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CHARACTERIZATION OF THE NOVEL INTERACTION BETWEEN NNOS AND CLAUDIN-3.

CARACTERIZACIÓN DE LA NOVEDOSA INTERACCIÓN ENTRE LA NNOS Y LA CLAUDINA-3.

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ABSTRACT

Neuronal Nitric Oxide Synthase (nNOS) differs from the inducible (iNOS) and endothelial (eNOS) isoforms in a PDZ domain involved in subcellular targeting located at its N-terminus that displays a hydrophobic binding groove that accommodates the C-termini of various cellular proteins. The specific polypeptides involved in these interactions remain mostly undiscovered so we are particularly interested in deciphering new PDZ ligands. By means of *in vitro* binding techniques and confocal microscopy we have characterized the novel interaction between the PDZ domain of nNOS and Claudin-3, a tight-junction tetraspan membrane protein essential in the paracelullar barrier. Finally, we have proposed tyrosine phosphorylation as one of the possible binding regulation mechanisms.

Keywords: Claudin-3, nNOS, PDZ domain, tight-junctions, nitric oxide.

RESUMEN

La Óxido Nítrico Sintasa neuronal se diferencia de las isoformas inducible (iNOS) y endotelial (eNOS) en la presencia de un dominio PDZ en su extremo N-terminal implicado en procesos de localización subcelular. Este dominio posee un surco hidrofóbico capaz de acomodar el extremo C-terminal de diversas proteínas celulares. Muchas de las cadenas polipeptídicas implicadas en estas interacciones con el domino PDZ de la nNOS no han sido identificadas por lo que estamos especialmente interesados en la identificación de nuevos ligandos de este dominio PDZ. Utilizando técnicas de unión *in vitro* y microscopía confocal hemos caracterizado la interacción entre el dominio PDZ de la nNOS y la Claudina-3, una proteína presente en las uniones estrechas que posee cuatro segmentos transmembrana y es esencial en la barrera paracelular. Finalmente, nuestros datos muestran que esta interacción se ve regulada a través de un posible ciclo de fosforilación-desfosforilación de residuos de Tyr localizados en el extremo C-terminal de la Claudina-3.

Palabras clave: Claudina-3, nNOS, dominio PDZ, uniones estrechas, óxido nítrico.

INTRODUCTION

Neuronal NOS is expressed constitutively in specific neurons of the brain and also in the spinal cord, peripheral nitrergic nerves, epithelial cells of various organs, pancreatic islet cells and in the vascular smooth muscle (Forsterman & Sessan, 2011). nNOS differs from the other two mammalian isoforms, eNOS and iNOS by an additional ~300 residue N-terminal extension mostly involved in subcellular targeting, This N-terminus extension comprises a PDZ domain (Bredt et al, 1991). PDZ domains (first found in the proteins PSD-95 Dlg-1 and ZO-1) are modular protein interaction domains which display remarkable selectivity towards their cellular native targets, which are usually the C-termini of proteins (Hung & Sheng, 2002. Stiffler et al, 2007). The conserved structure of PDZ domains consists on 6 β-strands and 2 α-helices forming a β-sandwich that displays a hydrophobic groove, where C-termini peptides can bind as antiparallel β-strands. We describe herein the binding of the PDZ domain of nNOS towards the C-terminus of a novel interaction partner: Claudin-3, a tight junction protein. Claudins are tetraspan membrane proteins essential components of the paracelular barriers found in tight junctions of all epithelia and endothelia which contain a longer intracellular C-terminus through which the majority of human claudins interact with PDZ domains of various proteins (Gunzel & Yu, 2013). Claudin-3 is expressed in a wide variety of epithelia, such as respiratory, urinary

and gastrointestinal tract, where it acts as a barrier-forming tight junction protein. By means of yeast two hybrid, isothermal titration calorimetry, circular dichroism, confocal microscopy and pull-down assays we have demonstrated the interaction between Claudin-3 and the PDZ domain of nNOS and proposed tyrosine phosphorylation as a binding regulation mechanism.

Experimental Procedures

Constructs- The cDNA corresponding to residues 14-131 of rat nNOS N-terminus was amplified and cloned into the yeast two-hybrid vectors pGAD and pGBT9 as well as in the recombinant expression vector pKLSLt. The cDNA corresponding to C-terminal sequence of Claudin-3 was created using long annealing oligonucleotides and ligated with protruding overhangs into pGBT9 or pGAD vectors. YFP-tagged and mCherry-Claudin-3 were obtained by inserting the Claudin-3 cDNA between BsrGI and BamHI restriction sites. GFP (Green Fluorescent Protein)-tagged nNOS clones were obtained after amplification of the desired fragment and its insertion in the mammalian expression vector pEGFP-C2.

