.8 nA ($N = 6$)

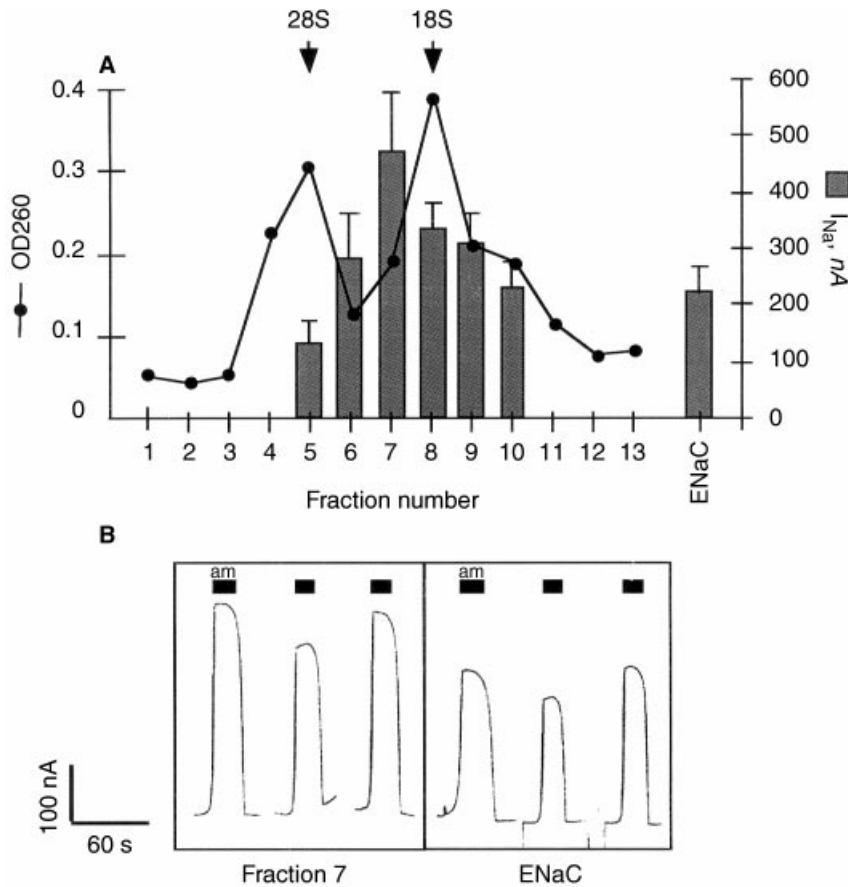


Fig. 1. Screening of the mRNA sucrose gradient. (A) A6 mRNA was size fractionated by sucrose gradient centrifugation, and the amount contained in each fraction was quantified by measuring the absorbance at 260 nm (OD₂₆₀; ●). The fractions containing the 18S and 28S rRNA in the corresponding polyA⁻ gradient were indicated. Fractions 5 to 10 were injected in oocytes to test their ability to increase epithelial Na channel (ENaC) activity (measured as amiloride-sensitive Na current, I_{Na+}; ■). Each bar represents the mean of six oocytes. Errors bars represent SEM. (B) original current recordings obtained in oocytes expressing ENaC alone (right panel) or ENaC plus mRNA from the fraction seven (left panel). The current sensitive to 5 mM amiloride (am) was measured at -100 mV. Oocytes coinjected with fraction seven mRNA exhibit larger current.

was observed in oocytes injected with ENaC cRNA alone. This activity was roughly doubled in oocytes coinjected with the mRNA from fraction seven (466.6 ± 109.9 nA; $N = 6$; $P < 0.07$). The other fractions had no significant potential effect on channel activity or, in some fractions, even decreased ENaC activity (Fig. 1). The fraction seven (mRNA size 1 to 2 kb) was used to construct a cDNA oligo(dT)-primed directional library by cloning into the pSPORT1 expression vector. To minimize species differences, all probes were prepared from *Xenopus laevis*: the cDNA library was constructed from the A6 *Xenopus* kidney cell line and screened by coinjection of the *Xenopus* ENaC subunits into *Xenopus* oocytes.

Our cloning assay consisted of coexpression into oocytes of the cRNA encoding the three ENaC subunits together with cRNA transcribed from a library, followed by measurements of ENaC activity as an amiloride-sensitive Na current by the two-electrode voltage-clamp method [6] (Fig. 2). The total library was divided into 10 different cDNA pools. Each pool was transcribed *in vitro* and coinjected into oocytes together with the three (α , β , and γ) ENaC subunits, and ENaC activity was measured. The pool associated with the highest amiloride-sensitive Na current was then subdivided for further screening.

