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Identification of a Potential Regulator of the Gap Junction Protein Pannexin1

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Recent studies have revealed a second class of gap-junction-forming proteins in vertebrates. These genes are termed pannexins, and it has been suggested that they perform similar functions as connexins. Pannexin1 is expressed in diverse tissues including the central nervous system and seems to form gap junction channels in the *Xenopus* oocyte expression system. Since protein interacting partners have frequently been described for connexins, the most prominent family of gap junction forming proteins, we thus started to search for candidate genes of pannexin interacting partners. $K_v\beta3$, a protein belonging to the family of regulatory β -subunits of the voltage-dependent potassium channels, was identified as a binding partner of pannexin1 in an *E. coli* two-hybrid system. This result was verified by confocal laser scanning microscopy using double transfected Neuro2A cells. The colocalization of both proteins at the plasma membrane is suggestive of functional interaction.

Keywords. Gap junction, pannexin, two-hybrid system, hemichannels, intercellular communication

INTRODUCTION

Recently, a novel group of potential gap junction proteins termed pannexins was identified. They were discovered because of their predicted structural homology to the innexins, the major group of gap junction forming proteins in invertebrates. In contrast to innexins, pannexins occur in both phyla (1). Pannexin1 (Panx1) is abundantly expressed in different parts of the central nervous system (CNS) and in various nonneural tissues (2–4).

Bruzzzone et al. (3, 5) were able to demonstrate the formation of homomeric Panx1 hemichannels and homotypic intercellular channels in the *Xeno-*

pus oocyte expression system. Furthermore, the formation of heteromeric hemichannels and intercellular channels in combination with Pannexin2 (Panx2) was shown (3). Based on these findings, Panx1 has been suggested to account for an additional neuronal gap junction forming protein (3). This assumption has been strengthened by a comparative study of expression levels of neuronal connexins and Panx1 in the hippocampus and the inferior olive which reported high expression levels for Panx1 in both brain areas (6). At present the issue of whether pannexins play a substantial role as gap junction forming proteins has not been settled. The discovery that Panx1 hemichannels are mechanosensitive and ATP

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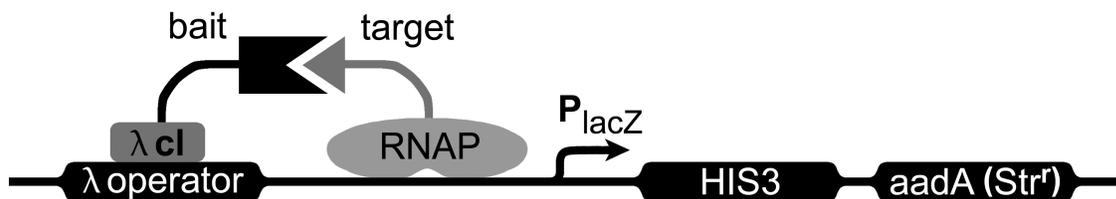


Figure 1. Cartoon of the *E. coli* two-hybrid system. The DNA of the reporter gene construct is represented through the black line. λ cl is the repressor protein of the bacteriophage λ , and RNAP the N-terminal domain of the α -subunit of the bacterial RNA-polymerase. The promoter (P_{lacZ}) is a modified form of the *lac* promoter, which is not inducible by IPTG. “bait” is the protein of interest (in this case the C-terminus of Panx1), while the potential interacting partner is called “target.” The expression of the two reporter genes *HIS3* and *aadA* is induced through the depicted interaction of the two hybrid structures (adapted from Stratagene).

permeant (7) suggested additional roles for these channel-forming proteins which involve functional regulation by interacting partners.

We, therefore, focused on a two-hybrid system originally developed by Joung and coworkers (8) (Figure 1). A stringent two-step selection procedure was used to identify putative binding proteins. The strategy led to the identification of $K_v\beta 3$, a protein belonging to the family of regulatory β -subunits of the voltage-dependent potassium channels. Confocal laser scanning microscopy of Panx1 and $K_v\beta 3$ transfected Neuro2A-cells confirmed colocalization of both proteins in the plasma membrane, supporting functional interaction.

