MAP4K4 A POSSIBLE NEW BIOMARKER IN CANCER THERAPY

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Abstract: MAP4K4 is a member of the germinal center kinase GCK-IV group and is involved in controlling cellular processes that include cell motility, rearrangement of the cytoskeleton and cell proliferation. MAP4K4 proved to be an upstream activator of the cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase. TNFα and p53 are two of the most important factors that have the capacity to increase the expression of MAP4K4. MAP4K4 is involved in a very complex network of signaling pathways and interactions that are involved in diseases like diabetes or cancer. The recent findings place MAP4K4 as a novel target that may provide insights into new therapies, in the effort to prevent or even treat these diseases.

INTRODUCTION

A protein kinase is an enzyme able to reassign a phosphate group from a donor molecule (usually ATP) to an amino acid residue of a protein. The mechanism used by protein kinases is involved in signal transduction for the modulation of enzymes. The activity of an enzyme can be activated or inhibited by the phosphorylation process.

Mitogen-activated protein kinases (MAPK) are components of a strictly conserved cascade of serine / threonine protein kinases that contain a Thr-x-Tyr motif within the activation loop in the kinase domain (Huangm *et al.*, 2004) and are involved in many signal transduction pathways (Johnson and Lapadat, 2002). They activate phosphorylation of transcription factors due to the signals received from the extracellular stimuli such as ultraviolet light, growth factors, cytokines and stress-inducing agents (Chang and Karin, 2001; Widmann *et al.*, 1999). There are a lot of essential cellular functions, such as differentiation (Aouadi *et al.*, 2006; Aouadi *et al.*, 2006; Bost *et al.,* 2005), proliferation (Roux and Blenis, 2004), apoptosis (Kyosseva, 2004; Willaime-Morawek *et al.,* 2003; Kolch *et al.,* 2005), that are regulated by MAPK pathways after their activation. It has been demonstrated that MAPKs play key roles in inflammation, stress responses and oncogenesis, (Huangm *et al.*, 2004), but there is recent evidence that this family is also determinant for the process of cell migration.

The main role of MAPK proteins in signal transduction, led to their involvement in the progression of cancer and autoimmune diseases. This is the reason for their election as new targets for drug development (Huangm *et al.*, 2004). The fact that MAPK pathways are very well conserved over the eukaryotic kingdom, made them eligible for the study of their function, structure and interconnectivity through genetic analysis of model organisms (Widmann *et al.*, 1999).

The complex protein interactions that involve MAPK signal transduction network has been supplemented by the recent work of Bandyopadhyay *et al*., (2010) who added new data as a resource for the future investigations. The main reaction through which all MAPK pathways act is the phosphorylation. During this cascade of events the phosphorylation of transcription factors leads to the regulation of gene expression and the phosphorylation of the cytosolic targets directs to the regulation of intracellular events. MAPKs are phosphorylated at the level of the threonine and tyrosine residues within the activation loop, through a kinase cascade, by MAPK kinases (MKKs), which in turn are phosphorylated and activated by MKK kinases (Raf and MKKK). The main purpose of these correlated cascades is the modulation of cellular proliferation and motility, cell cycle and differentiation, development, and transmission of oncogenic signals through gene transcription. This pathway is integrated in a rich net formed by correlations with a lot of other components, such as transcription factors, membrane receptors, and kinase scaffolds, and has many interactions with other activators and inhibitors of signaling (Kolch*.,* 2005).

Based on their type of activation loop, the MAPK family can be separated into three groups: extracellular signal - regulated protein kinase (Erk / MAPK), which has a Thr-Glu-Tyr motif; p38, which has a Thr-Ala-Tyr motif; and Jun N-terminus kinase (JNK), which has a Thr-Pro-Tyr motif (Johnson and Lapadat, 2002). Due to the diverse nature of the MAPK superfamily of enzymes, MAPK subfamilies ERK1 and ERK2 were the first group to be investigated with the purpose to understand MAPK signaling, but now the majority of studies are focusing on the role of the stress-activated kinases, especially p38 and JNK. There are many sequence similarities among constituents of each MAPK module used for stimulation of ERK1/2, JNKs and p38 but there is a very high fidelity and selective adaptation of each MAPK module in the processes of translation of the distinctive extracellular signals into physiological responses. Analyzing the specificity of all these processes and the importance of all interconnections inside each signaling cascade, is a detrimental problem that is now being investigated by specialists.

