Revisiting the heteromultimeric structure of ENaC

Rosanna De Nuccio, Laurent Schild

Department of Pharmacology and Toxicology, University of Lausanne, Switzerland

FONDS NATIONAL SUISSE DE LA RECHERCHE SCIENTIFIQUE

INTRODUCTION

The epithelial sodium channel (ENaC) plays a critical role in the renal regulation of sodium excretion and blood pressure.
ENaC is a heteromeric channel formed by three homologous α-, β- and γ-subunits. Several functional and biochemical studies have provided evidence that ENaC is a heterotetramer formed by 2α, 1β and 1γ subunits(1,2). Recently a high-resolution crystal structure has been obtained from an ortholog of ENaC, the acid-sensing ion channel ASIC1 that shows a homotrimeric channel. This discrepancy between ENaC and ASIC motivated us to revisit the subunit oligomerization of ENaC. For this purpose we affinity purified the ENaC channel complex carrying the His-6 Tag or HA Tag on different subunits. The subunits composition was determine by western blotting.

METHODS

Rat ENaC constructs: rat α-,β- and γ-ENaC subunits were cloned into pSD5. A 6 histidin tag (His-6) was added to the COOH and NH3 terminus of α-, β- and γ- ENaC cDNAs (α^{H6}, β^{H6} and γ^{H6} respectively). A HA tag was added in COOH terminus for α-subunits (α^{HA}) and into the loop for β- and γ-subunit (β^{HA} and γ^{HA} respectively).

Amiloride sensitive current: complementary RNAs of each subunit were synthesized *in vitro*. Stage V and VI Xenopus oocytes were injected with 100 nl of a solution containing equal amounts of ENaC subunits at a total concentration of 100 ng/µl. Electrophysiological measurements were made 16–30 h after injection. ENaC current (INa) was recorded using the two-electrodes voltage-clamp technique (model TEV-200; Dagan Corp.). Holding potential was 100 mV.

Pull down assay with NTA-Ni^{+2} agarose beads: the oocytes were injected with tagged and untagged subunits. The total protein were obtained by solubilization in triton X-100 buffer. Copurification was performed by incubation with Ni-NTA beads agarose at 4 C followed by elution with solutions of different imidazole concentrations (50mM, 100mM and 300mM) and finally with a EDTA containing solution (100mM). After SDS-PAGE (10%), proteins were transferred for immunostaining onto nitrocellulose membranes (Schleicher & Schuell) and immunoblotted using anti–rat ENaC and anti-HA as primary antibodies and anti-rabbit IRDye 800 CW (Licor®) and anti-mause IRDye 700 CW (Licor®) as secondary antibodies.

Biotinylation Assay: after protein expression, the injected Xenopus oocytes were incubated on ice for 15 min with 0,1mg/ml of EZ-Link Sulfo-NHS-Biotin (Pierce). The oocytes were subjected to lysis and incubated with Ni-NTA beads to purified the ENaC complex. The purified proteins were precipitated with streptavidin-coated and analyzed on Western blot.

Size exclusion chromatography. ENaC proteins were purified on NTA-Ni^{+2} agarose beads and applied to the Sephadex G-200 column equilibrated with 50 mM Tris-HCl, 100mM NaCl, 1mM βDDM buffer pH 7 at a flow rate of 0.5 ml/min.

RESULTS

1. Amiloride sensitive currents expressed by the ENaC constructs

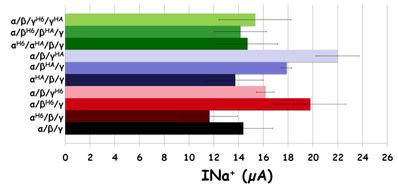

Fig 1. Two-electrodes voltage-clamp recordings from *Xenopus* oocytes expressing ENaC wild-type carrying or not his 6 tag and HA tag to asses the functionality of the constructs.

