The scaffold protein CNK1 in Raf-dependent signalling

Rafael D. Fritz, Algirdas Ziogas, Karin Moelling and Gerald Radziwill

Institute of Medical Virology, University of Zürich, Gloriastrasse 30/32, 8006 Zurich, Switzerland

Abstract

The multidomain protein CNK1 (connector enhancer of Ksr) interacts with kinases involved in different cellular processes such as proliferation, differentiation and apoptosis. We identified CNK1 as a scaffold protein in the Raf/MEK/ERK pathway. According to its scaffold function CNK1 mediates Src-dependent Raf-1 activation in a dose-dependent manner typical for scaffold proteins. Downregulation of endogenous CNK1 by short hairpin RNA interfered with Src-dependent activation of ERK. Thus, CNK1 is a scaffold coupling Src- and Raf-dependent signaling pathways.

In addition, we identified CNK1 as a binding partner of the angiotensin II type 2 (AT_2) receptor. The AT_2 receptor is a seven transmembrane protein that plays a dual role by stimulating or inhibiting the Raf/MEK/ERK pathway depending on the cellular context. On the one hand the AT_2 receptor promotes differentiation of neuronal cells by activation of the Rap1/B-Raf/MEK/ERK pathway. On the other hand the AT_2 receptor represses growth factor-induced MAPK cascade activation via several protein phosphatases.

We found two domains of CNK1, the SAM and the CRIC domain, to interact with the AT_2 receptor. In an overexpression system we could show that this interaction is specific for the ubiquitously expressed CNK1 but not for the neuronal isoform CNK2. We characterized this interaction by analysing mutant CNK1 proteins and found mutants with increased or decreased binding affinity towards the AT_2 receptor depending on the introduced mutation. The biological significance of the interaction between CNK1 and the AT_2 receptor was further supported by coprecipitation of both proteins from murine heart extracts. We suggest that CNK1 may play a role in the AT_2 receptor-mediated signalling pathways, either in the MAPK cascade activating branch or in the repressive branch via protein phosphatase involvement.

Schematic representation of CNK proteins

Fig. 1: **Human CNK proteins contain several protein-protein interaction domains:** SAM (sterile alpha motif) domain; CRIC (conserved region in CNK) domain; PDZ (PSD-95/Dlg-1/ZO-1) domain; PH (pleckstrin homology) domain, a prolin-rich region (PRC) and a potential coiled coil region. CNK2A exhibits the C-terminal PDZ domain ligand sequence ETHV. CNK2B encodes a short splice variant of CNK2A. Numbers denote amino acid positions.

CNK1 mediates Src-dependent Raf-1 activation

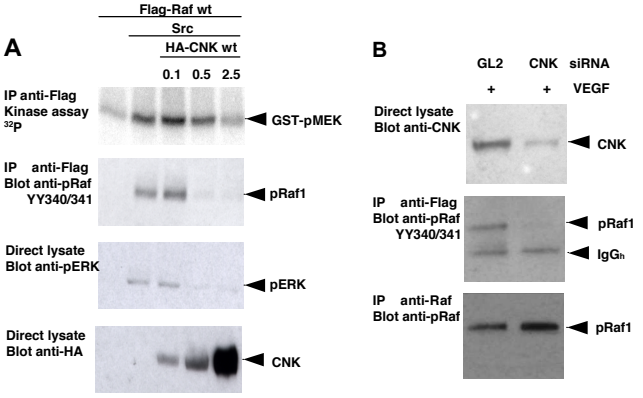

Fig. 2: (**A**) **CNK1 mediates Src-dependent Raf activation in a dose-dependent manner.** Flag-Raf wt (1 μg), Src (0.5 μg) and increasing amounts of HA-CNK wt (0.1; 0. 5, 2.5 μg) were expressed in HEK293T cells. Immunopurified Raf-1 was analysed by in vitro kinase assays using GST-MEK as specific substrate for Raf. In addition phosphorylation of Raf and ERK was analysed by the antibodies anti- pYpY340/341 Raf and anti-pERK, respectively. (**B**) **Downregulation of endogenous CNK1 impairs VEGF-dependent Raf activation.** HEp2 cells stably expressing shRNA directed against CNK1 or control shRNA (GL2) were serum-starved and treated with or without VEGF (100 ng/ml) for 15 min. Cells were lysed an immunoprecipitated with anti-Raf antibody and immunoblotted with anti-pYY340/341 Raf and anti-Raf antibody. Downregulation of CNK1 was controlled by anti-CNK1 blot.