Lately, a lot of researchers have focused their interest on discovering new upstream kinases that modulate the downstream effector MAP kinases. Recently, a new class of MAP4Ks homologous to the Ste20 kinase (an upstream

constituent of the MAPK signaling pathway implicated in the pheromone response pathway in yeast - *Saccharomyces cerevisiae*) (Cowan and Storey, 2003), was identified analyzed and characterized.

This group of protein kinases can exert their action upstream of MAP3Ks. STE20p kinases can be separated into two groups, the germinal center protein kinases (GCK) and the p21 activated protein kinases (Cowan and Storey, 2003).

These group called MAP4K proteins bring a new level of modulation for the MAPK / JNK signaling cascade and maybe a connection to regulatory proteins that are located at the plasma membrane. The MAP4K group includes: HPK1 (Hematopoietic Progenitor Kinase-1), GCK (Germinal Center Kinase), GLK (GCK-Like Kinase), MAP4K4 (Mitogen Activated Protein Kinase Kinase Kinase Kinase 4), kinase homologous to Ste20/Sps1, GCKR (GCK-Related Kinase), (Cowan and Storey, 2003).

MAP4K4 – THE MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE KINASE 4

MAP4K4 (mitogen activated protein kinase kinase kinase kinase 4) is a member of the germinal center kinase GCK-IV group (Tang *et al*., 2006) of the sterile 20 protein (STE20p) kinases and is involved in controlling cellular processes that include cell motility, rearrangement of the cytoskeleton and cell proliferation (Collins *et al*., 2006; Zohn *et al*, 2006; Taira *et al*., 2004; Nishigaki *et al*., 2003; Hu *et al*., 2004; Wright *et al*. 2003).

The studies that investigate MAP4K4 have demonstrated that MAP4K4 is an upstream activator of the cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase (Collins *et al*., 2006; Zohn *et al*, 2006; Wright *et al*. 2003; Bouzakri and Zierath, 2007)

For the first time, MAP4K4 was identified in correlation with NCK adaptor protein 1 (NCK) which is a receptor tyrosine kinase adaptor protein (Su *et al.,* 1997). Su *et al*. demonstrated through their *in vitro* expression study that the MEKK1, MKK4 and JNK cellular signaling cascade is activated by MAP4K4. Later, the activation of JNK by MAP4K4 was confirmed in TNFα signaling in human cell lines.

TNFα is a cytokine involved in inflammation and in insulin resistance by decreasing the expression of PPARγand GLUT4 glucose transporter. This action has been demonstrated to be mediated also by MAP4K4.

Tesz *et al.* (2007) showed that TNFα stimulates the mRNA expression of MAP4K4 through a mechanism that involves its receptor TNFR1 and aims the transcription factors cJUN and ATF2.

In their study on cultured adypocites, Tesz *et al.* (2007) show that while TNFα up-regulates the expression of MAP4K4, it has little or no effect on the expression of the protein kinases MKK4, MKK7, p38 SAP kinase, ERK1/2, JNK1/2. They also prove that other cytokines such as LPS, IL-1β and IL-6 have no effect upon the mRNA expression level of MAP4K4 in the same conditions in which TNFα increases its mRNA expression level 3 times. By inhibition with siRNA of the two receptors of TNFα - TNFα receptor 1 (TNFR1) and TNFα receptor 2 (TNFR2), the authors showed that only TNFR1 acts as a mediator of the MAP4K4 gene expression up-regulation. This TNFR1 receptor is also involved in the stimulating effect that TNFα has upon the phosphorylation of JNK1/2 and p38 SAP kinase and their downstream transcription factor substrates cJUN and ATF2. In conclusion, TNFα which modulated the expression of MAP4K4 had a much stronger effect on the phosphorylation of c-JUN and ATF2, than the effect of the other citokine IL-1β, which did not modify the mRNA expression of MAP4K4. Tesz *et al*., proved that TNFα is the only cytokine among the ones they studied with the capacity to increase