Protein Expression and Purification- Rat nNOS cDNA was cloned into the recombinant expression vector pKLSLt and expressed in *E. coli* BL21 DE3. Purification was performed following published protocol for this tag. Recombinant full-length nNOS tagged with a $(His)_6$ sequence was expressed and purified as previously described (Rodriguez-Crespo et al, 1998).

Yeast two-hybrid assays-We followed our published protocol (Navarro-Lérida et al, 2006).

Isothermal Titration Calorimetry (ITC)- The interaction between the PDZ domain of nNOS and C-terminal peptides was measured using a VP-ITC MicroCalorimeter in 20 mM Hepes buffer, pH 7.0, containing 0.15 M NaCl at 25 °C. The protein solutions were introduced into the sample cells, whereas the peptides were in the syringe (0.5 – 1 mM concentration). The heat released in each injection was calculated from the raw data by integration of the peaks after subtraction of the baseline using the Origin®7 software program.

Circular Dichroism- Far-UV circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter at 20 nm/min scanning speed. Proteins were dissolved in 15 mM MOPS buffer, pH 7.0, containing 100 mM NaCl (0.2 mg/mL protein concentration). CD measurements were also employed to study the thermal stability. T_m values correspond to the temperature at the midpoint of the monophasic thermal denaturation transition.

Cell transfection, immunofluorescence and pull-down assays- We followed the procedures described previously in our group (Navarro-Lérida et al, 2006).

RESULTS

A previous high-scale analysis suggested a novel interacting protein that could associate to the nNOS PDZ domain: the tight junction protein Claudin-3 (Stiffler et al, 2007). To address if Claudin-3 could indeed bind to the PDZ domain of nNOS we used the biophysical techniques described in experimental procedures. Claudin-3 bound to the nNOS PDZ domain in a yeast two hybrid assay and could displace the T_m of recombinant nNOS PDZ domain by 1.3℃ (Fig. 1A & 1B & 2D). Our ITC analysis also showed a strong binding, displaying a K_d value of 25 μM (Fig. 1C). Given these finding, we wondered if nNOS PDZ and Claudin-3 could indeed interact in a cellular environment.

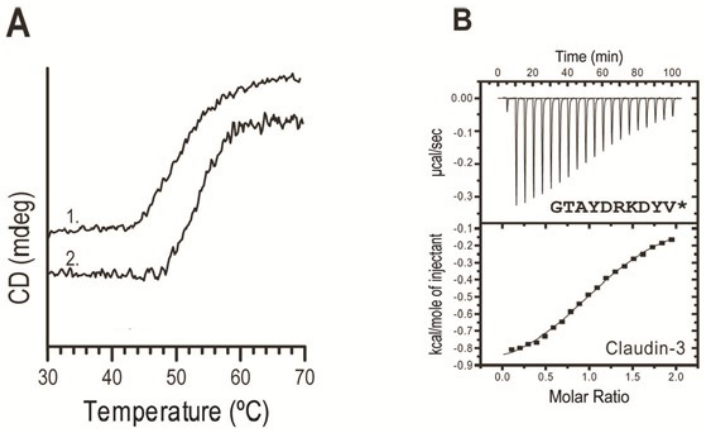

Figure 1: **Binding of the PDZ domain of nNOS to the C-terminus of claudin-3.** A, melting curve of the PDZ domain of nNOS in the absence (trace 1) or presence of 50 μM claudin-3 peptide (GTAYDRKDYV*) (trace 2), in the 20-80 °C range recording the circular dichroism signal at 222 nm. B, Isothermal titration calorimetric analysis of the binding of the C-terminal peptide of claudin-3 to the PDZ domain of nNOS.

To address if nNOS and Claudin-3 colocalized in mammalian cells we transfected HEK293 cells with mCherry-tagged Claudin-3 and GFP nNOS (residues 1-771). In the absence of Claudin-3 coexpression, nNOS(1-771) displayed a cytoplasmic distribution but became translocated to the membrane tight junctions when associated to Claudin-3. Neither GFP by itself nor a nNOS construct lacking the PDZ domain could reproduce this behaviour (Fig. 2A). Although a Claudin-3 C-terminal peptide bound tightly to the nNOS PDZ domain (Fig. 1), we questioned if this interaction could occur in a full-length context. YFP-tagged Claudin-3 bound

to the full length nNOS sepharose beads in a typical pull-down assay, supporting a possible *in vivo* interaction (Fig. 2B).

