MEKK1 AND MEKK3 INVOLVEMENT IN TNF-alpha SIGNALING IN CERVICAL CARCINOMA HELA CELLS

by
SERKAN İSMAİL GÖKTUNA

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
August 2005

MEKK1 AND MEKK3 INVOLVEMENT IN TNF-alpha SIGNALING IN CERVICAL CARCINOMA HELA CELLS

APPROVED BY:

Prof. Dr. Hüveyda Başağa …….
(Thesis Supervisor)

Assist. Prof. Dr. Alpay Taralp …….

Assoc. Prof. Dr. Batu Erman …….

DATE OF APPROVAL: …….

ABSTRACT

In this study, we have investigated the role of MEKK1 and MEKK3 in response to tumor necrosis factor-alpha (TNF-alpha) induction. It was known that MEKK1 and MEKK3 are good candidates for this moderation and they have been already shown to act in the downstream of TNF-alpha signaling or related cytokine responses. For these reasons, we studied MAPK activities and nuclear factor-kappaB (NF-kappaB) activation in response to TNF-alpha induction in the presence and in the absence of MEKK1 and MEKK3 to ascertain their involvement in the overall picture.

In untransfected cells, we obtained MAPK profiles upon TNF-alpha treatment. JNK, p38 and ERK MAP kinases activation profiles via phosphorylation was obtained. Then we observed NF-kappaB activation via IkappaB subunit degradation. In order to activate or exclude MAP3K molecules, continuously active and dominant negative forms of the MEKK1 and MEKK3 were overexpressed to understand their roles on MAPK activities and NF-kappaB activation in cervical carcinoma HeLa cells.

MEKK1 was found to be involved as the moderator of the TNF-alpha signaling in the upstream of MAP kinases. Termination of JNK phosphorylation in dominant negative MEKK1 overexpression was a good evidence for this phenomenon. On the other hand, MEKK3 overexpression gave us clues about its functional similarity to MEKK1 in this system but these results should be clarified with further experimentation. Our findings suggest that both MEKK1 and MEKK3 are involved in the TNF-alpha signaling in the upstream of MAPKs and NF-kappaB which is an important result to understand the nature of signaling events in cell death or cell survival.

ÖZET

Bu çalışmada MEKK1 ve MEKK3'ün tümör nekroz faktörü-alfa (kısaca TNF-alfa) uyarımındaki rolü araştırıldı. MEKK1 ve MEKK3 proteinleri TNF-alfa ve benzeri sitokin sinyal mekanizmalarında rol oynadıkları bilindiğinden dolayı bu moderasyon mekanizmasının en uygun adayları arasında yer almaktadırlar. Bundan dolayı bu proteinlerin varlığında ve yokluğunda MAPK profilleri ve nükleer faktör-kappaB aktivasyonu üzerindeki değişimleri gözlemlemeyi ve bu proteinlerin TNF-alfa sinyal mekanizmasında aldıkları rolleri tayin etmeyi amaç edindik.

İlk olarak TNF-alfa uyarımında MAPK ve NF-kappaB değişimleri transfekt edilmemiş hücrelerde gözlemledikten sonra devamlı aktif ve dominant negatif MEKK1 ve MEKK3 formlarını serviks karsinom HeLa hücrelerinde aşırı derecede ifade ettirerek (overexpression) bu proteinlerin MAPK ve NF-kappaB etkinleşmesindeki rolü incelenmiştir. JNK, p38 ve ERK MAP kinazlarının fosforilasyonla aktivasyon profilleri incelenmiş, NF-kappaB aktivasyonun iyi bir göstergesi olan IkappaB alt ünitelerinin fosforilasyonu ve yıkımı gözlenmiştir.

Bu çalışmalardan MEKK1 proteininin TNF-alfa sinyal mekanizmasında MAP kinazların üst kademesinde etkili bir moderatör olduğu bulunmuştur. Bunun en iyi kanıtı olarak dominant negatif MEKK1 geninin aşırı ifadesinde JNK fosforilasyonunun tamamen kesildiği gözlenmiştir. Diğer tarafta MEKK3'ün de benzer şekilde etkili olduğu konusunda ipuçları elde edilmiştir. Bu bulgular daha ileri çalışmalar ve kanıtlarla kesinleştirilecektir. Bu bulgular bize MEKK1 ve MEKK3 aynı anda TNF-a sinyal mekanizmasında MAPK ve NF-kappaB etkinleşmesinin üzerinde etkili olduklarını düşündürmektedir ki bu sonuç hücre ölümü ve kurtuluşunda etkin sinyal mekanizmalarının daha iyi anlaşılmasında bizlere faydalı olacaktır.

ACKNOWLEDGEMENTS

First of all, I am grateful to Prof. Hüveyda Başağa for her guidance and supervision throughout my study. Moreover, she provided such a good research atmosphere that promoted my creative and practical skills in research. I am thankful to her since she gave us the opportunity to be involved in her studies. Besides, it was a great pleasure to work with her and I consider that the experience I have obtained here will have utmost importance in my future research career.

Moreover, I am grateful to Dr. Alpay Taralp, Dr. Batu Erman and Dr. Uğur Sezerman for giving their time, assistance, guidance and support in this study and throughout my stay at Sabancı University. Especially, Dr. Alpay Taralp has promoted my enthusiasm and self-confidence in scientific works. Moreover, he encouraged me against any problems I have faced with his perfect personality that I chose as a model for my future academic life.

I am also very thankful to Dr. Özgür Kütük, since he helped me like a co-advisor. I didn't only benefited from his theoretical and practical knowledge on the subject while planning and concluding the project but also he helped me in such a way that an elder-brother does. He also taught me the responsibilities of a researcher in the social context. I am grateful to him for his giving time, sharing his experiences and being patient towards me.

My special thanks go to my beloved friends Çetin Baloğlu and Günseli Bayram Akçapınar. They always supported me technically and emotionally and they have one of the greatest shares in completing this work.

I haven't forget my six years long classmates, roommates, labmates, homemates, and most importantly my friends at the same time; Işıl, Sema and İbrahim. We have shared lectures, laboratory sessions, living in the same house and many other occasions during that years. They also assisted and supported my work in every way they were able to. I am very grateful to them for their unforgettable friendship.

Additionally, I thank to Ufuk Bayburt and Özgür Şahin since they have always dealt with my problems and guided me in different cases.

I also thank to Özgür Gül, Fahriye Ertuğrul, Özlem Sezerman, Mehmet Alper Arslan, Elif Damla Büyüktuncer, Ayşegül Verim and others from the Biological Sciences for their friendship and helps.

Finally, I thank to my family since they grew me up and they did everything to provide a good education, social environment, happy and peaceful family atmosphere for me regardless of their economic possibilities. Hopefully I won't disappoint them in the future. Of course, I dedicate my thesis to my dear family.

INDEX

INDEX OF FIGURES

1. INTRODUCTION

It is well established that signal transduction mechanisms are very significant since all the communicative interactions of the cell with outside and within interior occurs by signaling cascades. Hormones, chemical ligands or electric signals may induce signaling facilities. Hence, signal transduction is used in communication between different organ systems, tissues and cellular groups. Furthermore, signaling responses may only affect any single cell or a group of subcellular compartments within. Signal transduction is an important issue in development, immune responses, metabolic pathways and other versatile cellular responses leading to cell growth and survival. For these purposes, many laboratories throughout the world devoted themselves to identify signaling pathways in a vast amount of mechanisms concerning cell cycle and its regulation; programmed cell death (apoptosis) in cancer and inflammatory diseases; steps in embryonic development; immune responses in infection, cancer or inflammation; neuronal responses and neurodegenerative diseases. In all of these subjects and in many others, signaling and signal transduction is the key function to understand these mechanisms and to develop therapeutic solutions.

Each of the above processes is controlled via myriads of signaling pathways in the cells. Clearly seen, any cancer may not result without the accumulation of different errors in the cells since cells have different mechanisms to prevent any disturbance towards proliferation. A single mutation, deletion or defect usually is not enough for tumorigenesis. Indeed, there should be at least a few others to omit cellular control

mechanisms like adhesion, DNA repair, interferon signaling or apoptosis, which may prevent any malfunction leading to proliferation, tumorigenesis or malignancy.

Here we will mainly focus on the signal mechanisms leading to cervical cancer in HeLa cells. To understand the signaling pathways leading to cancer, we will start from the signaling pathways in relation to cell cycle since we know that cell cycle control lies at the heart of tumorigenesis. For this purpose, in the following parts we will review cell cycle and its control, apoptotic machinery in response any disturbance to cell cycle regulation and the most significant signaling events in cancer formation. We will conclude our discussion with TNF-α signaling and the involvement of MEKKs in the overall picture.

2. OVERVIEW

2.1. Cell Cycle

From the studies of biology pioneers like Pasteur and Wirchow, it had become clear that all organisms come from preexisting ones, while they also showed that all organisms are consisted of cells. As a consequence, one can also say that any cell should also come from an existing cell and this sequence can be extended to the beginning of the life. From unicellular to multicellular, all organisms use the same strategy to increase their number. This is what we know as cell division. Cell division is controlled via myriads of different mechanisms. In the development, growth, cell fate determination and in the death, either internal or external signals in terms of chemicals, hormones or various physical stresses act on the cell to force it to give decisions concerning its fate. Here we will shortly describe the importance of these signals and then we will continue with details of cell cycle and its control [Alberts et al. 2001].

Signaling cascades sit on the top of many other mechanisms concerning cell growth, survival, proliferation and programmed cell death. By versatile signal transduction pathways these mechanisms are regulated. Early questions in the signal transduction studies were addressed to the cell cycle and its regulation. Different phases in the cell cycle, diverse regulatory molecules and the mechanisms they used were

extensively studied by developmental biologists. When it had become clear that cell cycle and its control is important in proliferation of the cell and the cancer formation, then cancer researchers were also involved in the studies to understand the cell cycle regulation and the borderline between survival and death.

Cell cycle can be divided into different parts from G_1 to M phase. The parts other than mitosis can be grouped as interphase. In the interphase, the cell makes appropriate preparations for the mitosis. These preparations include the DNA replication and increase in the amount of cellular proteins that the two daughter cells will be using. Moreover, proteins that will govern the mitosis and its control are continuously produced and degraded. Not every kind of cell goes into mitosis while some cell types are arrested in a phase called G_0. Nerve cells are a good example this kind of dormant cells. Every step in cell division is controlled with a diverse array of molecules that we will describe shortly. In these steps different signal mechanisms take part and each of these mechanisms are important in giving decision to differentiate, proliferate or to turn into tumors.