the expression of MAP4K4 as a member of its signaling pathway and also MAP4K4 is the only MAP kinase they found to have this type of response to the action of TNFα.

Another key factor that was found to up-regulate MAP4K4 and to activate the JNK signaling pathway directing to apoptosis of the cells is p53 (Miled *et al.*, 2005). p53 is a tumor suppressor gene that modulates cell response to stress and is involved in cell progression in cancer. The activation of p53 stops cell cycle in G1 phase and can lead to senescence or apoptosis. That is why mutations of p53 are correlated with the majority types of cancer. Miled *et al.,* showed that MAP4K4 has four p53 binding sites. When p53 protein binds to these sites it up-regulates the mRNA expression of MAP4K4 gene 2 folds, and determines an increased level of the phospho-c-Jun protein through the activation of JNK signaling pathway. Also, the siRNA knockdown of MAP4K4 led to a reduction of p53 induced apoptosis. All these finding suggest that JNK signaling pathway is involved in p53 induced apoptosis through the modulator effect of MAP4K4.

JNK signal transduction pathway in which MAP4K4 is involved, is implicated in multiple physiological processes. JNKs were originally identified as the major kinases responsible for the phosphorylation of c-Jun, leading to increased activity of the AP1 transcription factor. Currently, there are new nuclear transcription factors that are also known to be targets: ATF2 Myc, Elk1, SMAD3, NFAT4, p53, MADD, DPC4.

All these transcription factors respond to different stimuli such as different types of stress, citokines or growth factors by modulating the expression of different genes. The activation of this signaling pathway can lead either to apoptosis of the cell or to tumorigenesis and inflammation.

Clarifying all the interactions and mechanisms that function inside this signaling pathway can lead to new therapies for the diseases in which this pathway is involved, including cancer and diabetes.

MAP4K4 A POSSIBLE TARGET FOR THERAPY

MAP4K4 is involved in a very complex network of signaling pathways, interactions and interconnectivities and its multiple implications in this net of mechanisms is yet to be discovered.

Yao *et al.* analyzed the MAP4K4 cDNA and they found two isoforms of the corresponding protein, one of the isoforms had a deletion in the region that contains two proline rich domains. The long isoform containing the two proline rich domains was predominantly expressed in the brain and the short isoform carrying the deletion was specific to other types of tissue such as human liver, skeletal muscle, and placenta (Yao *et al.,* 1999). The authors concluded that this variety of expression of different isoforms in different tissues could indicate a tissue or cell specificity of functions and mechanisms regulations.

MAP4K4 AND ITS ROLE IN CELL MIGRATION

Su *et al.,* (1998) have demonstrated that the gene MAP4K4 is required for *Drosophila* flies dorsal closure. Also in mice, the deletion of MAP4K4 determined the lack of migration of the mesodermal cells during gastrulation (Xue *et al.,* 2001). All these developmental processes are dominated by cell migration. In later studies, inhibition of MAP4K4 by siRNA transfection reduced the capacity of cancer cell lines to migrate (Collins *et al.,* 2006). However, the results about the activated pathway are controversy because some studies show that MAP4K4 acts

upstream of JNK, but others mention the activation of p38 in this process. Unfortunately the null MAP4K4 mice cannot survive, and because of that the role of MAP4K4 could not overcome the *in vivo* embryogenesis stage.