2. Post-translation modifications of the ENaC subunits when associated in a αβγ functional complex

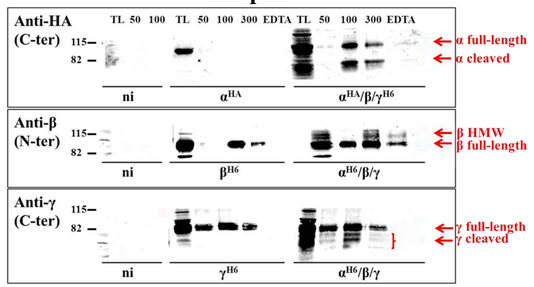

Fig. 2: Upper panel: Western blots obtained from total lysate (TL) showed a cleaved form of α-ENaC when associated in the αβγ complex. Lower panels: ENaC proteins were purified on Ni^{+2}-NTA beads. When associated in the αβγ complex the β-ENaC show a higher molecular weight, and the γ-subunit cleaved products

3. Purification of the α-, β- and γ-subunits in the ENaC complex

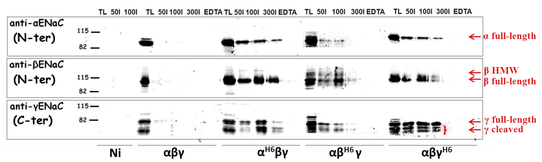

Fig.3: Affinity purification of ENaC protein on Ni^{+2}-NTA beads followed by western blot analysis and immunostaining with anti–rat α-ENaC antibody that recognizes the N-terminal region and only the full length form, anti-rat β-ENaC recognized the C-terminal region and anti-rat γ-ENaC recognized the C-terminal region
The α-, β- and γ-ENaC subunits copurify in the ENaC channel complex

4. Evidence for 2α 1β 1γ ENaC channel complex

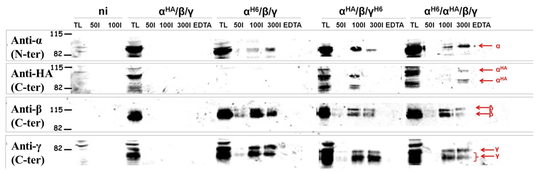

Fig. 4: Western blot of affinity purified ENaC proteins on NTA-Ni^{+2} beads from non-injected oocytes (ni), oocytes injected with $\alpha^{HA}/\beta/\gamma$, $\alpha^{H6}/\beta/\gamma$ and $\alpha^{HA}/\beta/\gamma^{H6}$,or $\alpha^{H6}/\alpha^{HA}/\beta/\gamma$. The α^{HA} copurify with β- and γ-ENaC in a α^{H6}-ENaC complex immobilized NTA-Ni^{+2} beads, indicating the presence of at least 2 α-ENaC subunits in the channel complex

5. Copurification of differentially tagged α-, β- and γ-ENaC subunits from the channel complex

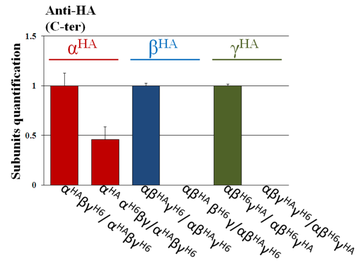

Fig. 5: Relative band intensity of HA-tagged α- or β- or γ-ENaC subunits expressed alone or together with α^{H6}-, β^{H6}- and γ^{H6}-subunit respectively in the purified αβγ ENaC complex(n=4)
α ENaC: in the purified complexes, α^{HA} co-purifies with α^{H6}; the quantification of the obtained signal for HA on WB represents 50 % compared with the HA signal for the ENaC complexes made of α^{HA} alone.
β, γ ENaC: no evidence of copurification of differentially tagged β or γ ENaC
This data are consistent with the presence of 2 different α-subunits (with and without HA tag), we don't have evidence for ENaC channel complex made of 2 different β- and/or γ-subunits, suggesting that the functional ENaC channel is made of 2α-, 1β- and 1γ-subunit