METHODS

E. coli Two-hybrid System

The BacterioMatch[®] II two-hybrid system including plasmids pBT, pTRG and the positive control plasmids pBT-LGF2 and pTRG-Gal11^P, the BacterioMatch[®] II Rat Brain Library, and BacterioMatch[®] II Reporter Cells were purchased from Stratagene (La Jolla, CA, USA). (All media and procedures are described in detail at <http://www.stratagene.com>)

A sequence corresponding to the C-terminal amino acids 297–426 of mouse Panx1 (gi:18043025) was cloned into the pBT-vector, giving rise to plasmid pBT-Panx1-CT. This vector expresses the full-length bacteriophage λ repressor protein fused to

the inserted bait. A rat brain cDNA library was fused to the N-terminal domain of the α -subunit of the prokaryotic RNA polymerase. The screening transformation, selection steps, and isolation of the target plasmids were performed according to the manufacturer’s recommendation. The inserts of positive clones were sequenced and analyzed using the BLASTX-algorithm (9).

Expression Analysis and Confocal Imaging

The full-length mouse Panx1 cDNA (gi:18043025; nt 373-1650) excluding the stop codon was cloned into the plasmid pEYFP-N1 (BD Biosciences). The full-length mouse $K_v\beta 3$ cDNA (gi:17028407; nt 351-1562) excluding the stop codon was cloned into the plasmid pECFP-N1 (BD Biosciences). The expression of Panx1 and $K_v\beta 3$ expression vector constructs was confirmed by Western blot analysis using transiently transfected Neuro2A cells (10). Neuro2A cells were obtained from the ATCC collection (Manassas, VA, USA), cultured and transfected as described (10).

Confocal laser scanning microscopy was performed 48h after transient transfection on samples fixed with 4% paraformaldehyde. Samples were mounted using ProLong[®] Antifade Kit (Invitrogen, Karlsruhe, Germany), coverslipped, and analyzed with a Zeiss LSM 510-Meta inverted confocal microscope (Zeiss, Göttingen, Germany). Data were collected as Z-stacks using the “emission fingerprinting mode” scanning module and analyzed using

the Zeiss LSM Image Browser software. Colocalization was calculated from TIFF files using the RG2B plugin program implemented into the ImageJ software (<http://rsb.info.nih.gov/ij/>) with default settings.

RESULTS

Interacting partners of the protein Panx1 were identified by a library screen using a rat brain cDNA library as target and the carboxy-terminus of Panx1 as bait (Figure 1). Self-activation of the reporters was excluded by several cotransformations. The known interaction of the dimerization domain of the yeast transcriptional activator Gal4 with a domain derived from a mutant form of Gal11 protein (Gal11^P) was used to verify that the experimental conditions applied were permissive for protein interaction in *E. coli* cells on selective medium (11). Pilot experiments demonstrated that one screening transformation produces sufficient double transformants to cover the whole rat brain library containing 2.1×10^6 primary clones. In the following screening transformation more than 3000 double transformants were obtained on selective medium lacking histidine and containing the inhibitor 5 mM 3-aminotriazol (3-AT), increasing the necessary amount of *HIS3* gene product. Thereafter, 169 fast growing colonies were chosen for testing the activation of the second reporter gene *aadA*. This selection step led to a further reduction to 82 clones. The pTRG-cDNA plasmid of each colony was isolated and subjected to a second round of transformation including pBT-Panx1-CT (positive control) and pBT (negative control). Finally, 46 plasmids were chosen for sequencing. All sequences were compared using the basic local alignment search tools (BLASTX 2.2.9) (9). A total of 25 different peptide sequences were obtained. Out of these, four sequences were identified which led to the synthesis of annotated fusion proteins with the α -subunit of the RNA-polymerase. Among the four positive sequences was the protein $K_v\beta 3$. The cDNA clone recovered had 100% identity with the last 58 amino acids of the $K_v\beta 3$ protein.

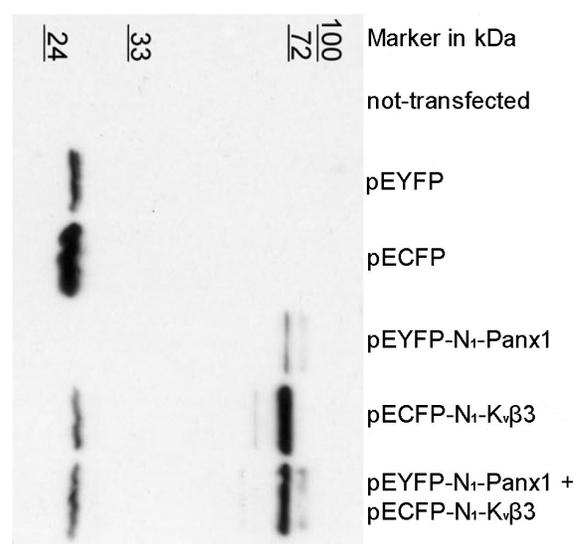


Figure 2. Western-blot analyses of transfected Neuro2A cells. Neuro2A cells were transfected with 400ng DNA and lysed 48 h later. 12 μ g protein was loaded in case of double-transfected cells and 8 μ g in case of single-transfected cells. Immunodetection was carried out using BD-Living Colors A.v. Peptide antibody (BD Biosciences).

