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Analysis of Two Newly Identified Protease-Activated Receptor-2-Interacting Proteins, Jab1 and p24A, and their Role in Receptor Signalling

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## ABSTRACT

### Search for novel signalling proteins interacting with PAR-2

Protease-activated receptor-2 (PAR-2), a seven-transmembrane domain G protein-coupled receptor (GPCR), relays signals from extracellular serine proteases, like trypsin, tryptase and others, to intracellular targets. PAR-2 has been shown to exert important physiological and pathological functions in multiple systems. However, the role of PAR-2 in the central nervous system (CNS) is largely unknown. The PAR-2-mediated intracellular signal transduction pathways are also not yet understood. To improve our understanding of PAR-2 functions in the CNS, we used full-length human PAR-2 as bait to search for PAR-2-interacting partner proteins in the yeast two-hybrid system. We screened $1.6 \times 10^6$ clones of a human brain cDNA library. From there, 308 colonies were found positive for the selection markers histidine, adenine and LacZ. Subsequent sequencing and BLAST analysis revealed that the cDNAs from 34 colonies encoded 19 potential PAR-2-interacting proteins. The further yeast two-hybrid tests confirmed that 12 candidate proteins were truly positive in yeast.

### Jab1 regulates PAR-2-dependent gene expression

Importantly, here we demonstrate the functional interaction of PAR-2 with Jab1, a interacting protein found in yeast. Jab1 was initially identified as a coactivator of c-Jun, and was later shown to be the fifth subunit of COP9 signalosome. Our data from *in vitro* glutathione *S*-transferase (GST) pull-down assays and *in vivo* co-immunoprecipitation assays clearly revealed that Jab1 physically interacted with PAR-2 within cells. Moreover, several intracellular domains of PAR-2 are required for the interaction with Jab1. Jab1 was also shown to be colocalized with PAR-2 both in transfected HEK293 cells and in normal primary human astrocytes by double immunofluorescence staining. We then showed that stimulation with PAR-2 agonists (trypsin or specific PAR-2-activating peptide) dissociated Jab1 from PAR-2 in a time-dependent manner. This dissociation could be prevented by the inhibitor of receptor endocytosis phenylarsine oxide, but not by the lysosomal protease inhibitor ZPAD. Interestingly, we found that activation of PAR-2 induced the redistribution of Jab1 from the plasma membrane to the cytosol, but had no effect on expression of Jab1. Furthermore, Jab1 mediated PAR-2-induced c-Jun activation, which was followed by increased activation of activator protein-1 (AP-1). Loss-of-function studies, using Jab1 small interfering RNA, demonstrated that Jab1 knockdown significantly blocked PAR-2-induced AP-1 activation.

Therefore, these data demonstrate that Jab1 is an important effector that mediates a novel signal transduction pathway for PAR-2-dependent gene expression.

**p24A might regulate post-Golgi sorting of PAR-2 to the plasma membrane**

Furthermore, we characterized another PAR-2-interacting protein, p24A, as also found in the yeast screening. p24A belongs to the p24 family of coated vesicle membrane proteins. Binding studies by *in vitro* GST pull-down assays clearly demonstrate that the second extracellular loop of PAR-2 strongly bound to the aa 1-125 of p24A at its N-terminus. However, the intracellular domains and transmembrane domains of PAR-2 failed to interact with p24A. The physical interaction of PAR-2 with p24A was further confirmed by co-immunoprecipitation assays *in vivo*. Interestingly, p24A was shown by double immunofluorescence staining to be colocalized with the intracellular PAR-2 at the Golgi stores, but not with cell surface receptors in double transfected HEK293 cells. We then demonstrate the interaction of PAR-2 with p23, another member of p24 family, by co-immunoprecipitation assays *in vivo*. When we studied the functional significance of the PAR-2-p24A interaction, we found that activation of PAR-2 resulted in protein dissociation of p24A from PAR-2 at the time, when the intracellular receptor was sorted to the plasma membrane for resensitization. In contrast, p23 was dissociated from PAR-2 later only at 60 min after agonist stimulation, when PAR-2 is completely resensitized. The dissociation between PAR-2 and p24A was completely inhibited by brefeldin A, which has been known to attenuate resensitization of PAR-2. Brefeldin A is an inhibitor of guanine nucleotide exchange factor that was known to prevent conversion of inactive ADP-ribosylation factor 1 (ARF1)-GDP to active ARF1-GTP. Activation of ARF1 results in the dissociation of hetero-oligomeric complexes of p24A and p23. Therefore, our data imply that p23 and p24A trap the PAR-2 at the Golgi apparatus, which is essential for the intracellular PAR-2 pool formation. Upon PAR-2 activation, p24A releases the PAR-2 and regulates receptor resensitization. On the other side, p23 assists PAR-2 sorting to the plasma membrane. Here, we also demonstrate that p24A strongly interacted with PAR-1 and $P2Y_1$ receptor, suggesting that our model might explain the molecular mechanism underlying post-Golgi transport of certain GPCRs to the plasma membrane.