SCREENING THE LIBRARY

The whole cDNA library (named A8) contained approximately 30,000 independent cDNA clones. For screening, plasmids were extracted, linearized using the *NotI* enzyme, and *in vitro* transcribed to synthesized cRNA. When the cRNA from the unfractionated cDNA library was coinjected together with ENaC subunits in the oocytes, there was no increase in ENaC activity (Fig. 3). In the first round of screening, the library was subdivided into 10 pools, each containing about 2,500 clones (A8.1–A8.10). The functional assays showed that the A8.1 pool increased ENaC activity by 187%. This pool was subdivided into a further 10 pools (A8.1.1–A8.1.10) that were screened in the second round. The A8.1.8 pool, containing about 200 clones, increased ENaC activity by 300%. This pool was then subdivided into seven pools (A8.1.8.1–A8.1.8.7), each of 30 clones. The A8.1.8.1 pool increased ENaC activity by 378%. To overcome the problem of day-to-day variability of the expression system, the functional assay was performed three times in three different batches of oocytes for each of the three rounds of screening. Additionally, at least two different cRNA preparations were injected, and at least 10 oocytes were measured in each experiment. In the

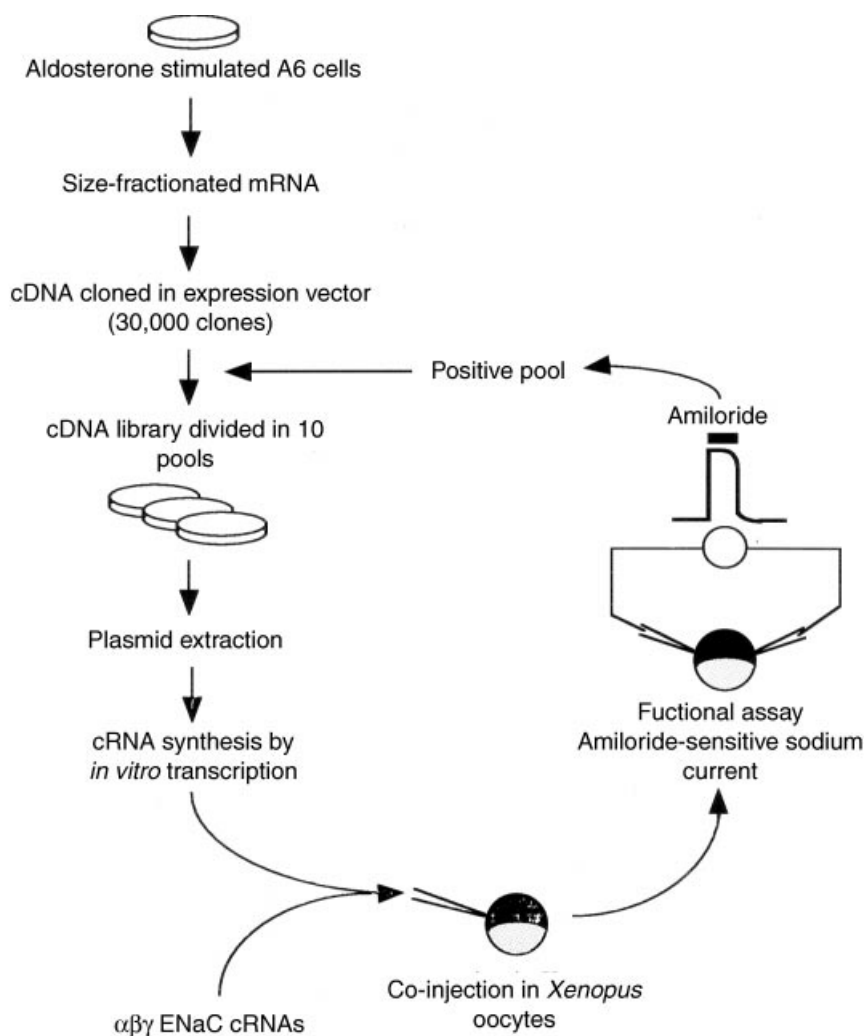


Fig. 2. Strategy of cloning by functional complementation assay. mRNA extracted from aldosterone stimulated A6 cells was size fractionated by sucrose gradient centrifugation. The fraction of interest was used to construct a cDNA library in pSPORT1 expression vector. The library was divided in 10 pools. The plasmid DNA of each pool was isolated, linearized, and *in vitro* transcribed to synthesize cRNA. The cRNA was then injected in oocytes, together with the three cRNA encoding α -, β -, and γ -epithelial Na channel (ENaC) subunits. ENaC activity was measured as an amiloride-sensitive Na current. The positive pool, leading to an increase of ENaC activity, was then subdivided and screened in a further round of functional complementation assays.

fourth and final round, the 30 clones contained in the A8.1.8.1 pool were isolated, and the plasmid of each clone was digested by restriction enzymes to check the size of the insert. Only 12 clones contained an insert allowing the synthesis of a protein. The cRNA of these 12 clones was then coinjected separately into oocytes. Only one of these clones, the clone A8.1.8.1.3, was able to increase ENaC activity by 330%. The overall screening procedure took 12 months.