2.1.1. Phases of the Cell Cycle

2.1.1.1. Parts of the interphase

As we mentioned above the cell cycle consists of several distinct phases which are classified by the physiologic and morphologic differences occurring during the cell growth. After a daughter cells separates from its sister it undergoes a similar cycle of events that ends up with two identical daughter cells again (in meiosis the case can be different). Here we will go into further details in the cell cycle and its control.

The overall period between two divisions is called as interphase as we described previously. Interphase is marked by continuous growth and accumulation of proteins

that will be used later on in the daughter cell growth. The first growth phase is called as G_1 and it is marked by increase in cell volume and mass while there is no significant change in the genetic material. After a desirable level of growth is attained the cell has two fates to choose from; either it ceases growth and goes into a dormant phase know as G_0 or it follows another cycle to divide. Here the decision is given at an important phase called as G_1 checkpoint. This checkpoint is governed by myriad of molecules ending up with cyclin D and their corresponding kinase, CDK4/6 (in vertebrates). This complex determine the exit from G_1 phase while cyclin A and pair CDK2 is involved progression from G_1 to S and further progress in S phase [Herr 1993]. However, we will mention about the role of cyclins and their kinases after describing overall picture. Once, the decision is given then there is no return and cell enters into a new phase; S phase (S stands for DNA synthesis) [Alberts et al. 2001; Murray et al. 1993; Lewin et al. 2000].

The S phase in the cell cycle has a characteristic property of increase in the amount of the genetic material in a very short time. After the phase is attained, cell concentrates on vigorous DNA synthesis to form two copies of the genome that will be shared by the two daughter cells. In mitosis this process is unique and provides conservation of genetic material and somatic or gametocytes cells obtain the same number of chromosomes as their parent cells have (2n in only diploid organisms whereas in haploids, triploids, hexaploids etc. the process also leads to conservation of genetic material in n, 3n, 6n chromosomes). However, in meiosis the second S phase is omitted in consecutive divisions so that the number chromosomes are halved, which is the process that secures the conservation of the amount of genetic material after fertilization. After completing the DNA synthesis another important decision remains to be made in proceeding to next step in the cell cycle. However, the cell should guarantee that that there is no disruption in the structure of the DNA and the replication is made properly, if the check fails then the cellular mechanisms corrects any errors that prevent advancement. Otherwise, it is assumed that the molecular stability of the replicated and template genome is attained and the cell is ready for a new phase [Hayles et al. 1994; Nurse 1994].

The next phase is called as G_2 phase and it is similar to the G_1 phase in terms of "growth". Nevertheless, this time the cell has two fold DNA of a G_1 cell. Still expression of the proteins that the daughter cells will use continues while the focus in

on the expression of the proteins that will be used in mitosis/meiosis. Here comes another important instance for the fate of the cell, the G_2 checkpoint, which will further determine whether cell go into division or it will be arrested and die eventually [Hartwell and Weinert 1989]. The decision is totally dependent to another pair of proteins, cyclinB1 and its kinase CDK1. The downstream activity of this complex renders many different physiologic and morphologic events in mitosis. Also degradations of other cyclin/CDK complexes are involved in the overall picture as we will describe in cell cycle control. Thus, at the G_2 checkpoint, the fate of the cell is determined and it is now ready to undergo cell division to complete its cycle [Sherr 1994].

2.1.1.2. Mitosis

The early studies on cell division addressed either ovary maturation or embryonic development in the animals. The cell is division is considerably different from the early phases and it is still different in mitosis and meiosis. The mitosis is also called the "M" phase as we see in the figure 2.1. The cyclin B1/CDK1 complex (also known as MPF) triggers the events in the mitosis [Dunphy et al. 1988]. This complex appears near the end of G_2 phase and remains intact to the end of M phase. The induction of cell division genes by the MPF leads to diverse morphologic events in the cell. First, in the metaphase, chromosomes condense, then nuclear envelope disappears, the centrosome duplicates while forming spindle fibers. In the metaphase, sister chromatids are attached to these spindles from their kinetochores and they are aligned on the equatorial plane by the end of prophase. Until that time MPF should also induce the expression of an important factor called as APC (anaphase promoting complex). This control system can also be considered as another checkpoint and specifically called as M exit. APC induction leads to progression in the M phase and the aligned sister chromatids are detached and pulled towards the poles of the cell. Finally in the telophase the nuclear envelopes reform around the chromatids while chromatids unpacked and form chromatin. The cytokinesis may be continuous in the M phase or it may be limited to telophase and triggered by the APC [Zachariae and Nasmyth 1999]. In the end cyclin B is degraded and CDK1 is released from the complex. Starting from the association of cyclin B with CDK1, the M phase involve great physiological events

ending up with two identical daughter cells which are ready to continue cell cycles for other rounds. In the next chapter we will look the details of the events governing the cell cycle control.

2.1.2. Cell Cycle Control

Control of the cell cycle is essential for any organisms. While it guarantees the passage of genetic material to their offspring, it is also indispensable to avoid deadly consequences like apoptosis or cancer. In unicellular organisms it is only important in the continuation of the genetic material in the progeny whereas it is essential in multicellular organisms to survive. Loss of the cell cycle can lead cell one of the different scenarios; either it should kill itself for the sake of the whole organism or should try to repair the problem causing mechanisms. When the control over this decision is also lost, then the cell may proliferate and form cancers depending on the cell type. For these purposes, the cell cycle control is the central issue in the cellular mechanisms and it should be handled with care in every cell for the survival of the organism.

Early studies to understand the cell cycle was performed by the use of amphibian oocytes and sea urchin embryos since one can already see the changes in the cell easily by the use of microscope. Moreover, manipulating such large cells was far much easier than small oocytes of other animals. Once the different phases in the interphase are determined via microscopic observations of different morphological states, the next step was to determine the physiological events leading to these changes. The first tool was the injection of cytoplasm samples from different phases. By the use of the injection experiments it was understood that some factors in S, G_2 and M phases were able to induce either progression through that phases or directly into mitotic division. One of the important molecules was described as MPF (maturation promoting factor) which stands for the maturation of the oocyte. In this experiment, the cytoplasm taken from

prophase II arrested oocytes were injected into immature eggs and it induced the maturation of the egg [Masui et al. 1971; Nurse et al. 1976; Blow and Nurse 1990]. The factor is called as MPF due to this activity. Similarly, by the use of fusion experiments, where two oocytes with different cell cycle phases were fused together, it was understood that there are more than three factors involved in the cell cycle regulation [Rao and Johnson 1970; Gautier et al. 1988]. Later on genetic studies utilizing fusion (*S. pombe*) and budding yeast (*S. cerevisae*) promoted our understanding about the molecular basis of the control [Johnston et al. 1977; Simanis et al. 1986]. Now we know that the MPF is cyclin B/CDK1 complex and it is involved in the induction of APC (anaphase promoting complex) [Zachariae and Nasmyth 1999]. Cyclins were among the very first molecules that regulate the function of cell cycle. From different experiments varying from gene knockout studies to yeast two hybrid, we are now able to know hundreds of others molecules and yet dozens of others to be identified. Below we will start our discussion of the cell cycle control with cyclins and proto-oncogenes and we will continue our survey of the cell cycle control in other chapters in depth.

2.1.2.1. Cyclins

First potent molecules in these signal mechanisms were the ones that regulate cell cycle checkpoints. These molecules are known as cyclins and their associated kinase counterparts [Evans et al. 1983; Simanis and Nurse 1986; Draetta et al. 1989]. Both cyclins (regulator) and Cdk (cyclin dependent kinases-catalytic subunit) control and regulate different phases in the cell cycle. Once they are associated they promote the progression of the cell from one phase to another. In this way they utilize the advancement of the cell towards division [Reed 1992; Sherr 1994]. Once cancer scientists know cell division has an important step in proliferation, they addressed their questions to the molecules that regulate the function of cyclins and other regulatory proteins that are important in the cell cycle. For example, CDK2/cyclin E and CDK2/cyclin A complexes control the S phase entry and progression and cdk1 complexes that mediate mitosis [Reinmuth et al. 2004; Cordon-Cardo et al. 1995]. On the other hand, the cdk inhibitor kinase p21, regulate cdks to maintain the completeness

of each cycle before progressing to the next [Hartwell et al. 1994]. There are also similar proteins that regulate the functioning of the cell cycle.

After the discovery of the yeast cyclins regulating the cell cycle, the attention was turned into find the responsible counterparts in the vertebrates and eventually in mammals. From the studies with mammalian cell cultures it was understood that progression through the cell cycle was also regulated by similar structured cyclins and their kinase pairs [Riabowol et al. 1989; Herr 1993]. This occurs in such a way that each step through the cell cycle requires the involvement of different cyclin-CDK pairs. As we mentioned above, CDK1/cyclinB1 pair (M-Cdk) is responsible from the G_2 to M transition while exit from G_1 was observed to be under the control of CDK4/6-cyclin D pairings (G_1-Cdks). Moreover, cyclins A (S-Cdk) and E (G_1/S-Cdk) are found to be paired with CDK2 which is required for the G_1 to S transition and progression through the S phase [Sherr and Roberts 1995]. Experiments by the use of dominant and negative mutants or by specific antibodies not only provided genes that governs the cell cycle, the cell survival and viability but also provided us with the functioning and compartmentalization information about each CDK-cyclin pair have. Moreover, in other cases, it has been shown that other CDKs may be involved in place of a particular cyclin/CDK complex. The approach has also provided us with the knowledge of novel tissue-specific functions for cyclins and CDKs [Reinmuth et al. 2004].