For further verification of the interaction between $K_v\beta 3$ and Panx1, we decided to perform colocalization studies on double transfected Neuro2A cells using pEYFP-Panx1 and pECFP- $K_v\beta 3$ expression vector constructs. Neuro2A cells were transiently transfected and the expression of the two vector constructs verified by Western blot analysis as shown in Figure 2. This experiment demonstrated the expression of the fusion protein products, since immunoreactive bands were detected close to the expected sizes (Panx1-EYFP, 75 kDa, Figure 2, lane 5; $K_v\beta 3$ -ECFP, Figure 2, lane 6, 71 kDa). Furthermore, coexpression was confirmed in double transfected cells (Figure 2, lane 7).

Next, the intracellular distribution of the two fusion proteins was analyzed in single and double transfected cells by confocal laser scanning microscopy making use of the distinct expression spectra of the ECFP and EYFP fusion proteins. The single transfected cells were used to record reference spectra. Confocal pictures showed that Panx1-EYFP was mainly located in the plasma membrane while

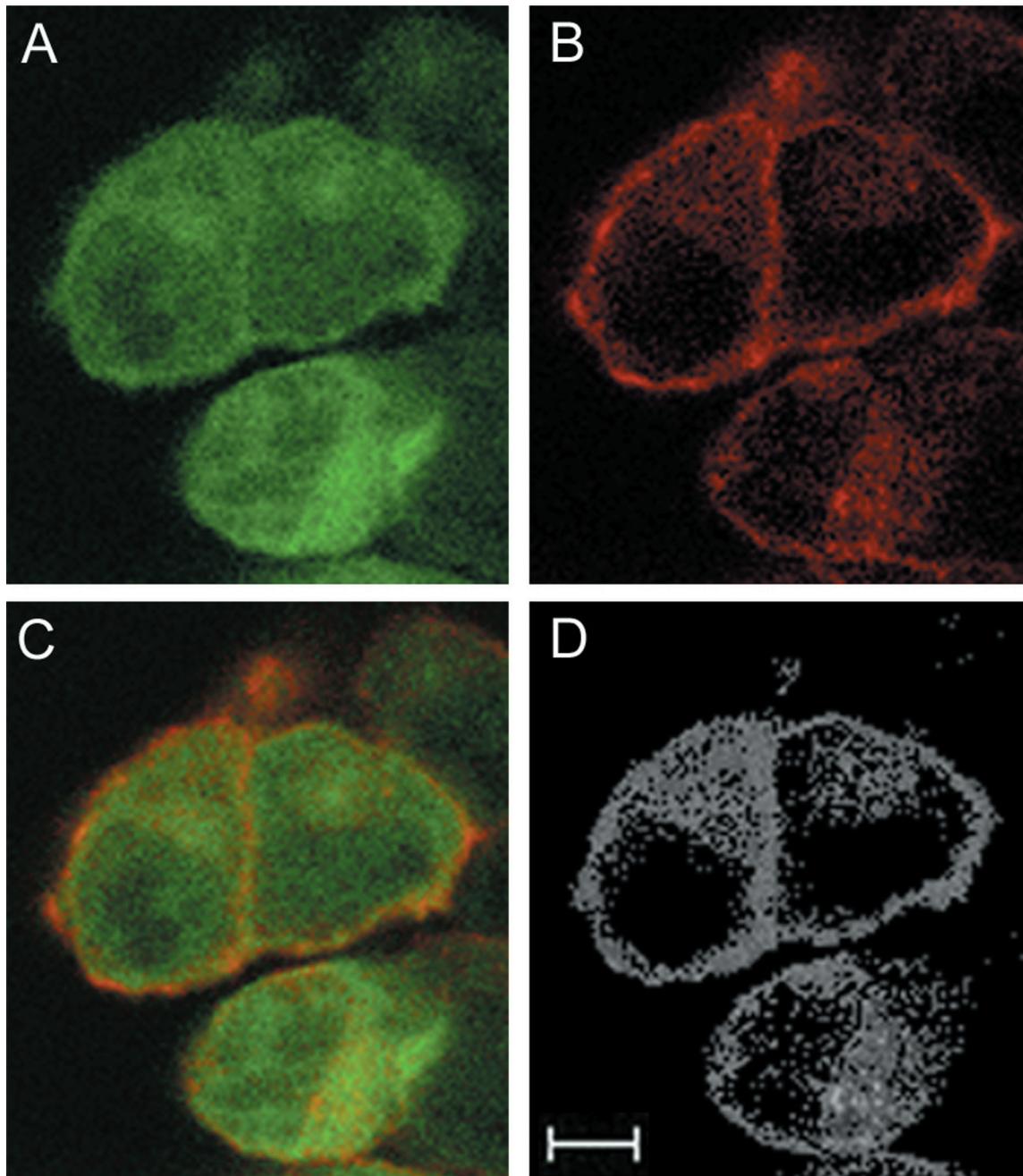


Figure 3. Confocal imaging of Panx1 and $K_v\beta 3$. (A) $K_v\beta 3$ -ECFP, (B) Panx1-EYFP, (C) merged image of (A) and (B). Images represent a single plane derived from a Z-stack recording. (D) Colocalization analyses result using the original RGB-picture (C) and the ImageJ software suite. Scale bar = 5 μm

$K_v\beta 3$ -ECFP was found in the cytoplasm (data not shown).

Colocalization was investigated in double transfected cells. Recorded spectra of Z-stacks were unmixed using the Panx1-EYFP and $K_v\beta 3$ -ECFP reference spectra to identify the intracellular localization of both proteins. Figure 3 summarizes a typical distribution of $K_v\beta 3$ (Figure 3A) and Panx1 (Figure 3B) fluorescence. Panx1 appeared to colocalize with $K_v\beta 3$ almost exclusively in plasma membrane regions in case of double transfected cells as shown in the merged image (Figure 3C). Finally, colocalization information was extracted from raw data using the ImageJ software. This analysis (Figure 3D) demonstrated Panx1 and $K_v\beta 3$ colocalization in membranous structures. In summary, our results provide evidence that an interaction between the two proteins may exist in both bacteria and eukaryotic cells, making the $K_v\beta 3$ protein the first candidate for a Panx1 interacting partner.

DISCUSSION