The clone A8.1.8.1.3 was renamed CAP-1 for channel activating protease. CAP-1 cDNA is 1340 bp long, and sequence analysis revealed a 987-bp open reading frame encoding a 329-amino acid protein. This protein shares homologies with several members of the serine protease family. The effect of CAP-1 on ENaC activity has been characterized [18], and results indicate that CAP-1 specifically increases ENaC activity about threefold. In many respects, the activity of CAP-1 mimics that of extracellular trypsin, as recently reported [19]. Experimental evidences

indicate that the action of CAP-1 occurs at the extracellular surface of the cells, suggesting an effect on the large extracellular loop of ENaC proteins, although proteolytic cleavage of ENaC itself has not been demonstrated.

In summary, functional complementation cloning has allowed us to identify a novel regulator of ENaC activity. We have also identified a new pathway in which ENaC activity can be modulated by an extracellular serine protease activity. This novel mechanism may be investigated in the regulation of the activity of other members of ENaC family, such as homologues expressed in neurons (FaNaCh) [20], BNaC or ASIC/DRASIC [21–24], or mec-4 and mec-10, components of the mechanosensory complex in *Caenorhabditis elegans* neurons [25]. We are currently characterizing the biochemical and physiological properties of CAP-1. Of interest, CAP-1 does not appear to be regulated by aldosterone as we had anticipated from our cloning strategy. Rather, CAP-1 seems to be constitutively expressed in A6 cells and could be a limiting factor in

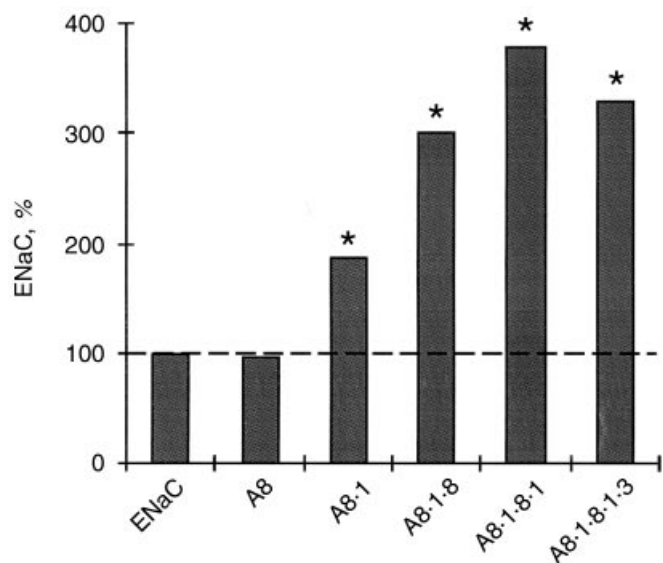


Fig. 3. Screening of the A8 library. Epithelial Na channel (ENaC) activities measured in the positive fractions during the different rounds of the screening. Activities of the fractions are expressed as a percent of ENaC activity obtained in oocytes injected with ENaC alone in the same experiments. Each bar represents the mean of three independent experiments, each experiment containing at least 10 oocytes. * $P < 0.02$ versus ENaC alone.

the physiological activation of ENaC. This idea is supported by the finding that aprotinin, a serine protease inhibitor, fully inhibits amiloride-sensitive electrogenic Na transport when present on the apical side of the monolayer (Ki 0.6 μM) [19].

Our cloning strategy should allow us to isolate in the future other regulatory proteins up- or down-regulating ENaC activity. Loss or gain of functional mutations in the genes responsible could lead to dysregulation of blood volume or blood pressure as already shown for ENaC [2]. The approach has both pitfalls and advantages. On the negative side, the method is time consuming and requires high motivation. The well-known variability of the oocyte expression system cannot be overcome without a statistical approach that predicts the number of independent experiments that have to be performed. The sensitivity of the assay is not very high, but a 40% change in ENaC activity in a defined mRNA fraction was enough to clone CAP-1, providing that the cDNA library was restricted to a rather small number of independent clones. In this study, 30,000 clones were counted, but probably only 50% contained an insert capable of coding for a protein. On the positive side, the technique allows the cloning of a fully functional (by definition) cDNA so that the difficult problem of assigning a function to a gene cloned by a classic method does not arise. On the other hand, functional cloning does not give access to the biochemistry of the identified molecule. This step may be difficult, especially for a channel protein like

ENaC, but may possibly be less arduous for a serine protease such as CAP-1.

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