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Mass-spectrometry based profiling of PKR-interaction partners identifies KSRP as a novel protein regulator of PKR

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Introduction

The RNA-dependent protein kinase (PKR) is an interferon inducible, double-strand RNA-activated protein kinase that plays a significant role in innate antiviral immunity. Due to its key role in antiviral immunity many viruses have evolved mechanisms to avoid PKR initiated effects. We and others have previously described the influenza virus non-structural protein 1 (NS1) as an antagonist of PKR. We are interested in further elucidating the role of cellular and viral factors in regulating PKR activation in the context of influenza virus infection.

We used a SILAC approach followed by LC-MS/MS analysis to identify precipitable interaction partners of PKR upon influenza A virus (IAV) wildtype or ΔNS1 infection and validated selected binding partners in independent biochemical assays.

We detected 47 cellular PKR binding proteins involved in various cellular pathways including RNA processing and stress response. We validated the interaction between PKR and a subset of candidates in co-immunoprecipitation experiments and could show that some of the identified proteins induced PKR phosphorylation upon overexpression. Hereby, the KH-type-splicing regulatory protein (KSRP) was identified as a novel regulator of PKR. Overexpression and knockdown experiments indicated that KSRP is able to support antiviral signaling by enhancing PKR activation in a process that involves direct protein-protein-interaction.

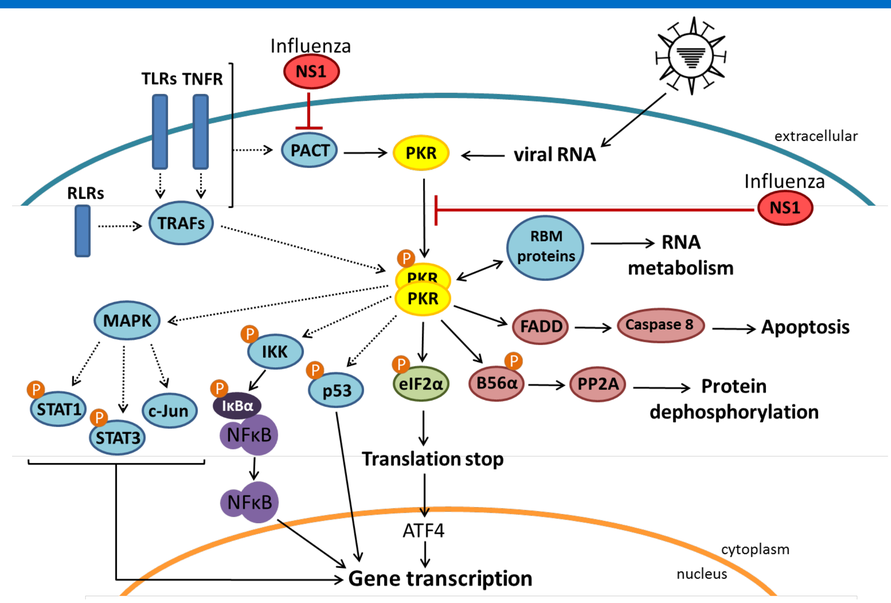

Fig. 1 The RNA-dependent Protein Kinase (PKR).

PKR is an antiviral effector protein. Upon recognition of viral RNA it leads to inhibition of translation, apoptosis and production of type I IFN. The NS1 protein of influenza A and B viruses can block PKR activation.

Methods

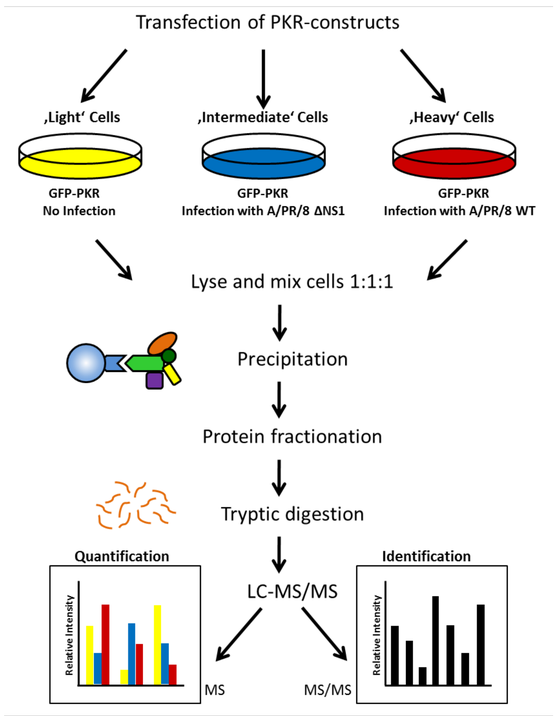

Fig. 2 Experimental setup.