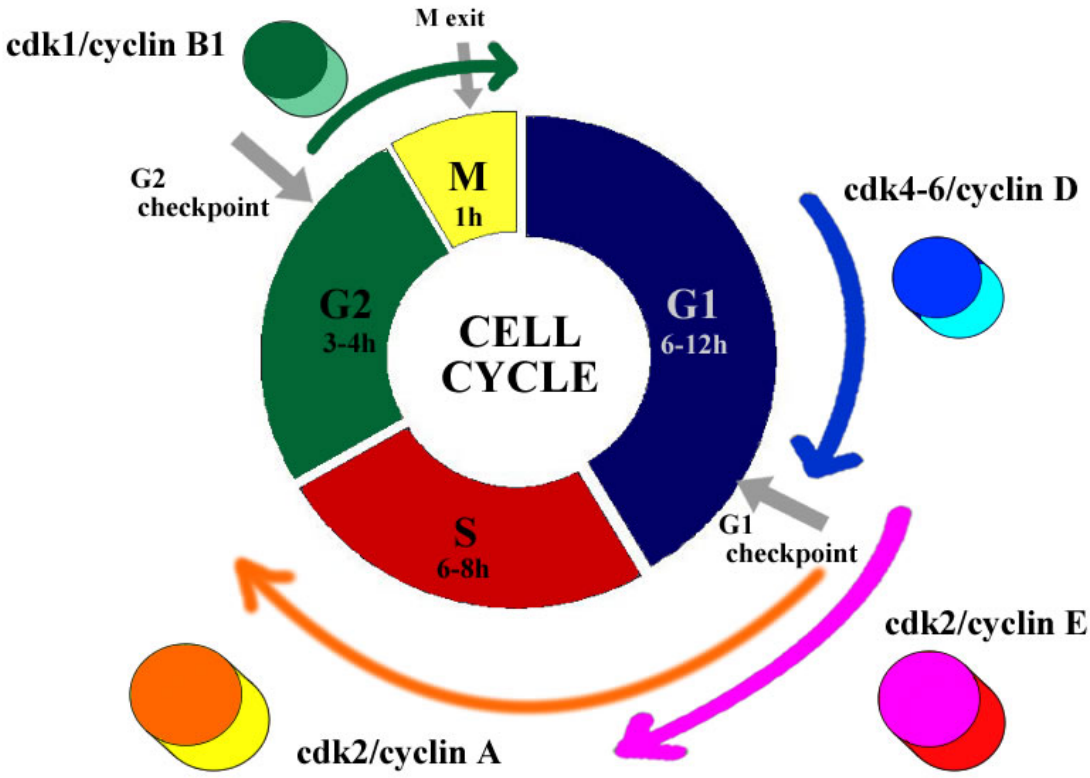


Figure 2.1: Control of the cell cycle via cyclins and their kinase counterparts. The arrows indicate the time of association of the complex. The arrow heads shows the approximate time of the cyclin degradation.

2.1.2.2. Tumor Suppressors and Proto-oncogenes

Important proteins in the proliferation may be classified as tumor suppressor genes and proto-oncogenes which express related regulatory proteins. Tumor suppressors are usually the proteins involved in cell cycle regulation and any defect in them may lead to proliferation since the control over the cell cycle is diminished [Donehower et al. 1992]. Proto-oncogenes, on the other hand, activate the systems leading to cell division. Though proto-oncogenes are normally functioning or may be essential in the cellular mechanisms, any disturbance to their concentration such as overexpression or constitutive activation may lead to dysfunction in the cell cycle regulation and uncontrolled cell division may result [Downward et al. 2003].

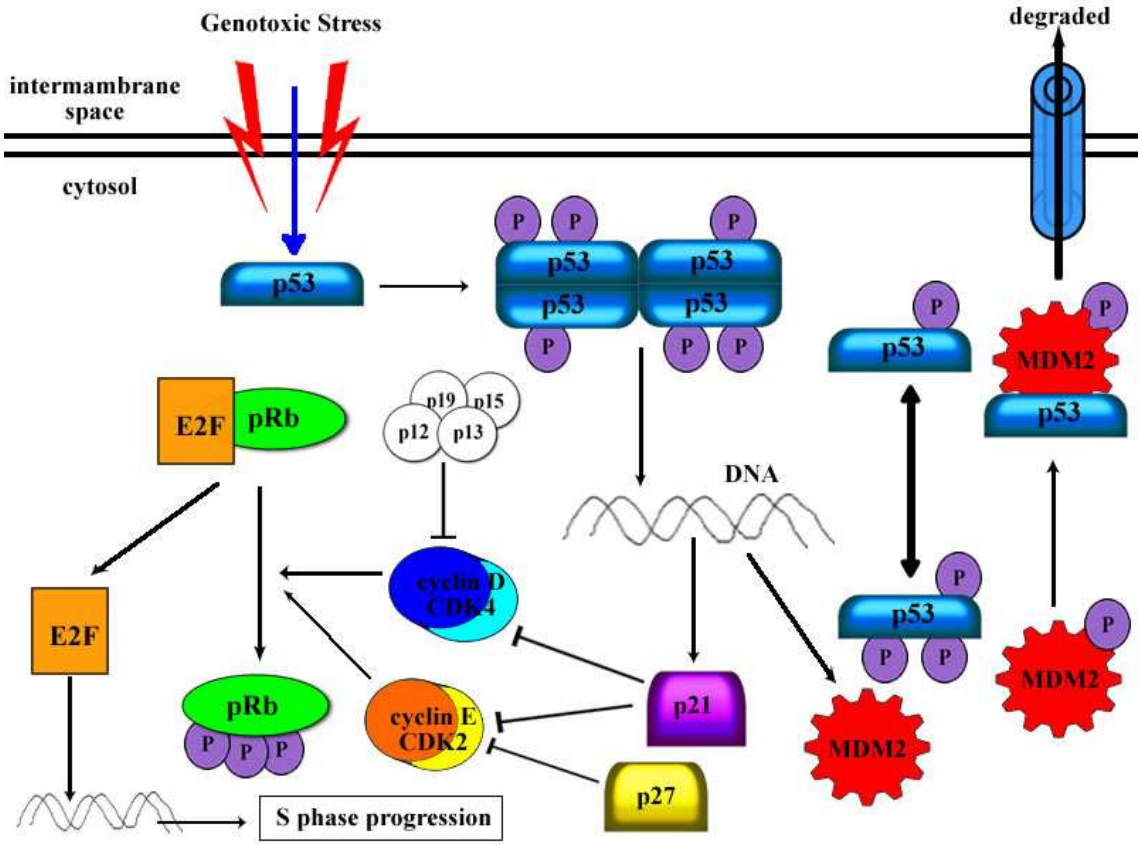


Figure 2.2: Involvement of tumor suppressors in the cell cycle control system.

On the other hand, a well known example of tumor suppressors is the transcription factor p53. p53 acts as a DNA-binding protein and transcription factor controlling the expression of a vast amount of gene products involving in growth control, apoptosis, angiogenesis, and other cellular functions [Volgenstein et al. 2000]. p53 and similar functioning genes are called as tumor suppressor genes since any alteration may lead to cancer along with other modifications [Iggo et al. 1990]. After the DNA damage or cellular injury, p53 protein is concentrated in the cell which is associated to mdm2 degradation [Haupt et al., 1997]. The accumulated p53 is an active transcription factor and it induces either the cell-cycle arrest or apoptosis [Levine 1997]. This arrest is mediated by the cyclin inhibitory protein p21 we discussed above [Attardi et al. 1996; Hansen et al. 1997]. If p53 gene is mutated or deleted, then the cell becomes susceptible to DNA damage and following dysregulation in the cell cycle. Hence, p53 is an important regulatory molecule which sits at the junction of many related signal

transduction pathways leading to apoptosis or proliferation. Its absence the common feature of many cancer types and it is a hot topic to associate p53 related treatments, mainly including gene therapy in accordance with chemotherapy experiences [Han et al., 2002; Fang et al. 2003].

Rb (retinoblastoma) gene is another well known example of the tumor suppressor genes. Similarly, hypophosphorylated Rb gene prevents the activation of E2F, which is a transcription factor that induces transcription of proteins for the cell progression of the cell cycle. However, the phosphorylation of Rb by first cyclin D-CDK4/6 and later in the G_1 by cyclin2/CDK E frees the E2F leading to the S phase entry [Harbour et al. 2000]. For, this control activity, Rb is an important regulator of the cell cycle and in the absence of the RB gene the embryo may not survive (while in heterozygous child (Rb+/-) any somatic mutation to active gene may lead to retinoblastoma.) due to lack of control during the cell cycles [Weinberg et al. 1995].

2.2. Apoptosis

The cell death was first observed about 150 years ago in the microscope specimens of plants. Today what we know programmed cell death, apoptosis, is a general mechanism of the signal cascade that is involved in many processes in development, growth, immune responses and cancer prevention [Alberts et al. 2001]. Apoptosis is utilized extensively in development of organs and the fine tuning of details of body parts in developing embryos; such as, the digit formation in mammals, the brain formation from extremely numerous nerve cells and the tadpole tail disappearance makes use of apoptosis with different mechanisms. Furthermore, apoptosis is the general machinery in the regeneration of injured body parts and the response of the cells to loose their control over cell division to save the overall organism [Lewin 2000]. Apoptosis differs from acute cell death, necrosis, since it occurs via cell shrinkage and disruptions of cellular components under control. Moreover, apoptotic cells contain

membrane surface tags for phagocytes to be engulfed. However, necrosis is an acute response of cells to tissue damage, injury or high levels of cytokines. Moreover, it may harm the tissues considerably due to the swelling and burst of the cells leading to the diffusion of cellular components containing lytic enzymes to the neighboring cells. Finally, the neighbors are extensively damaged in a very short time period, which may lead further damage by inflammation. Putting them together, apoptosis is superior to necrosis in many aspects and it is routine in life activities of the multicellular organisms both in the development and in the survival (in the later periods of life).

In our context, understanding apoptosis is an extremely powerful tool in developing therapeutic solutions to diverse array of health problems. Though apoptosis is an essential mechanism in cancer prevention and treating tumor without damaging healthy tissues, excessive apoptosis is also a problem in inflammatory diseases like atherosclerosis, rheumatoid arthritis and countless autoimmune problems. Therefore, it is not possible to develop solutions towards cancer without considering deadly consequences of the inflammation. Hence, we will need tissue specific apoptotic activity and their control profile to draw more logical pictures of the whole mechanism. Only after then, we will be equipped with the ultimate tools towards any kind of problem associated with apoptotic machinery if we learn how to manipulate its function in different tissues as we desire.

2.2.1. General Apoptotic Scheme

In the following parts, we will discuss the apoptotic machinery and signaling pathways in different systems. Apoptosis may be the result of one two major mechanisms. In the extrinsic pathway, any death signal through death receptors with Fas or similar accessory elements activated via hormones, cytokines or chemokines or association of the toll-like receptors (in the case of killer T-cells may induce apoptotic machinery by the help of procaspase activation through adaptor proteins). The other path is the intrinsic path and it involves the activation of caspase cascade via internal stimuli. Here the signaling is triggered via internal signals as in the case of the cell cycle

control lost due to the oncogene activation, mutation in tumor suppressors or related dysregulation that may lead to proliferation. Ras activation by a mutation will render proliferation of the cell and Myc also promotes the proliferation in the cell. However, it has been shown that, both Ras and Myc activated cells surprisingly undergo apoptosis and this is a good analogy for the regulation of cell cycle in case of the existence of abnormal proliferation signals. Similarly, MDM2 is known to downregulate p53 activity while p19 triggers p53 to commit cell-cycle arrest leading to apoptosis. The result of these stimuli is the cytochrome c release from the mitochondria and this event further provokes the caspase activation [Degterev et al. 2003].