6. The $\alpha^{HA}\beta\gamma$ and $\alpha^{H6}\beta\gamma$ channels don't form the aggregates

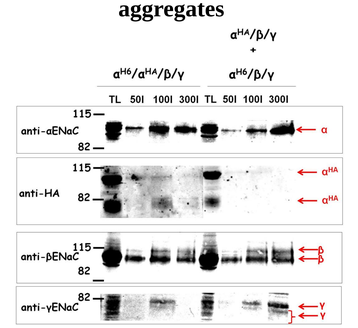

Fig. 6: Oocytes were separately injected with $\alpha^{H6}/\alpha^{HA}/\beta/\gamma$, or with $\alpha^{HA}/\beta/\gamma$ or with $\alpha^{H6}/\beta/\gamma$. The TL of oocytes expressing either $\alpha^{HA}/\beta/\gamma$ or $\alpha^{H6}/\beta/\gamma$ was mixed and the ENaC proteins purified on Ni^{+2}-NTA beads. The α^{HA} copurify with β- and γ-ENaC in a α^{H6}-ENaC complex purified on Ni^{+2}-NTA beads but not when expressed separately (in different oocytes) from α^{H6}-subunits.

7. The cleaved ENaC channel complex is expressed at the cell surface

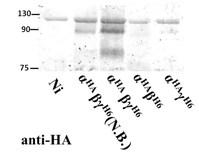

Fig. 7: Western blot of affinity purified α_{HA}-ENaC proteins on NTA-Ni^{+2} beads from biotinylated non injected oocytes (Ni) , injected with $\alpha^{HA}/\beta/\gamma^{H6}$, or α^{HA} / β^{H6} ,or α^{HA} /γ^{H6} and from not biotinylated oocytes injected with $\alpha^{HA}/\beta/\gamma^{H6}$ as a negative control for specific streptavidine binding.
The proteins were further purified on streptavidine beads.
At the cell surface the α_{HA} ENaC subunit is predominantly found in the cleaved form only in the αβγ complex

8. Estimation of the molecular weight of the functional ENaC channel complex by size-exclusion chromatography

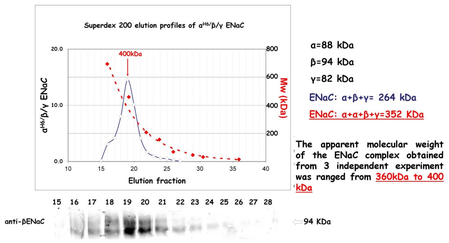

Fig. 8: $\alpha^{H6}\beta\gamma$ ENaC protein was affinity purified on Ni^{+2}-NTA beads and runned on superdex G-200 column. The elution fractions were collected and analyzed with immunoblotting using anti-β Ab. Most of ENaC protein eluted in the fraction 19. The blue line represents the band intensity of each elution fraction corresponding to β-ENaC; the red curve represents the standard curve obtained from elution of protein with known molecular weight.

CONCLUSIONS

- We have copurified the functional multimeric ENaC channel made of α-, β- and γ-subunits.
- The cleaved form of ENaC channel expressed at the cell surface likely represents the functional form of the channel (3).
- The ENaC channel complex is made of at least two α-, but only one β- and one γ-ENaC subunits.
- Purification of the ENaC channel complex by size exclusion chromatography reveals a 360-400 kDa channel protein consistent with a heterotetrameric channel complex.
- Compared to αβγ, the α-subunit, in the αβ and αγ ENaC complexes doesn't show a cleaved form, this means the co-purification of cleaved α_{HA} with α_{H6} β and γ can not result from the presence of heterogeneous channels made of αβα or αγα.
- These observations suggest that the functional ENaC channel complex is made of 2α-, 1β- and1γ-subunits

REFERENCES

1. **Anantharam A, Palmer LG**. Determination of epithelial Na+ channel subunit stoichiometry from single-channel conductances. J Gen Physiol. 2007 Jul;130(1):55-70. Epub 2007 Jun 11.
2. **Firsov D, Gautschi I, Merillat AM, Rossier BC, Schild L**. The heterotetrameric architecture of the epithelial sodium channel (ENaC).EMBO J. 1998 Jan 15;17(2):344-52.
3. **Frindt G, Palmer LG**. Surface expression of sodium channels and transporters in rat kidney: effects of dietary sodium. Am J Physiol Renal Physiol. 2009 Nov;297(5):F1249-55. Epub 2009 Sep 9.