MAPKKK6 is a Negative Regulator of Apoptosis

Elisabeth Ortner and Karin Moelling

Institute of Medical Virology, University of Zurich, CH-8006 Zurich

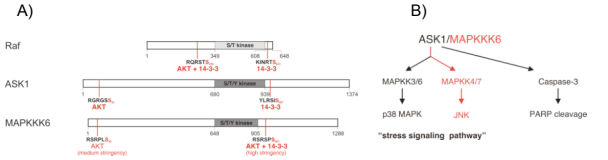
Abstract

MAP kinase kinase kinase 6 (MAPKKK6) belongs to the family of MAPKKK, which comprises 20 members including the Raf kinase. It was initially identified as an interaction partner of another MAPKKK, the apoptosis signal-regulating kinase 1 (ASK1). These two kinases share a similar structure with a central kinase domain and a C-terminal coiled coil sequence and have 45% amino acid (aa) identity. MAPKKK6 is shorter than ASK1 lacking 32aa near the N-terminus and 54aa near the C-terminus. Both kinases are ubiquitously expressed regarding tissue distribution and show a similar intracellular localization in the cytoplasm, nucleus and mitochondria. Due to the homology with ASK1, MAPKKK6 was suggested also to be an apoptosis inducing kinase. This is true for the overexpression of MAPKKK6, which leads to apoptosis similar to overexpressed ASK1. However, we show that MAPKKK6 differs from ASK1 by exhibiting an anti-apoptotic function.

Endogenously expressed and overexpressed MAPKKK6 can form homo-oligomers similar to ASK1. MAPKKK6 and ASK1 also exist as hetero-oligomers when expressed at their endogenous levels. Matched levels of overexpression of both kinases abolish induction of apoptosis after starvation. Furthermore, knockdown of MAPKKK6 by two different siRNAs to about a third of protein expression also induces apoptosis already in non-stressed cells, which is increased by stress induction by starvation, TNFα treatment or oxidative stress. This is in contrast to the behaviour of ASK1, since ASK1 knockdown results in inhibition of apoptosis activation upon stress induction. The relative levels of these two kinases decide on the biological outcome of the cell. We propose a model in which ASK1 is negatively regulated by hetero-oligomerization with MAPKKK6.

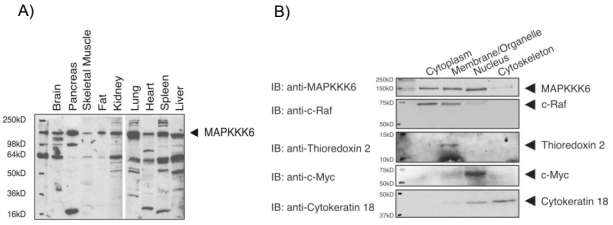
ASK1 is negatively regulated by AKT phosphorylation at Ser83 near the N-terminus. MAPKKK6 exhibits two putative phosphorylation sites near the N- and C-termini. Whether one of these sites is phosphorylated by AKT and which biological role this phosphorylation could play is currently under investigation.

Comparison of MAPKKK6 and ASK1



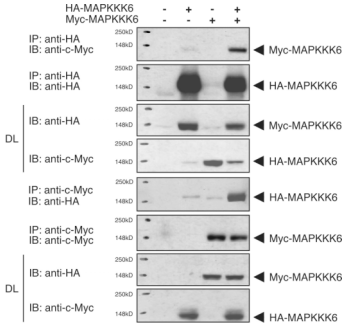
A) Sequence comparison of the MAPKKK family members Raf, MAPKKK6 and ASK1 focusing on AKT phosphorylation- and 14-3-3 binding sites. For MAPKKK6 the putative sites were analyzed by the ScanSite program. *B)* ASK1 induces apoptosis via the stress signaling pathway leading to the phosphorylation of JNK and p38 MAPK and by the activation of Caspase-3, which itself cleaves several substrates such as PARP. For MAPKKK6 only a weak activation of JNK has already been published.