MAP4K4 AND THE METABOLIC REGULATION

Tang *et al.,* (2006) have recently discovered that the kinase MAP4K4 is a negative modulator of adipogenesis. They trasfected cultured adipocytes with siRNA corresponding to all the protein kinases expressed in adipocytes and they discovered that MAP4K4 is one of the four negative regulators of the insulin-responsive glucose transport. By attenuating MAP4K4 with siRNA, they increased the expression of PPARγ, the capacity of adipocytes to store triglycerides and the insulin stimulated transport of glucose. The experiments also proved that the silencing of MAP4K4 protected the inhibitory effects of TNFα on the expression of PPARγ and GLUT4 genes. This particular finding shows the involvement of MAP4k4 in mediating the effect of TNF α in adipocites.

The same function was noticed in human muscle explans (Bouzakri and Zierath, 2007). MAP4K4 activated ERK1/2 and silencing of MAP4K4 prevented the event of insulin resistance induced by the TNFα citokyne.

All these findings are also confirmed through genomic investigations. In a 5914 single nucleotide polimorphysm study on 1344 individuals Elbein *et al*., (2009) discovered MAP4K4 among 11 potential candidate genes correlated with type 2 diabetes in African American families.

MAP4K4 AS A MEDIATOR IN THE INFLAMMATORY PROCESSES

It has been demonstrated that MAP4K4 is a kinase involved in the activation of T-cells. Mack *et al.* (2005) could prevent the activation of primary mouse T-cells, by silencing MAP4K4. The authors showed that MAP4K4 is necessary for the control of the activation of TNFα promoter, but the mechanism of this control wasn't completely characterized.

Earlier, Yao and his collaborators have shown, using dominant-negative MAP4K4 mutants on 293T cells, that MAP4K4 mediates the TNFα-induced JNK activation (Yao *et al.,* 1999).

All these data show that MAP4K4 is involved in the regulation of TNFα signals in the immunological cells. Taking into account that TNFα cytokine has an important role in insulin resistance, MAP4K4 could be an interesting study target for metabolic diseases.

MAP4K4 IN CANCER

Metastasis is the biggest cause for death in cancer patients. Cell migration and motility are highly correlated with tumor invasion and metastasis. Later studies have revealed that many members of key signaling pathways involved in cell migration are also activated in cancer cells by overexpression or different types of mutations (Collins *et al.,* 2006). This is why, understanding the mechanisms behind tumor cell motility can lead to new clues in analyzing the insights of metastatic development.

MAP4K4 proved to be highly overexpressed in different types of cancers, such as ovarian cancer, hepatocellular carcinoma, lung cancer, pancreatic or prostate cancer (Collins *et*

al., 2006; Wright *et al.,* 2003; Han *et al*., 2010; Liang *et al.,* 2008). In these types of tumors MAP4K4 is associated with the processes of cell migration, invasiveness and adhesion.

The experiments on SKOV-3 ovarian carcinoma cell line (Collins *et al.,* 2006) show that the kinase MAP4K4 is involved in the motility of cancer cells by activation of JNK signaling pathway in this type of cancer. Collins *et al*. investigated the three possible transduction pathways on which MAP4K4 could direct its effect: c-Jun N-terminal kinase (JNK), p38, and Erk (1/2). The knockdown of MAP4K4 by specific siRNA did not have any effect on the phosphorylation of p38 or ERK, but a highly significant decrease of the phosphorylation of JNK could be noticed. The authors could not determine the intermediate kinases implicated in the activation of JNK by MAP4K4 and the downstream factors through which MAP4K4-JNK mediates its effects.

Recombinant retroviruses based experiments on HepG2 cultured cells show that knockdown of MAP4K4 in this type of hepatocarcinoma cells inhibits the adhesion and cell growth, compared with the control cultures that had no inhibition of MAP4K4 expression (Han *et al*., 2010). MAP4K4 was also found to be highly overexpressed in tumoral versus nontumoral adjacent tissue in hepatocellular carcinoma patients (Liu *et al.,* 2011). MAP4K4 overexpression could be correlated with worse overall survival and high recurrence rate, larger tumor size, metastasis and advanced tumor stage. The *in vitro* knockdown experiments on HepG2 and Hep3B HCC cell lines highlight the role of MAP4K4 in activating proliferation of the cells, stimulating cell cycle and inhibiting apoptosis and repressing multiple signaling pathways such as JNK and NFκB. *In vivo* silencing of MAP4K4 determined a retarded tumor xenograft growth (Liu *et al.,* 2011).