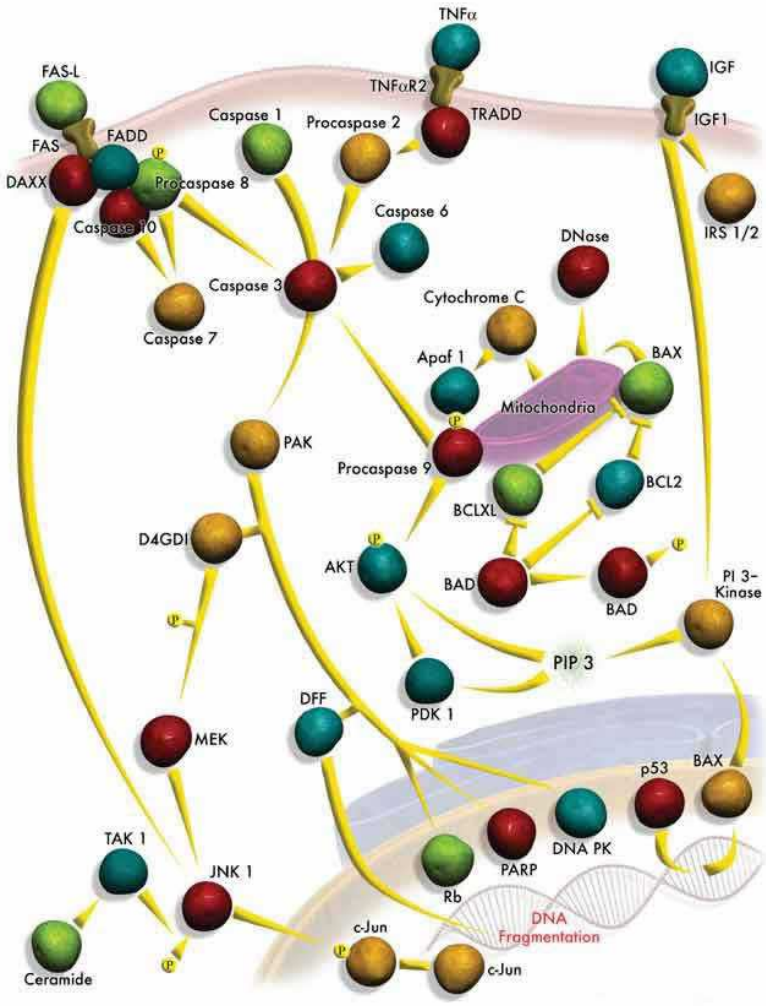


Figure 2.3: Overview of apoptosis [Retrieved from Upstate cell signaling site]

Having learned about the two different paths leading to caspase activation, we will go into further details of this mechanism. In the extrinsic pathway, death signal receptors (Fas or TNFRP) have associated proteins like Raf or TRADD which are also linked to procaspase 8 or 2 by noncovalent interactions [Suda et al. 1993]. When the death signal arrive the adaptor proteins are involved in the activation of procaspase by aggregation of them on the receptors and the resulting cleavage of one another. The cleavage of procaspase 8 or 2 renders their activation through caspase 8 or 2 formation and functional caspases initiate the caspase cascade by cleaving other caspases in the downstream. When cells are damaged, they may also induce the production of Fas ligand and Fas protein or caspase aggregates to kill them selves. On the other hand, intrinsic pathway uses other systems to activate procaspases. When there is a problem in the cell cycle and the proliferation is unavoidable, the internal stimulus is forwarded to mitochondria in one of many ways. Then the usual response is the release of cytochrome c from the mitochondria to cytoplasm. Cytochrome c existence in the cytosol triggers procaspase 9 activation through adaptor protein Apaf-1 [Degretev et al. 2003]. Aggregation of Apaf-1/caspase 9 complexes accelerates the caspase cascade involving other species of the caspases. Through the action of caspases, the nuclear lamina is disintegrated irreversibly and then there is no way to return. The cell shrinkage and the disappearance of nucleus follows these events ending up with a cell consisting of dozens of apoptotic bodies ready for the engulfment (figure 2.4) [Alberts et al. 2001; Ellis et al. 1991; Li and Yuan 1999].

However, the molecules we discussed above are not the only ones involved. There are also dozens of other mechanisms controlling the apoptotic scheme. For example, Bcl-2 family of proteins is such kind of regulatory molecules that control the activity of procaspases. Bcl-2 and Bcl-X_L directly acts on the system by blockage of cytochrome c from the mitochondria resulting in inhibition of the apoptosis [Chao and Korsmeyer 1998]. On the other hand, other factors from the same family may act adversely and activate apoptosis instead. Such as Bax and Bak promote cytochrome c release whereas Bad activate procaspase by blocking death-inhibitor members of the family [cited in Alberts et al. 2001]. Therefore, Bax and Bak mutants are quite resistant to apoptotic induction [Adams et al. 1998]. Still there are other regulators of the machinery and they are known as IAP family of proteins. They either act on procaspase

deactivation of preventing action of active caspases [cited in Alberts et al. 2001; Wang et al. 1998].

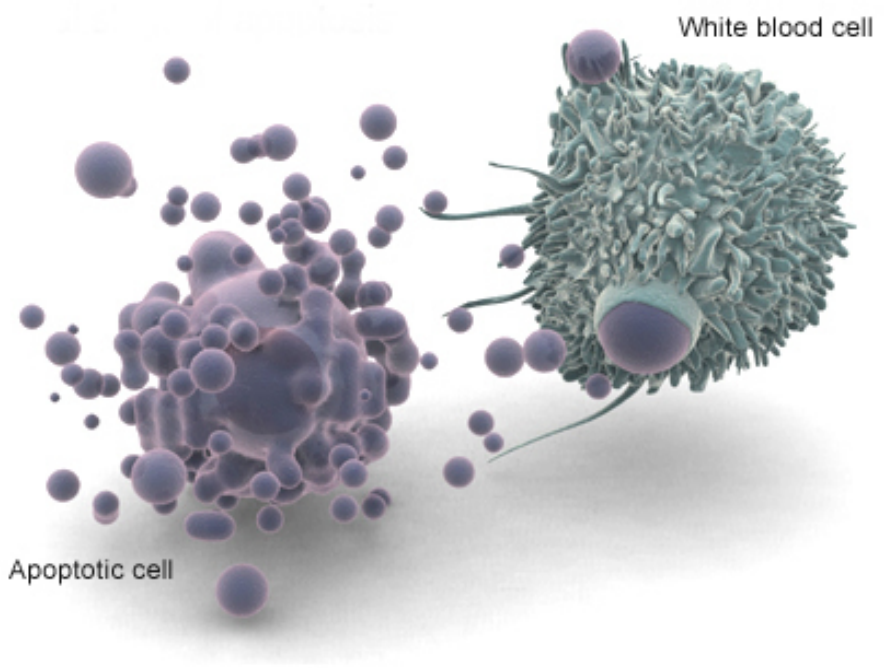


Figure 2.4: Final stage of apoptosis. The apoptotic cells is ready to be phagocyted by a macrophage [US NLM website]

2.3. Cancer

2.3.1. Overview of the Cancer

2.3.1.1. Physiology of the cancer

From all the above discussions we clearly understand that the control over cell division is the essential role of any mechanism. For that reason, many accessory

pathways try to compensate the function of lost machinery. Regardless of the diversity of these extra paths the control over the cell may be lost. If the normal functioning of the cell cannot be attained again, then cell may choose two routes without return. We explained the first path in the apoptotic scheme. Then the remaining path will not be beneficial for the overall health of the multicellular organisms. If the balance over this control is lost and apoptotic machinery is shut down for any reason, the result is the proliferation of the cell (uncontrolled cell division). When a group of proliferated cells form an abnormal structure, this is called a tumor and the process is tumorigenesis. The tumor may be benign and the morphology is the solid neoplasm. Otherwise, a malign tumor may gain additional properties to endanger the organism. To illustrate, the attraction of blood vessels and the formation of extra ones is such kind of a process and it is called angiogenesis. If one or more of the cells lose their contact and gain motility, they diffuse into blood stream from the walls of vessels and travel into other locations in the body, then our tumor is called malign and the process is metastasis [Alberts et al. 2001; Lewin 2000; Gutkind 2000].

In all of the above steps several important mechanisms are involved to block progression towards malignancy. In the first step cell cycle control is strictly regulated via myriads of tumor suppressor proteins such as p53 ad Rb. If these molecules become useless due to the inhibition or some other dominant regulation then the internal signals may lead to the caspase activation to turn on the apoptotic machinery. Regulators of these mechanisms such as active NF-κB may inhibit this activity. Strongly activated NF-κB is not sensitive to the extracellular signals regardless of the cell and the cell may continue to survive via DNA repair or required machinery. For the next step in the road to malignancy, the proliferation of the cell required to proceed into this goal. This may be achieved by oversensitive growth hormone receptors or other sensors like TGF-β. The numbers of such receptors are abnormally increased in the proliferating cells. Once the control over the cell division is overcome, then the cell starts to divide uncontrollably. However, the resources will not be sufficient to support such an increase in the cell cycle, either the tumor of the proliferated cells will suffer from starvation or they will find alternative ways to feed themselves. Here tumor cells may produce short range hormones to attract blood vessels. Upon angiogenesis, the tumor cells can increase their number logarithmically. However, some of the naughty cells from the

neoplasm will gain other characteristics due to the chromosomal instability and lack of the DNA repair. They may lose their contacts since they have lost the control over the expression of cell adhesion factors. These kind of metastatic cells are not prone to divide but they move around in the tumor and eventually they fuse into the blood-stream and travel into far distances in the body. In the end, the metastatic cells get plugged in the blood-stream and they get out of the vessels where they continue to proliferate and spread the tumors in the other organs or tissues. When the number of the cancer cells exceeds a limit that the body cannot handle, then the death of the organism follows [Alberts et al. 2001; Lewin 2000; Gutkind 2000].

2.3.1.2. Morphology of cancer

Except for the physiological features cancer cells demonstrate a variety of morphological differences that make them distinguishable from the cells of normal tissues. First, cancer cells appear to show irregular tissue formation that can be recognized in the pathological specimens. They may have increased number of growth receptors or proto-oncogene expression that will be evident in immunological staining of the same specimens. Their nuclei are continuously involved in the M phase due to high rate of divisions and this causes the observation of highly condensed chromatin, even chromosomes can be distinguished. When the chromosomes are examined the aneploidy is a common feature and chromosomal aberrations are frequent. Therefore, some chromosomes can be lessened while other can be found in multiples; chimerical chromosomes are evident in the late phase while it is a common feature in Californian phenotype [Alberts et al. 2001].