293T cells were labeled by cultivation in medium containing light (R0K0), intermediate (R6K4) or heavy (R10K8) amino acid isotopes of lysine and arginine. Cells were transfected with a GFP-PKR construct and either mock infected or infected with WT or ΔNS1 IAV. After cell lysis, same amounts of proteins were mixed and affinity-precipitated with GFP-Trap® matrix. The eluted proteins were fractionated and digested with trypsin. The resulting peptide solution was analysed using an LTQ-Orbitrap (Discovery; Thermo Scientific™) equipped with a Nano-LC (Thermo Scientific™). Data was acquired using Xcalibur software (Thermo Scientific™). The MS/MS data was searched using the SEQUEST algorithm in Proteome Discoverer 1.4 (Thermo Scientific™) against the Homo Sapiens (ncbi) database.

PKR phosphorylation

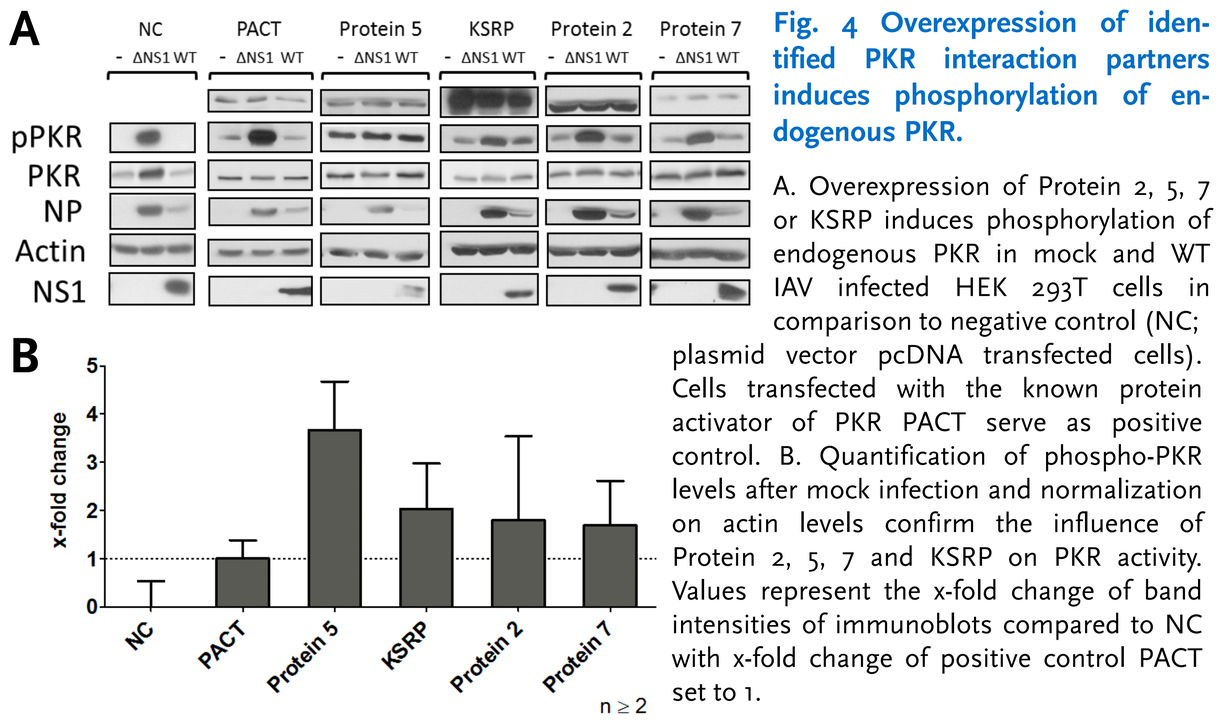

Fig. 4 Overexpression of identified PKR interaction partners induces phosphorylation of endogenous PKR.