MAP4K4 was found to be among the 42 genes up-regulated in nonmelanoma skin cancer, in a microarray based study verified through quantitative real-time PCR, on normal versus tumoral tissue biopsies (Nindl *et al.,* 2006).

Microarrays validated through quantitative real-time PCR and immunohistochemical analysis of the protein expression in formalin-fixed, paraffin-embedded colorectal tumour samples, found MAP4K4 to be a component of the five-gene signature as a potential predictor of lymph node metastasis and overall survival in colorectal cancer patients (Hao *et al.,* 2010).

MAP kinases pathway was recently discovered to be involved in the progresion and metastatic process of prostate cancer (Chandran *et al.,* 2007). Comparing the expression of certain genes in primary tumors and metastatic prostate cancer samples, Chandran and his collaborators found MAP4K4 among the 3 fold overexpressed genes, correlated with the progression and metastasis of the tumors.

Little is known about the involvement of MAP4K4 in the progression of pancreatic cancer. Recent studies show that members of the MAP kinases family such as MAP4K4 and MAPK9 have an aberrant expression in pancreatic ductal adenocarcinoma (PDAC), (Ammerpohl and Kalthoff, 2007). The microarray based study on a group of 36 romanian patients with PDAC revealed MAP4K4 to be one of the overexpressed genes correlated with survival in tumor compared with normal pancreatic tissue (Badea *et al*., 2008).

After discovering the TNFα – MAP4K4 interaction pattern in human skeletal muscle, Bouzakri *et al* (2009) investigated the effect of TNFα treatment on primary beta pancreatic cells. They showed that by treating mouse primary beta cells with TNFα, MAP4K4 gene had a correlated increased mRNA expression. Also, the knockdown of MAP4K4 by siRNA transfection, inhibited the TNFα induced peripheral insulin resistance on beta cells (Bouzakri *et al.*, 2009).

Liang *et al.* (2008), investigated the expression of MAP4K4 protein in 66 stage II PDAC, and their pair benign tissue, 48 chronic pancreatitis and 14 normal tissue samples. They found that MAP4K4 was overexperssed in 46% of the PDAC samples, but also in 23 % of chronic pancreatitis tissue samples, these results indicating that MAP4K4 could be correlated not only with the stage II PDAC but also with chronic pancreatitis. The authors could also correlate the MAP4K4 protein overexpression in stage II PDAC with poor overall survival, metastasis and high rate of tumor recurrence, larger tumor size and higher number of positive lymph nodes. They also suggested MAP4K4 as a prognosis marker for stage II pancreatic ductal adenocarcinoma (Liang *et al.,* 2008; Liang *et al.,* 2009).

CONCLUSIONS

MAP4K4 was found to be involved in cell migration, proliferation and adhesion but also in the inflammatory processes and metabolic regulation signaling pathways. Malignancies require events like cell motility and migration, and these processes are activated by TNFα through MAP4K4. These findings could be the premises that lead to a possible role of MAP4K4 in regulating tumor invasion (Collins *et al*., 2006; Wright *et al*., 2003). MAP4K4 could regulate the cytoskeletal elements involved in cell migration and adhesion, so the role of this kinase could extend not only into cancer cells but in modulating the normal function of moving cells like myeloid lineages.

The recent findings place MAP4K4 as a novel target that may provide insight into new therapies, in the effort to prevent or even treat many metabolic diseases like diabetes or even multiple types of cancer such as colon, prostate, breast, ovarian, pancreatic or hepatic cancer.

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