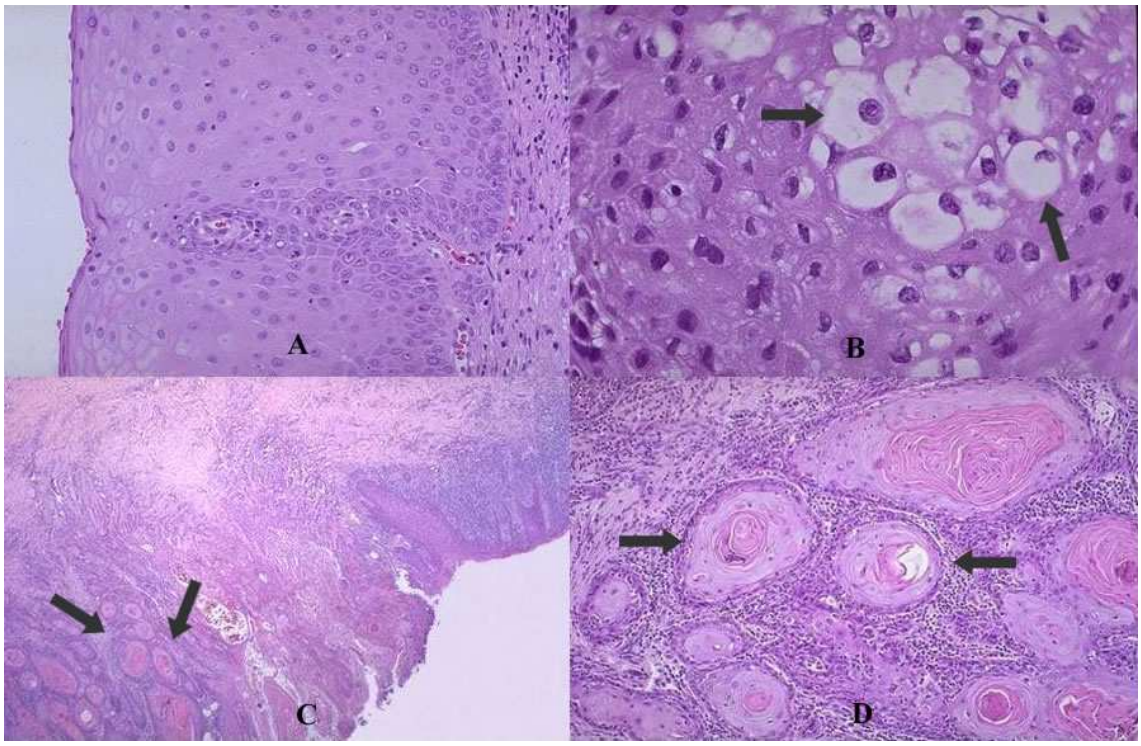


Figure 2.5: Different Morphologies of the Cervical Carcinoma. While we see the normal cervix (A) specimen in the first picture, in other pictures we observe cervix with HPV infection (B), infiltrating carcinoma of the cervix (C) and the squamous carcinoma (D) [Shashidhar 2005].

2.3.1. Signaling Mechanisms in Cancer

2.3.1.1. Receptor tyrosine kinase (RTK) signaling

Tyrosine kinase receptors are a large family of cell surface proteins that are related to diverse array of growth factors. These receptors are usually effective in epithelial tissues where the principal growth factor is the family of epidermal growth factors (EGF) and related signals. Since epithelial tissues are related to different carcinoma types and carcinomas are the leading cancer type, understanding about RTK signaling will improve our toolbox while developing therapeutic agents to different tumors [Reinmuth et al. 2004].

2.3.1.2. Ras-MAPK

Different growth factors, proto-oncogenes and cytokines activate cell cycle towards cell division via a small G protein called Ras. Here Ras is a potential proto-oncogene whose activation renders the cell to undergo proliferation. This property is shown by mice transformation studies by different forms of Ras in different viruses. It is an active proto-oncogene and some other types may actively acts as oncogene readily. Though Ras by itself is not enough for transformation, a 20 fold increase may be enough for transforming the cells. This protein promotes the stimulation of serine/threonine kinase Raf [Stewart et al 1984; Sinn et al. 1987; Evan et al. 2001; Hahn et al. 2002]. Ras utilize the recruitment of Raf to the plasma membrane and its phosphorylation [Kosako et al. 1992; Tzivion et al. 1998]. Upon this activation also mitogen activated protein kinases (MAPK) are affected due to MEK stimulation. This effect occurs by the phosphorylation and the activation of ERK (extracellular signal regulated kinase or the MAPK affected) by Raf. Numerous cell-adhesion receptors and growth factors take part in the control and regulation of anchorage-dependent cell-cycle entry and they trigger vast amount of signaling events, such as MEK–ERK cascades [Downward et al. 2003] and the phosphatidylinositol 3-kinase (PI3K) signaling. All of these events are strictly regulated by different mechanisms including transient ones and gene expression machinery.

By the association with their respective ligands, growth-factor receptor tyrosine kinases such as the vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR), the fibroblast growth factor receptor (FGFR), and the receptor ERBB are activated. This event is followed by the binding of adaptor proteins such as growth-factor-receptor-bound protein 2 (GRB2). With the help of GRB2; the guanine-nucleotide exchange factor SOS induces RAS activation via GTP replacement in Ras. Activated Ras further catalyze RAF induction and downstream events. As we told above, RAF phosphorylates MEK, which in turn activates ERK. This pathway is controlled via some regulatory proteins that inhibit the Ras of Raf activity in different ways. These regulators include RAF kinase inhibitor protein (RKIP) [Yeung et al 2000]; RAS and RAB interactor 1 (RIN1), [Wang et al. 2002]; impedes mitogenic signal propagation (IMP) [Matheny et al. 2004]; and the MKP (MKP1, 3) family of MAPK phosphatase [Shapiro et al. 1998; Farooq et al. 2004]. There are other negative

regulators such as AKT and serum/glucocorticoid inducible kinase (SGK). On the other hand, positive regulators of the pathway are protein kinase C (PKC), SRC, p21-activated kinase (PAK) and 14-3-3. All these molecules may induce RAF activity regardless of the concentration of Ras. Ras-Raf pathway also activated via adhesion of integrins to specific extracellular-matrix molecules. Such interactions activate focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K), which may also be activated by RAS and will also yield Raf activation via a different path [Reviewed in Arslan et al. 2005].

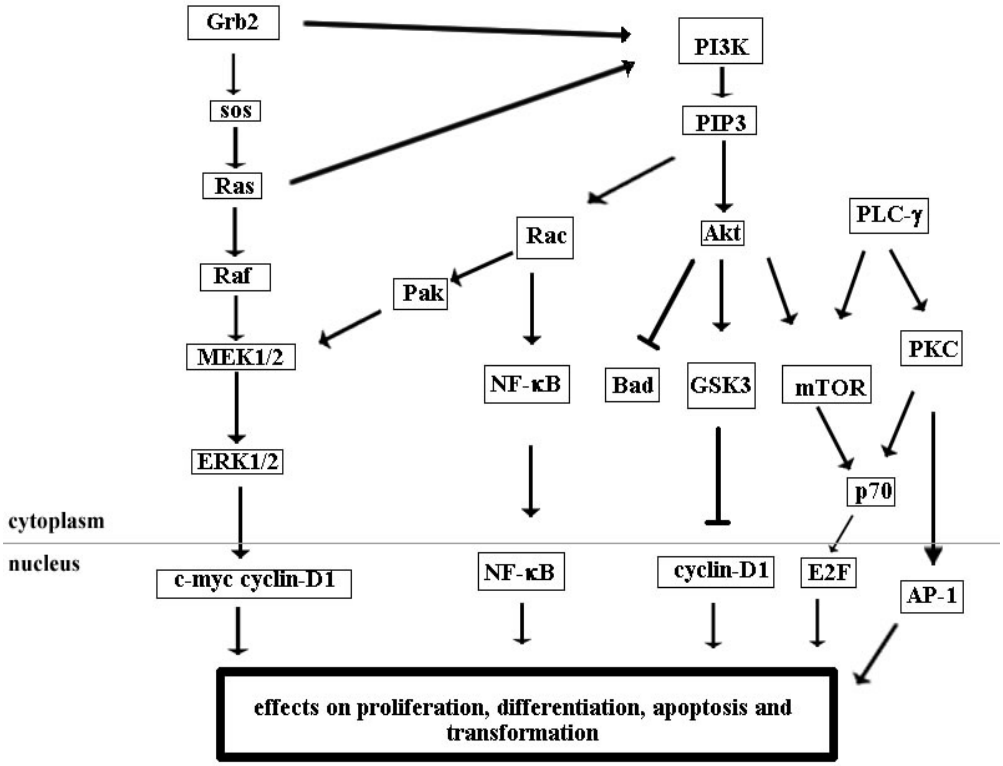


Figure 2.6: Overview of RAS related MAPK and PI3K pathways (adapted from Reinmuth et al. 2003; Arslan et al. 2005). Activation is indicated via arrows while bars indicate inhibition.

From the recent statistical data, it is obvious that activated Ras-MAPK pathway takes part (in 15-90% of different cancers active Ras pathway is observed [Downward et al. 2003]). Therefore, Ras activation is a common feature in melonomas, gliomas, adenocarcinomas, breast and ovarian cancer and other carcinomas where epithelial tissue is involved. Understanding about this pathway will surely lead us other common

features in these cancers and our job will obviously easier. Consequently, any treatment addressed to RTK signaling especial Ras-MAPK-ERK pathway will be promising in different types of cancers though not a single drug will possible useful to all these cancers since other mechanisms are involved in different ratios.

2.3.1.3. Mitogen activated protein kinases

Other than self phosphorylating tyrosine kinases, there exist other important groups of kinases in the form of serine-threonine kinases. Especially MAPK, MAP2K and MAP3K are important in versatile regulatory pathways and are included in the serine-threonine kinases and they are involved in the mechanisms regarding any change in the environmental conditions. Their activation pathways are conserved during the course of evolution and they are common in different kingdoms (fungi, animal and plants). They are activated through phosphorylation of one threonine and one tyrosine residue in the close proximity which further triggers conformational changes leading to functionality. This activation site is found near to catalytic site which was shut before the phosphorylation [Roger J. Davis cited in Gutkind 2001].

MAP kinases are subdivided into four; ERKs, p38, JNK/SAPK, ERK5 (BMK). ERKs (or ERK1 and ERK2) are mitogen activated kinases whereas p38 and JNK are activated upon cellular stress or proinlammatory stimulants. For example, G-protein activated pathway leads to MEKK activation, which is a regulator of ERK. Upon MEKK activation, ERK is phosphorylated and this renders many dysfunctions in the cell. Therefore, ERK activation is associated to cancer formation in the cell and it is a good indicator of cellular dysfunction. Similarly p38 and JNK pathways are controlled via phosphorylation and dephosphorylation and any failure in their regulation leads to basement of cancer formation [Johnson et al 2002; Schulte et al. 2003; Bruemmer et al 2003; Lee et al 2003; Filomenti et al. 2003; Ishikawa et al. 2003].