A. Overexpression of Protein 2, 5, 7 or KSRP induces phosphorylation of endogenous PKR in mock and WT IAV infected HEK 293T cells in comparison to negative control (NC; plasmid vector pcDNA transfected cells). Cells transfected with the known protein activator of PKR PACT serve as positive control. B. Quantification of phospho-PKR levels after mock infection and normalization on actin levels confirm the influence of Protein 2, 5, 7 and KSRP on PKR activity. Values represent the x-fold change of band intensities of immunoblots compared to NC with x-fold change of positive control PACT set to 1.

Bioinformatic evaluation and validation

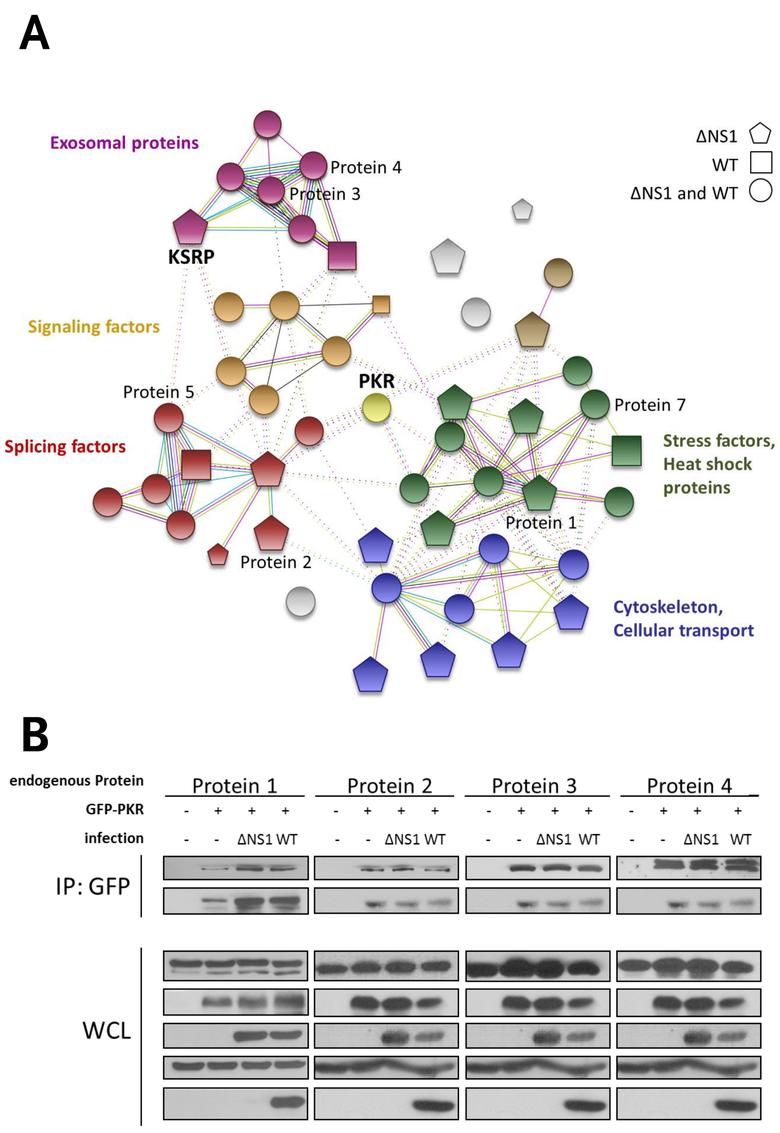

Fig. 3 Bioinformatic analysis of GFP-PKR bound proteins and exemplary validation by CoIP.

A. STRING database (STRING v10) analysis of GFP-PKR interacting proteins after infection with WT or ΔNS1 IAV with following criteria: protein found in two or more experiments, protein score ≥ 10, H/L or M/L ratio ≥ 1,5. String network was clustered (MCL algorithm = 2) to visualise related proteins. Protein clusters include exosomal proteins, RNA-splicing factors, ribosomal proteins and cellular stress factors. B. Exemplary candidates from the MS screen were validated by coprecipitation analyses of transfected GFP-PKR with the endogenous cellular proteins in HEK 293T cells. Bound proteins (IP) and cell lysates (WCL) were analyzed by SDS PAGE and immunoblotting using the indicated antibodies. Candidate proteins were chosen in a hypothesis driven process based on their molecular functions and reagents availability.