Expression analyses of endogenous MAPKKK6



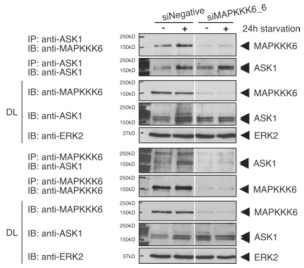
A) In a murine tissue blot MAPKKK6 was found to be ubiquitously expressed. *B)* In a subcellular fractionation assay MAPKKK6 was detected in the cytoplasmic, nuclear, and membrane/organelle fraction, which is similar to the localization of ASK1.

Homo-dimerization of overexpressed MAPKKK6



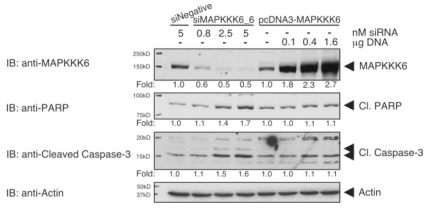
Co-immunoprecipitation analysis of overexpressed HA- and Myc-tagged MAPKKK6 in HEK-293 cells. Similar to ASK1, MAPKKK6 also formed homo-dimers.

Hetero-dimerization of endogenous MAPKKK6 and ASK1



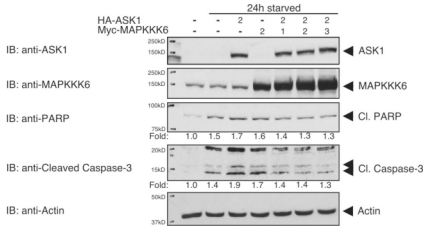
Co-immunoprecipitation analysis of endogenous MAPKKK6 and ASK1 in HeLa cells. Hetero-dimerization of these two kinases was detected in non-stressed (-) and also serum-deprived (+) cells. Specificity of interaction was verfied by siRNA mediated knockdown of MAPKKK6 (siMAPKKK6_6).

Overexpression and knockdown of MAPKKK6 lead to apoptosis



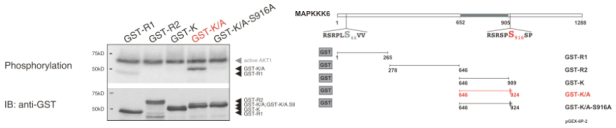
In HeLa cells knockdown (siMAPKKK6_6) and also overexpression of MAPKKK6 (pcDNA3-MAPKKK6) led to increased apoptosis after 24h serum withdrawal, which was analyzed by cleavage of PARP (Cl. PARP) and of Caspase-3 (Cl.Caspase-3) in immunoblots of direct lysates. This is in contrast to ASK1, since knockdown of this kinase prevents apoptosis after stress induction.

Concomitant overexpression of ASK1 and MAPKKK6 reduces apoptosis



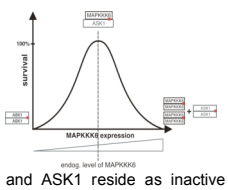
In HeLa cells overexpression of HA-ASK1 or Myc-MAPKKK6 led to apoptosis after 24h serum starvation, which could be reduced when both kinases were overexpressed concomitantly.

A GST-fusion protein of MAPKKK6 is phosphorylated by AKT in vitro



In a radioactive in vitro kinase assay using GST-fusion proteins of MAPKKK6, only the GST-construct containing Ser916 was phosphorylated by AKT (*red*), which could be inhibited by a point mutation to alanine. The GST-fusion protein GST-R1, which contains a second putative AKT phosphorylation site at Ser46, only showed a very weak phosphorylation.

Model for the negative regulation of apoptosis by MAPKKK6 mediated by hetero-dimerization with ASK1



MAPKKK6 is ubiquitously expressed and exhibits the same intracellular localization as ASK1. Overexpression of MAPKKK6 induces apoptosis as it is known for ASK1. When ASK1 and MAPKKK6 are overexpressed at matched levels, then apoptosis is inhibited. However, knockdown of MAPKKK6 also results in apoptosis.

Therefore, we propose a model where MAPKKK6 and ASK1 reside as inactive heterodimers when expressed at their endogenous levels. A change of the physiological amount of MAPKKK6 either by overexpression or by siRNA mediated knockdown causes ASK1 homo-dimerization and induction of apoptosis. A putative regulatory role of the PI3K-AKT pathway in the formation of either homo- or hetero-dimers of ASK1 is currently under investigation.