ERKs can utilize the phosphorylation and translocation of membrane associated and cytoplasmic components, such as RSK (ribosomal S6 kinase). Once they enter into nucleus, they phosphorylate related transcription factors which will further activate early genes including *fos, jun* and *myc* [Elion et al. 1998]. All of these molecules are related to one or more survival mechanisms in the cell cycle while myc is a well known

oncogene. Fos has been shown to function as a sensor for ERK signal duration. When ERK activation is transient, its activity fades away before fos protein accumulates. On the other hand, normal ERK signaling results in phosphorylation of fos by ERK and its stabilization. This event further affects progression into cell cycle entry [Murphy et al. 2002]. RSK modulate the function of different transcription factors including NF-κB and I-κB (nuclear transcription factor κB and inhitor of NF-κB respectively) [Ghoda et al. 1997], which will be discussed extensively in the following parts. Other than ERKs, there are also different groups of MAPKs which are classified as p38 and the JNKs (c-Jun N-terminal protein kinases). In their signaling mechanisms, unlike to ERKs, they are not activated by mitogens. However, they are stimulated via inflammatory cytokines, such as interleukins or tumor necrosis factors (TNF). These MAPKs are responsible from different signaling events in inflammation, differentiation, cell growth, cell-cycle progression, and cell death [Johnson et al. 2002].

These MAPKs are upregulated by different kinases including MAP2Ks, MAP3K and their upstream elements (possibly receptor associated proteins). These regulations may lead to different responses in diverse cellular machinery. For example, ERK, p38 or JNK group of MAPK kinases are all related to apoptosis since they regulate either of cell survival, growth, differentiation or repair. Hence, all of these MAPKs can be attributed to apoptotic machinery in one or more aspects. In previous studies, different MEKK (MAP3K) family of proteins found to be associated with different mechanism related to different MAPK regulatory mechanisms [Reviewed in Arslan et al. 2005; Johnson et al. 2002].

2.4. TNF-α Signaling

2.4.1. TNF-α involvement in NF-κB Signaling

There is an important group of transcription factors known as Rel or nuclear factor-kappa B (NF-κB) which takes part in the inflammatory response, the regulation of cell growth, apoptosis. These factors are activated via many different signals such as proinflammatory cytokines, bacterial lipopolysaccharide (LPS), UV radiation, oxidative stress, viral proteins, double-stranded RNA and mitogens [Siebenlist et al. 1994; Miyamoto et al. 1995; Ghosh et al. 1998; Pahl et al. 1999]. In the cell NF-κB may be found as a homodimer or heterodimer made up of Rel family member proteins p50/105, p52/p100, p65, RelB and c-Rel. However, it is not active in these forms since its activity is blocked by an inhibitory protein family called IκB which in turn regulate the activity of NF-κ B [Bauerle et al. 1996; Baldwin et al. 1996; Gilmore et al. 1999]. When a TNF-α molecule binds to its receptor TNFR1, this event will initiate a series of activations leading to apoptosis via caspase-8 induction along with NF-κB activation (Fig. 2.x) [Chinnaiyan et al. 1995; Hsu et al. 1995&1996; Ting et al. 1996; Arch et al. 1998; Wallach et al. 1999]. Different responses of TNF-α to apoptotic machinery (as pro or anti-apoptotic) are regulated by NF-κB, which was examined in clinical trials and in several cancer types inducible and constitutive forms of NF-κB are resistant to apoptosis tried to be induced by chemotherapy, radiotherapy and TNF treatment [Van Antwerp et al. 1996; Liu et al. 1996; Baichwal et al. 1997; Rayet et al. 1997]. NF-κB acts as by increasing the expression of inhibitory proteins of apoptotic machinery (c-IAP 1 and c-IAP 2) and as we told before it also affects the proteins involved in cell cycle [Chu et al. 1997; Wang et al. 1998; Guttride et al. 1999]. Moreover, NF-κB controls the expression of genes involved in invasion and metastasis while [Huang et al. 2000& 2001; Wang et al. 1996], recently, it was shown that NF-κB is also constitutively activated in human cervical carcinoma development [Nair et al. 2003; Kütük and Başağa 2004; reviewed in Arslan et al. 2005].

TNF-α informs the cell about any problems in the close proximity of the cell and the requirement of cell death. If this signal was not deliberately delivered (dose dependent) then the cell may go for apoptosis or may not. Here the significant element is nuclear transcription factor NF-κB. It is very well-known that NF-κB is a regulator of versatile mechanisms while acting as anti-apoptotic mediator in the cell. If there are other signals concerning its activity then NF-κB may act as proapoptotic mediator as in the inflammatory responses. In the upstream, NF-κB is regulated by the inhibitory protein IκB and the kinase of IκB (IκK) while in the upstream of IκK a series of MAP kinases and related proteins regulate the function of it. Among them we can count NIK and MEKKs. Here the important thing is the involvement of different mechanisms. To illustrate, MEKK3 is well-known with its RIP mediated reinforcement to TRAF2 (a TNFR1 associated protein) only then it becomes active and modify IκK upon phosphorylation which becomes inactive and cannot phosphorylate IκB and NF-κB cannot enter nucleoplasm anymore to block apoptosis. On the other hand, in UV triggered TNF-α signaling MEKK3 is missing but other pathways and regulators compensate its activity, like NIK and MEKK1. These are the examples of NIK independent activation of NF-κB.

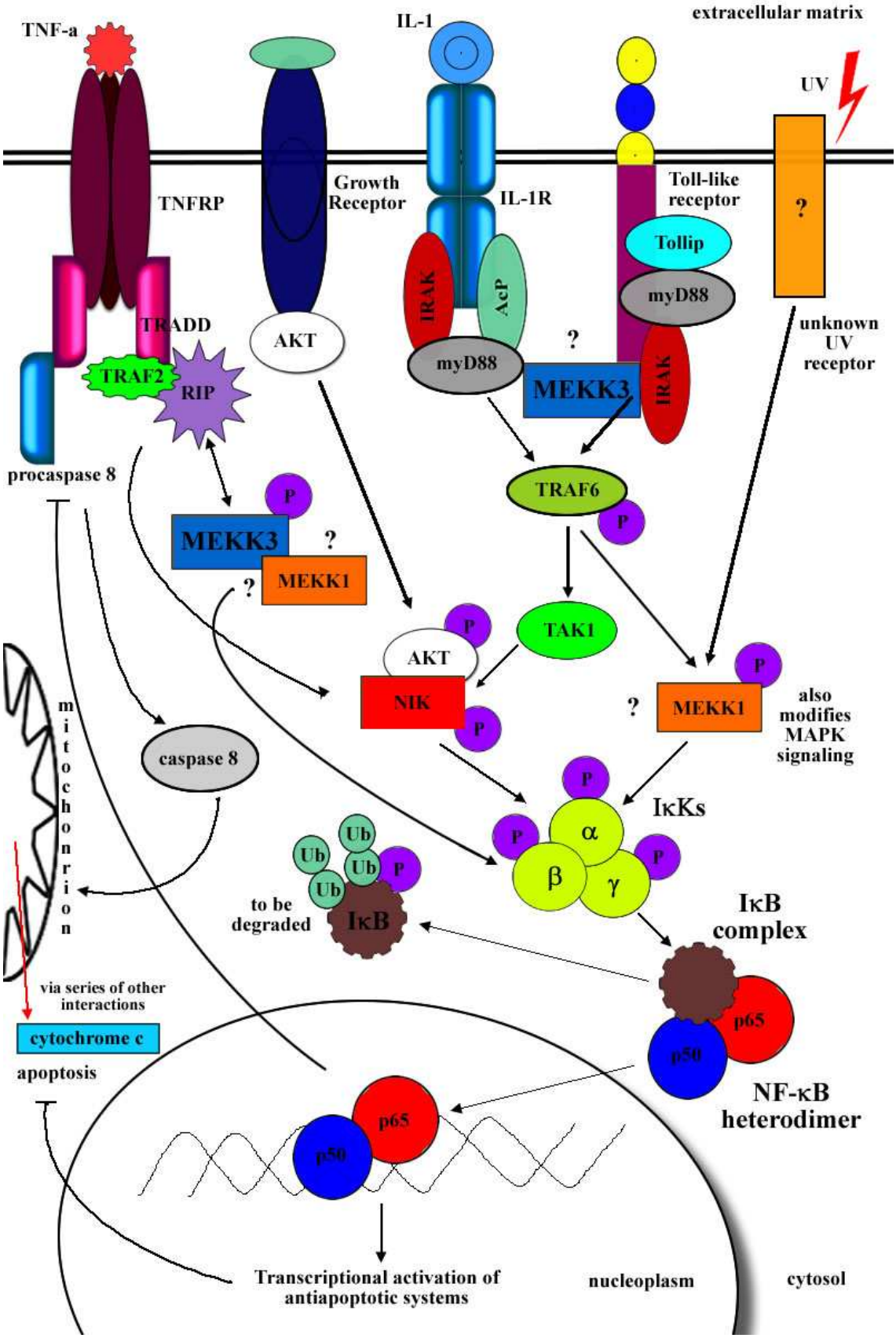


Figure 2.7: General proinflammatory and growth factor signaling in relation to NF-κB activation and apoptosis.

According to above diagram, TNF-α engages to its receptor, TNF-α receptor type 1 (TNFR1), leading to trimerization of the receptor. The clustering of TNFR1 promotes the recruitment of TNFR associated death domain (TRADD), which in turn recruits TNFR associated factor 2 (TRAF 2) and receptor-interacting protein (RIP). The recruitment of these adaptor molecules activates NF-κB-inducing kinase (NIK) and IKB signalsome complex, which leads to activation of NF-κB and potential anti-apoptotic gene expression. TNFR1 trimerization also favors the recruitment of Fas-associated death domain (FADD) and caspase-8 that results in activation of the TNF-α induced apoptotic pathway. The competition between two pathways determines the fate of a cell under TNF-α treatment. Similarly, growth factors may induce growth factor associated factor like Act which in return activated NIK via phosphorylation. IL-1 signaling system is involved in the overall picture by the use of IL-1RacP (IL-1 receptor accessory protein) induced activation of IRAK through adaptor protein myD88. IRAK further promotes the TRAF6 activation resulting in NIK regulation through TAK1. Again arrows represent activation whereas bars show inhibition. Toll like receptors activate TRAF6 in a similar fashion through myD88 and IRAK induction. Lastly, unknown UV receptor regulates MEKK1 and NIK activity which will further end up with NF-κB activation as in the previous cases.