We gained first evidence for an interacting partner of Panx1 by applying an *E. coli* two-hybrid system. Two stringent selection steps using two different reporter genes yielded four annotated clones. Furthermore, 21 clones were obtained generating fusion proteins of yet unknown identity. This result supports our experimental strategy to select the C-terminal domain of Panx1 as bait consistent with previous studies on connexins demonstrating that the C-terminus is a prominent candidate region for various protein interactions (12–15). One of the four clones expressed part of the C-terminal domain of the $K_v\beta 3$ protein. $K_v\beta 3$ belongs to the family of β -subunits of voltage-dependent potassium channels (16). The β -subunits are expressed in the central nervous system and are known to be oxoreductase enzymes with a NADPH-binding site as well as a binding site for an unknown substrate (17). Some of these proteins convey rapid inactivation to the otherwise very slowly inactivating delayed rectifier channels. The velocity of the

inactivation is dependent on the intracellular redox potential (16, 18).

With respect to our *in vitro* findings, we regarded an interaction of Panx1 with $K_v\beta 3$ feasible. We therefore employed confocal microscopy to analyze the localization of these two proteins in transfected Neuro2A cells. Confocal laser imaging is a well established technique to verify the interaction of connexins with cytoplasmic binding proteins (12, 14). Colocalization of the two fusion proteins was confirmed which occurred preferentially in the plasma membrane. This result suggests that a physical interaction between the two proteins is likely in eukaryotic cells. However, the present data are preliminary and additional experiments need to prove this interaction beyond doubt. Technically, coimmunoprecipitations using antibodies directed against tagged fusion proteins are capable of proving an interaction with high confidence. Since the large EYFP-/ECFP-tags used in the present study potentially could interfere with the binding of $K_v\beta 3$ and Panx1, it is necessary to introduce small tags at the amino terminus of the fusion proteins. These experiments are underway and first results indicate that this interaction does exist in transfected cells (data not shown).

It has been speculated that the interaction of some β -subunits with delayed rectifier channels resembles the ball and chain model known for alpha subunits of the A-type potassium channels (16, 18). A similar ball and chain model involving the C-terminus has been proposed in connexin functions (19, 20). We thus suggest that $K_v\beta 3$ binds to the carboxy-terminus of Panx1 and is likely to be involved in controlling inactivation mechanisms of Panx1 hemichannels or intercellular channels, possibly in response to changes of the intracellular redox potential.

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