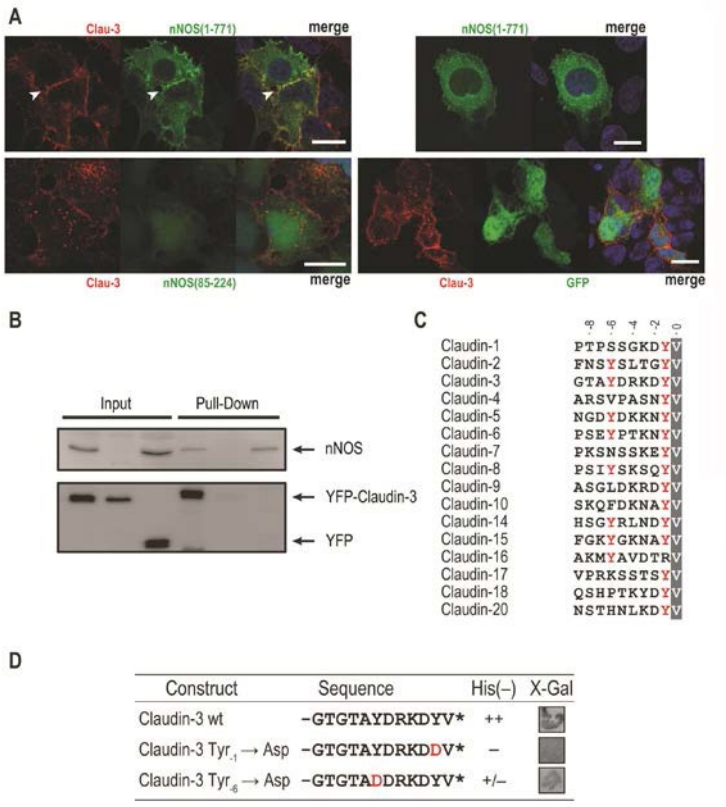

Figure 2. Interaction between claudin-3 and the nNOS PDZ domain. A, confocal microscopy immunofluorescence of HEK293 cells transfected with mCherry-tagged claudin-3 together with various GFP-tagged constructs. B, Claudin-3 appeared associated to nNOS(1-771) in a pull-down assay performed as described in the Experimental Procedures section. C, C-terminal sequences of selected claudin isoforms displaying phosphorylatable residues at the P_{-1} or P_{-6} D, yeast two-hybrid assay using the nNOS PDZ domain (residues 1-131) in the bait plasmid and the C-terminus of claudin-3 or its phosphomimetic mutants.

We also explored the role of tyrosine phosphorylation in this interaction as it has been described that phosphorylation of both Y_{-1} and Y_{-6} present in claudin-3 C-terminus (Fig. 2C) might regulate its association with yet unrecognized PDZ domains (Twiss et al, 2013). To further dissect if phosphorylation of Claudin-3 C-terminal could affect its association towards the nNOS PDZ domain we created the phosphomimetic mutations $Y_{-1}D$ and $Y_{-6}D$ and analysed

the binding of these Claudin-3 mutant sequences in a yeast two-hybrid assay. Whereas the $Y_{-1}D$ mutation completely abrogated binding, the binding of the $Y_{-6}D$ mutant was only marginally affected (Fig. 2D). In summary, Claudin-3 behaves as a novel ligand for the nNOS PDZ domain and this interaction might be regulated by the phosphorylation state of the two Tyr residues positioned at the Claudin-3 C-terminus.

DISCUSSION

Protein-protein interactions mediated by PDZ domains are vital for numerous cellular functions. In this work, we have dissected in detail the molecular determinants of the nNOS PDZ domain-Claudin-3 interaction, two proteins known to be present in the lung epithelium (Asano et al, 1994). We have shown that Claudin-3 is able to induce the translocation of nNOS to the plasma membrane when Claudin-3 is present. This association was also shown in pulldown assays. Furthermore, our results suggest that this association is regulated through the tyrosine phosphorylation of the Claudin-3 C-terminus. Although phosphorylation of C-terminal sequences and the concomitant disruption of binding to PDZ domains is perhaps better characterized in the case of Ser and Thr residues, several precedents exist in which Tyr residues are involved. (Birrane et al, 2003). Somehow expectedly, our results indicate that phosphorylation of Claudin-3 Y_{-1} might abrogate the binding of the C-terminus of Claudin-3 towards the nNOS PDZ domain. The partial inhibition in the binding observed in the phosphomimetic $Y_{-6}D$ construct indicates the presence of contacts of Claudin-3 with the PDZ domain of nNOS that include residues distal from the C terminus. This is reminiscent of other PDZs phosphorylation-dependent interactions (Nomme et al, 2015). Since nitric oxide is known to participate in post-translational modifications, it will be important to determine how Claudin-3 functionality becomes regulated when attached to active nNOS.

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