2.4.2. MEKK Involvement

Until recently the role of MAP3Ks in the cytokine signaling and in the NF-κB activation was not well established. Most of the studies were claiming that NIK was the principal element in the NF-κB activation but the evidence to its relation with TNF-α or similar cytokines were not clear (Nakano et al. 1998). First, findings suggested that MEKK1 is a good candidate of TNF-α associated NF-κB activation, since it was shown that MEKK1 was able to phosphorylate IκKβ in IκK complex which in turn leads to its activation [Lee et al 1997]. Active IkK further phosphorylates IκBα and NF-κB freely moves to nucleus [Zandi et al. 1997, see also in the figure 2.7]. Besides in many of the other studies it has been shown that MEKK1 induction leads to induction of other MAPKs via injury or other cases (where TNF-α signaling has been activated). Usually,

this phenomenon has been shown by JNK and p38 activation in survival, JNK and ERK1 activation in regrowth of the neuronal cells [Waetzig et al 2002]. Other studies forced us to think MEKK2 or MEKK3, which are structurally very similar, can also induce the NF-κB activation by IκKα and β-subunit phosphorylation [Lee et al. 1998]. Later on, studies shown that MEKK3 knockout mice embryos were unable to complete their development due to excessive apoptosis resulting from switched off NF-κB activity. Finally, MEKK3 was described with its role in RIP and TRAF2 activation leading to IκK phosphorylation [Yang et al. 2000]. However, last findings in MEKK3 mediated activities has claim that MEKK3 is actually acts under IL-1 and toll-like receptors and it exerts its antiapoptotic moderation activity via these signaling pathways [Huang et al. 2004]. These results have led us to return back to our starting point to define primary MAP3K under TNF-α signaling. Again we were left with MEKK1 as it was previously mentioned. Though MEKK3 is already associated with the IL-1 and toll-like receptor signaling, its role under TNF-a signaling cannot be underestimated. Moreover, MEKK1 involvement in those pathways has not been studied and MEKK1 has been reported to be involved in apoptotic induction via Smac/Diablo release from the release in a different mechanism that is not involved in cytochrome c release [Mendoza et al. 2005]. MEKK1 seems to be involved both in antiapoptotic and proapoptotic machinery but we have to clarify the mechanisms in which MEKK1 acts for the apoptotic induction while it is involved in the antiapoptotic machinery of NF-κB activation. Though this dual character of MEKK1 increase the complexity of the picture, any effort in finding its exact role under TNF-a signaling will prove to have utmost importance in understanding the downstream events.

2. PURPOSE OF THE STUDY

TNF-α signaling is a major cytokine signaling which may lead to diverse responses throughout downstream effectors. Two major pathways were described to be followed upon TNF excitation of the cell; one path leads to apoptosis while other goes to survival. Here the important factor is the control of downstream effectors via moderator proteins. Several of them have been described early as TRADD, RIP, TRAF2, NIK, procaspase 8 and finally MEKK1 or MEKK3. Some of the studies have claimed that MEKK3 is the principal effector while others attribute this role to MEKK1 as described in the previous section. For these reasons, our aim is to reveal

- whether MEKK1 or MEKK3 is the principal moderator in the downstream of the TNFRP and the action mechanisms these MAP3Ks in the cell survival or in the cell death

- possibility of MEKK1 and MEKK3 involvement in the TNF signaling at the same time

- how their overpression will effect the NF-κB activity or the apoptotic response by looking up to downstream elements

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals

All chemicals were supplied by Amresco, Applichem, Fluka (Switzerland), CalBiochem (USA), Merck (Germany), Riedel de Haen (Germany), and Sigma-Aldrich (Germany)

4.1.2. Enzymes

4.1.2.1. Restriction enzymes

EcoRI, EcoRV, SalI and ClaI (Fermentas and Promega)

4.1.3. Commercial Kits

Qiaprep Spin Miniprep Kit (250) (Qiagen)

Qiagen Plasmid Midi Kit (100) (Qiagen)

Fugene 6.1 Transfection kit (Roche)

Pierce chemiluminescence ECL kit

Advanced ECL kit (Amersham Pharmacia)

4.1.4. Plasmids

CA pcDNA3-flagMEKK1Δ,

DN (-/-) pcDNA3-flagMEKK1Δ (K432M),

SRα3HA–MEKK3

DN (-/-) SRα3HA–MEKK3 (K→M)

pmax-GFP

pE-GFP

4.1.5. Cells

HeLa An1 (HÜKÜK- Şap Enstitüsü)

E. coli (TOP10 and DH5) kindly provided by EMBL, Hamburg

E.coli TOP F' (Invitrogen)

4.1.6. Antibodies

rabbit anti-JNK1 (FL) (Santa Cruz Biotechnology Inc., USA) sc571

mouse anti-pJNK (G-7) (Santa Cruz Biotechnology Inc., USA) sc6254

rabbit anti-pSAPK/JNK (Cell Signaling Technology Inc., USA) 9251

mouse anti-p38 (A-12) (Santa Cruz Biotechnology Inc., USA) sc7972

mouse anti-pp38 (D-8) (Santa Cruz Biotechnology Inc., USA) sc7973

rabbit anti-ERK1(K-23) (Santa Cruz Biotechnology Inc., USA) sc94

mouse anti-pERK (E-4) (Santa Cruz Biotechnology Inc., USA) sc7383

rabbit anti-IκBα (FL) (Santa Cruz Biotechnology Inc., USA) sc847

mouse anti-pIκBα (B–9) (Santa Cruz Biotechnology Inc., USA) sc8404

rabbit anti-IκBβ (S-20) (Santa Cruz Biotechnology Inc., USA) sc946

rabbit anti-flag (Sigma, Germany) F7425

rabbit anti-HA (Abcam, USA) ab9110

anti-mouse HRP conjugated (Amersham Pharmacia, Germany)

anti-rabbit HRP conjugated (Amersham Pharmacia, Germany)

4.1.7. Buffers and Solutions

4.1.7.1. Culture media

4.1.7.1.1. MEM and added materials

The media is supplied as liquid and sterile. It is the optimum medium for the growth of HeLa cells. For the use in normal growth of HeLa cells, 10% FCS (Sigma), 2% penicillin-streptomycin (Sigma) and 2mM glycine (Sigma or Merck) are added by filter sterilization. For transfection, antibiotics are omitted and for the fugene mixture MEM is used without any additional material.

Liquid MEM with Hank's salt, NaCl and $NaHCO_3$ without glycine (Sigma)

Sterile FCS (Sigma)

Penicillin-Streptomycin (Sigma)

Sterile 1xPBS (Sigma or Biological Industries)

Sterile Trypsin (Sigma or Biological Industries)

Sterile DMSO (Sigma)

4.1.7.1.2. LB broth

Luria Bertani (LB) broth (Sigma, Merck or Applichem) is used as the liquid culture for the bacteria. It contains tryptone, yeast extract, and chemical salts in appropriate amounts. For 1l of LB broth, 20g of the LB powder is added to distilled water and mixed well. Then the culture is autoclaved for sterility. 100μM ampicillin or 50μM kanamycin is added for the selective media as required.

4.1.7.1.3. LB agar

LB agar is used as solid media for the bacteria. It is used for the differential screening of bacteria in transformation by the use of appropriate antibiotics. 1l LB agar is prepared by mixing 40g LB-agar with 1l distilled water. After sterilization in the autoclave, liquid agar is cooled for antibiotic addition if required. Before solidifying, the liquid agar must be poured into appropriate plates in 20-25ml amounts. Then they should be let harden in the fume hood. Then these plates are sealed and can be stored at 4°C for months. Again 100μM ampicillin or 50μM kanamycin is added for the selective media as required.

4.1.7.2. Buffers

4.1.7.2.1. Electrophoresis and western blot buffers

1xTE (Tris-EDTA) for dissolving DNA

1xTAE (Tris-Acetate-EDTA) (50x stock)

6xLoading Dye (Fermentas) for DNA

1xRunning Buffer Tris-glycine-SDS (10x stock)

1xTransfer Buffer (Tris-glycine-SDS and methanol)

2xLoading Buffer (Novex, Sigma) for proteins

10xPBS (Phosphate Buffered Saline) (Sigma)

1xPBS-Tween 20 (0.2% Tween 20)

4.2. Methods

4.2.1. Mammalian Cell Culturing

Cervical epithelloid carcinoma, HeLa, cells were obtained from HÜKÜK (Şap Enstitüsü Kurumu with catalog no: 90061901 strain An1 with passage number of 14). These cells were cultured in minimum essential medium Eagle (Sigma) with the addition of 10% FCS (sigma or israil), 2% penicillin-streptomycin and 2 mM glycine and grown in an incubator at 37°C and providing 5% CO_2. The cells were washed with 1xPBS prior to trypsinizing and they are detached at confluency by cell culture grade trypsin. As required they are frozen in FCS with 10% DMSO (sigma) after trypsinizing the cells and harvesting them in a Heraeus centrifuge at 300g. The frozen stocks were kept at -80°C for short term and in liquid nitrogen for longer periods. These stocks were freeze thawed in 37°C water bath to avoid damage from crystallization.

4.2.2. Transfection

4.2.2.1. Fugene

HeLa cells are transfected either by the use of fugene transfection kit (Roche) or by electroporation. For fugene transfection cells were inoculated into 6well (at $0.15 \cdot 10^6$ cells per well) or 60mm ($0.3 \cdot 10^6$ cells) to guarantee the cells will be in the log phase of their growth at the day of transfection. For the transfection antibiotic free medium is used in place of the medium we described above. The transfection is carried according to the manufacturer's protocol by putting the transfection reagent directly into 100-150µl serum and antibiotic free medium. Then appropriate amount of (3-5mg) the plasmid vector is added to the mixture and they are tapped gently to facilitate their mixing. Here we used CA pcDNA3-flagMEKK1Δ, DN (-/-) pcDNA3-flagMEKK1Δ (K432M), CA pSRα 3HA-MEKK3- and DN (-/-) pSRα3HA–MEKK3 plasmid vectors. Then the mixture was incubated at least 15 at most 45 minutes for the incorporation of the plasmids to the fugene reagent. This mixture is added into the medium containing the cells to be transfected drop wisely. The plate or the dish is moved back and forward to provide even dispersal. The cells were left to grow in an incubator for 36-48 hours for the expression of the vectors and reporter assays. The transfection efficiency is indirectly measured by the use of pmaxGFP plasmid transfection of other batches of the cells simultaneously. Then the transfection efficiency is assumed to be comparable to the cells expressing GFP which was observed under fluorescent microscope. Also, the success of the transfection was checked by antiFLAG antibodies in immunoblots of the proteins obtained from transfected cells.