KSRP is a protein regulator of PKR

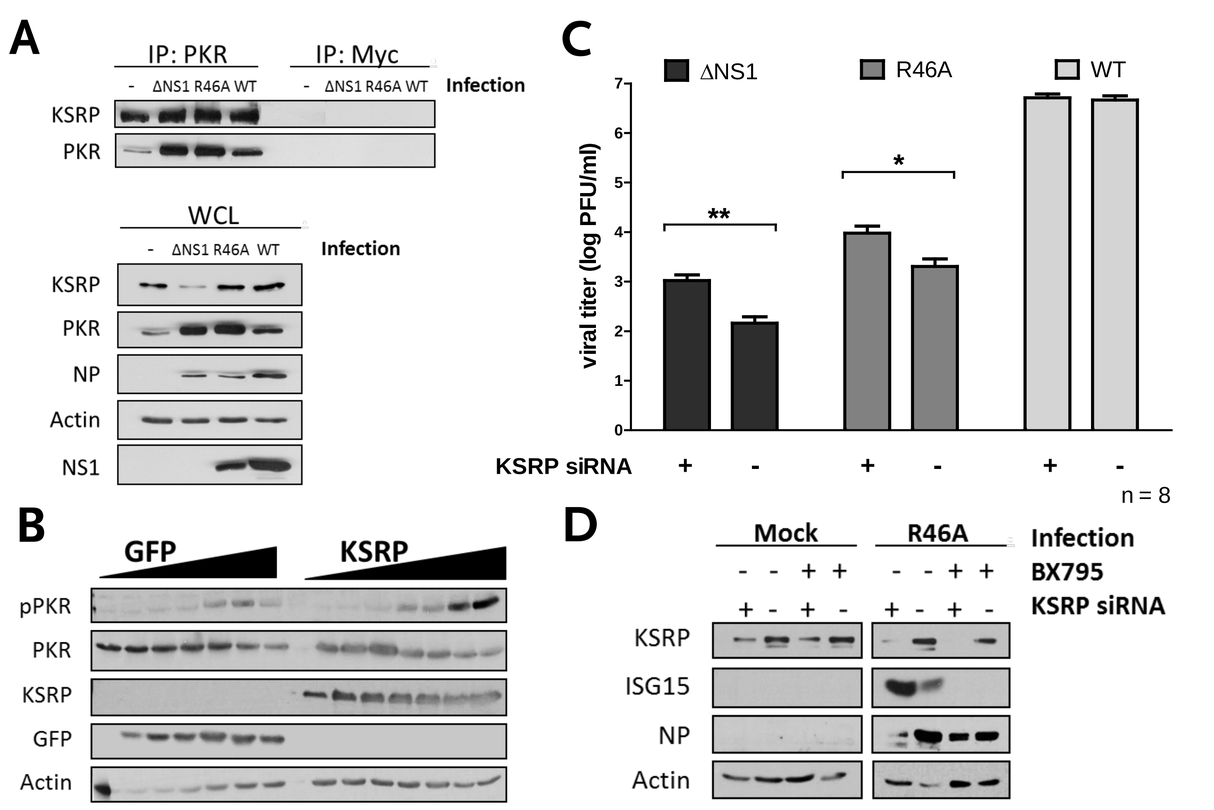

Fig. 5 KSRP interacts constitutively with PKR and is able to enhance PKR phosphorylation, which can negatively affect viral replication.

A. PKR and KSRP bind constitutively in non-infected and WT or mutant IAV infected human alveolar epithelia cells as seen by coprecipitation analysis of endogenous proteins. B. Overexpression of KSRP in HEK 293T cells induces phosphorylation of endogenous PKR in a dose-dependent manner. C + D. Viral replication of IAV mutants ΔNS1 and R46A, that are not able to inhibit PKR activation, is significantly enhanced 48hpi upon knockdown of KSRP in human alveolar epithelia cells treated with TBK1/IKKε-inhibitor BX795 to exclude effects caused by type I interferon expression. KSRP knockdown and effect of inhibitor were confirmed at 72hpi by SDS-PAGE and immunoblotting using the indicated antibodies.

Conclusion

Taken together, this study demonstrates the aptitude of quantitative mass spectrometry for elucidation of cellular antiviral response pathways. Precipitation of PKR and binding partners revealed KSRP as a novel protein regulator of PKR. The mechanism of KSRP mediated PKR activation seems to be complex and needs to be analyzed in greater detail in ongoing studies.

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