CNK1 but not CNK2 interacts with the AT_2 receptor

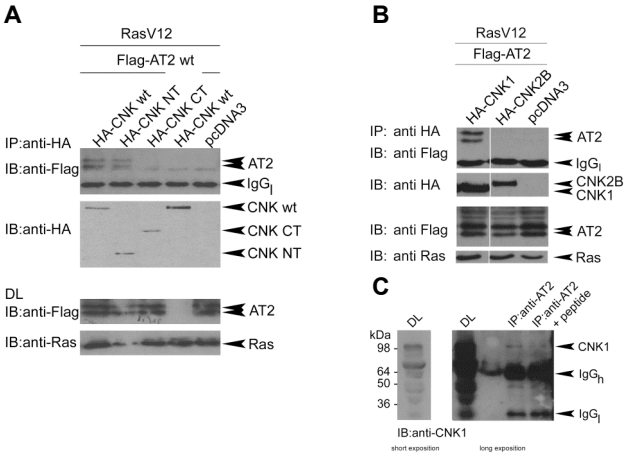

Fig. 3: (**A** and **B**) **The AT_2 receptor binds to the N-terminus of CNK1 but not of CNK2.** HEK293 cells were cotransfected as indicated. The interaction of proteins was analysed by immunoprecipitation (IP) with anti-HA followed by immunoblotting (IB) with anti-Flag antibody. The amount of precipitated CNK1 was monitored by immunoblotting with anti-HA antibody. Protein expression was examined in direct cell lysate (DL). CNK NT encompasses the SAM, CRIC and PDZ domain. (**C**) **CNK1 interacts with the AT_2 receptor at endogenous protein levels.** Heart tissue lysate was incubated with anti-AT2 antibody in the absence or presence of the

CNK1 binds to the AT_2 receptor through the SAM and the CRIC domain

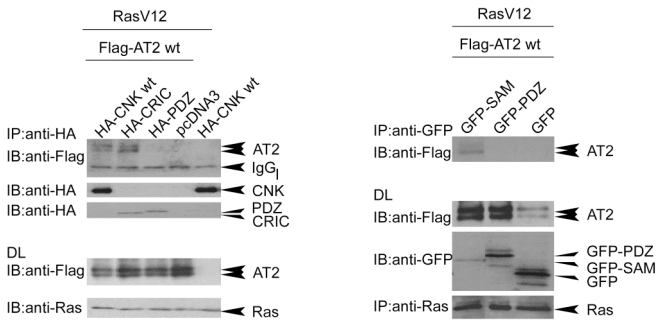

Fig.4: **The SAM and the CRIC domain mediate the interaction of CNK1 with the AT_2 receptor.** (Left) HEK293 cells were cotransfected as indicated. HA-CNK wt, HA-CRIC and HA-PDZ were immunoprecipitated (IP) with anti-HA antibody and subjected to immunoblotting (IB) using anti-Flag antibody to detect coimmunoprecipitated Flag-AT2 proteins. (Right) The SAM and the PDZ domain fused to the green fluorescent protein (GFP) or GFP alone were immunoprecipitated from cell lysates with anti-GFP antibody. Coimmunoprecipitated Flag-AT2 receptor was detected by immunoblotting using anti-Flag antibody. Protein expression levels were controlled in direct cell lysate (DL).

Mutation of the SAM or the CRIC domain alters CNK`s binding affinity towards the AT_2 receptor

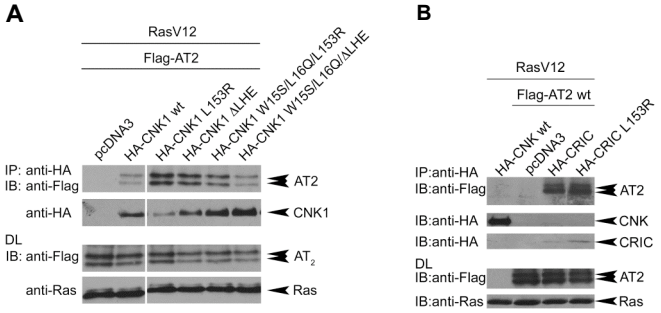

Fig. 5: (**A**) **Mutation of the CRIC domain increases SAM domain-dependent binding affinity towards the AT_2 receptor.** HEK293 cells were cotransfected as indicated and analysed as in Fig. 3. The amino acid substitutions W15S and L16Q were introduced in the SAM domain. The three amino acids Leu, His and Glu were deteled in the CRIC domain. Both types of mutations have been found in the CNK homolog from *Drosophila melanogaster*. The amino acid substitution L153R targets an Leu residue conserved in several species. (**B**) **The substitution L153R in the CRIC domain does not effect the binding of the isolated domain to the AT_2 receptor.** HEK293 cells were cotransfected as indicated and analysed as in Fig. 5A.

Putative role(s) of CNK1 in AT_2 receptor-dependent signalling pathways

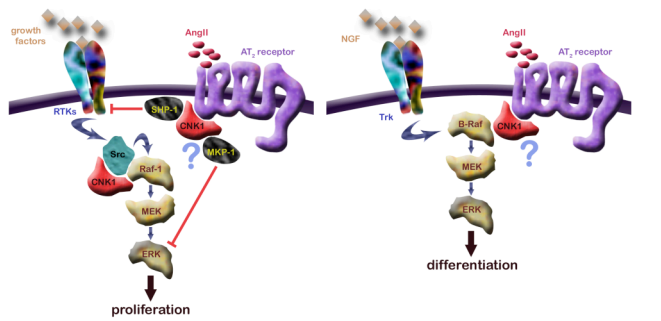

Fig. 6: **CNK1 may be involved in one or more AT_2 receptor-dependent signalling events.** (left) On the one hand CNK1 could mediate the AT_2 receptor-induced activation of protein phosphatases such as SHP-1 or MKP-1 thereby repressing the MAP kinase cascade and resulting in antiproliferative impulses. In this situation CNK1`s function would be contrary to it`s role in Src-dependent Raf-1 activation. (right) Stimulation of the AT_2 receptor with angiotensin II induces neurite outgrowth and differentiation. This process involves the Rap1/B-Raf/MEK/ERK module. Since CNK1 binds also B-Raf, it could be a modifier of AngII-induced differentiation.