4.2.3. TNF-α Treatments

After the transfection of the cells was complete and assumingly they are expressing the desired plasmids then the next step is to treat cells with our two cytokines. In stocks, TNF-α has concentrations of 10μg/ml (it was delivered in lyophilized powder and stocks were prepared by dissolving it in sterile distilled water). We added 2μl of these stocks to each 1ml liquid media so that we obtained a working concentration of 20ng/ml. For treatments, we usually started with 12 hours or 4 hours time periods. We applied following scheme *12h**→8h→*4h**→2h→1h→30m→15m and in the end of the treatment we removed the media to stop the cytokine treatments and we lysed all the samples at the same time.

*Both 0-4 hour and 0-12 hour scans were obtained for different experiments (will be explained in discussions.

4.2.4. Protein Isolation

4.2.4.1. Total protein isolation

Treated and control HeLa cells were washed throughout with ice-cold PBS and their wash-outs were discarded. Again appropriate amount of ice-cold PBS 1-1.5ml is added and plates are left in this position. Then the cells are scrapped from the plates, harvested at 13000rpm for 30 seconds. Wash-out is discarded again and they are lysed on ice in a solution containing 50mM Tris-HCl (pH 7.5), 150mM NaCl, Nonidet P-40 0.5%, (v/v), 1 mM EDTA, 0.5mM PMSF, 1mM DTT, protease inhibitor cocktail (Complete from Roche, Germany) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2, Sigma, Germany) for 20 minutes. After the cell lysis cell debris was removed by centrifugation 10 min at 13000rpm and protein and supernatant contains total proteins. These proteins can be stored at -20°C for short term and at -80°C for longer periods.

4.2.4.2. Differential protein isolation

The procedure was the same as total protein isolation till the addition of lysis buffer. For the differential isolation we used two different buffers. In the first step we used T1 buffer (hypotonic: 10mM HEPES-KOH pH 7.9, 2mM $MgCl_2 \cdot 6H_2O$, 0.1mM EDTA, 10mM KCl, 1.5% NP-40, DOC, Triton X100, or Tween20, 1mM DTT, 0.5mM PMSF, complete protease inhibitors (Roche) and phosphatase inhibitors (Sigma) as instructed in their manuals) in appropriate amounts (100-120µl) for the lysis of the cell membrane while the nuclei were remaining intact. First lysis step took 20 minutes on ice as above and the lysate was centrifuged at 13000rpm for 1 minute. Then the supernatant contains cytoplasmic fraction and removed to be stored in appropriate eppendorf tubes. The pellets were containing nuclei and they are lysed in 20-40ml T2 buffer (50mM HEPES-KOH pH 7.9, 2mM $MgCl_2 \cdot 6H_2O$, 0.1mM EDTA, 50mM KCl, 400mM NaCl, 10% glycerol, 1mM DTT, 0.5mM PMSF, complete protease and phosphatase inhibitors) for another 20 minutes on ice. Then the lysate is centrifuged at 13000rpm and at 4°C for 30 minutes. Finally, supernatant contained the nuclear proteins and they were transferred into their vials.

4.2.5. Protein Content Assay

Protein concentrations were determined with Bradford protein assay. Biorad Bradford reagent is diluted 1:5 with distilled water. This solution should be kept on ice while using in the laboratory. Otherwise, it should be stored in the fridge and be prevented from light since the active solution is light sensitive. 498ml of the diluted Bradford reagents were put into eppendorf tubes and 2ml of each protein samples are mixed to them. Additionally, a group of vials should be prepared as standard from known concentration of BSA solution. Then samples are transferred into a microtiter plate in 300µl amounts. The absorbance of each sample and standards were measured in a microtiter plate reader at 595nm. From the BSA standard, a standard curve was obtained and unknown concentrations of the samples were obtained by the use of this graph and its formula.

4.2.6. SDS-PAGE

Biorad mini protean gel systems were used as protein electrophoresis. 10% SDS-PAGE was prepared by mixing 4.1ml distilled water, 2.5ml pH 8.8 1.5M Tris-HCl, 50µl 20% SDS, 4.4ml 30%/0.8% acrylamide/bisacrylamide, 50µl 10% APS and 5µl TEMED in the given order (for 10.005ml or two SDS-PAGE gels in 1mm glasses) for the running gel. Then gels were overlaid with isopropanol to initiate polymerization. Once the gel was formed then the isopropanol was removed and stacking gel (3.075ml water, 1.25ml pH 6.8 0.5M Tris-HCl, 25µl 20% SDS, 0.67ml 30%/0.8% acrylamide/bisacrylamide, 25µl 10% APS and 5µl TEMED for 2 stacking gels or 5.05 ml) was poured onto the running gel. Finally the well combs were placed for the formation of wells. Fully polymerized gels were put into gel tanks and 10xrunning buffer is diluted to 1x and added into the tanks. On the other hand, 15-30mg proteins were pipetted for the sample preparation. Equal volumes of 2xloading dye (125mM Tris pH6.8, 4% SDS, 10% glycerol, 0.5% bromophenol blue and 1.8-2% mercaptoethanol) was added to each sample and proteins were denatured in a 95°C water bath for 1 minute. Then each of the samples was loaded to the SDS-PAGE gel along with a 10ml of the appropriate marker. Stacking of the gels was performed at 60V for 30 minutes while run of the proteins were achieved at 120V for 1-1.5 hours by the help of a power supply.

4.2.5. Immunoblots

Once the proteins were developed on a SDS-PAGE gel, they are removed from the electrophoresis tanks and put into a blotter surrounded by one PVDF membrane, two Watman papers and finally with sponges. Here all these components were prewetted with the transfer buffer (12mM Tris, 96mM glycine, 20% methanol) while hydrophobicity of the PVDF membrane was altered by 30 seconds methanol wash

followed by washing in the distilled water. Then proteins were blotted onto the PVDF membranes via application of 25V potential overnight. The membranes were then blocked with 5% dried milk in PBS-Tween 20 (0.2%) for 2 hours at RT and incubated with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Freiburg, Germany) in antibody buffer containing 10% (v/v) Milk Diluent/Blocking concentrate and 10% PBS-Tween20 (2 and 1.5 hours at RT relatively). After required washes with PBS-Tween 20 (3x5 minutes after blocking and primary antibody and 3x10 minutes after secondary antibody), proteins were finally analyzed using an enhanced chemiluminescence detection system (PIERCE or ECL-Advanced, Amersham Pharmacia Biotech) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech).

4.2.6. Competent Cell Preparation

We prepared an overnight culture of frozen cells in LB broth at 37C. The day after, 4x25ml mediums were inoculated with 4x250µl overnight culture and incubated till OD_{600} was 0.2 at 37°C at 280rpm. Then they were removed from incubator and put on ice for 15 minutes. They were centrifuged in the sterile Sorvall centrifuge at 5000rpm for 10 minutes at 4°C. The supernatants were discarded while the pellets were resuspended in 15ml ice cold RF1solution (RF1: 6g RbCl, 4.95g $MnCl_2 \cdot 4H_2O$, 15ml 1M potassium acetate pH 7.5, 0.75g $CaCl_2 \cdot 2H_2O$, 75g glycerol, pH is adjusted to 5.8 with acetic acid and volume was completed to 500ml). These suspensions were incubated on ice for 2 hours and they were centrifuged in the Sorvall tubes at 5000rpm for 10 minutes at 4°C. Again the supernatants were discarded and each of the pellets were carefully resuspended in 4ml RF2 solution (RF2: 4ml 500mM MOPS pH 6.8 [adjusted with NaOH], 240g RbCl, 2.2g $CaCl_2 \cdot H_2O$, 30g glycerol, pH was adjusted to 6.8 with 0.5M NaOH and the volume was completed to 200ml). They were incubated one more time for 15 minutes and they were aliquoted in 250µl into eppendorf tubes. These aliquots were freeze in liquid nitrogen and stored at -80°C for months.

4.2.7. Transformation of E.coli

TOP10, TOP10 F' or DH5 chemical competent cells were used for transformation. Competent stock were removed from freezer and thawed on ice. Each of the vials was divided into two prechilled eppendorf tubes and 10µl of each plasmid (unknown concentration since they were provided in very small amounts from our partner laboratories) were added to competent bacteria. These mixtures were incubated on ice for 45 minutes and they heat shocked at 42°C water bath for 2 minutes. After the heat shock, the tubes are immersed into ice and rested for 5 minutes. Then 1ml of LB broth was added to each tube and they were incubated 1 hour at 37°C at 280rpm for the expression of the plasmid DNA. Finally, the bacteria were plated onto selective LB agar for the selection of transformants. Transformants were compared with the control plates and stored in glycerol stocks at -80°C.

4.2.8. Mini and Midiprep of Plasmid DNAs

Transfectant bacterial cells were inoculated into appropriate amount of the selective medium with antibiotics (25-100ml) in 1l flasks. They were incubated overnight at 37°C at 280rpm. After an absorbance value of about 3 is obtained at OD_{600}, the cells were removed from the incubator and harvested at 6000g in a Sorvall centrifuge with SS-34 rotor. Then we followed the instruction manual's directions for Qiagen mini and midiprep kits for the isolation of the plasmid DNAs. However, for simplicity, we aliquoted midiprep eluants into eppendorf tubes (about 800µl eluants for each) before isopropanol and ethanol precipitations. Then DNA pellets were dissolved by 60µl TE buffer and each aliquot was collected in a single eppendorf tube for the identical plasmids.

4.2.9. Restriction Enzyme Digestion of Plasmids

To verify the identity of transformed plasmid, plasmids were digested with EcoRI, SalI or ClaI restriction enzymes as indicated in their instruction manual. Digestions were carried for 1 hour for EcoRI and 3 hours for other enzymes after

mixing DNA, enzyme, appropriate buffer and distilled water in an eppendorf tube in a 37°C water bath. The digestions were stopped by inserting reaction tubes into ice and the digests were analyzed on 0.8% agarose gel.

4.2.10. Agarose Gel Electrophoresis

Agarose gel was prepared by mixing 1xTAE buffer with 0.8% (w/v) agarose and by cooking the mixture in a microwave oven at 600W for 3 minutes (seldom shaken). The gel was left to cold-down to about 60°C for the addition of ethidium bromide (0.005%). Then immediately the gel was poured into its tray to cast and appropriate combs were placed for the formation of wells. On the other hand, samples were prepared by mixing 6xloading dye (Fermentas) with the appropriate plasmid DNA and water volumes (usually loading dye: DNA: water → 1:1:4 or loading dye: plasmid DNA → 1:5 mixtures were applied). Once the gel got hardened, the comb was removed and the gel was put into electrophoresis chamber. After the addition of 1xTAE to cover the gel, samples along with 10ml Fermentas High-range DNA ladder was loaded to wells and gel was run at 100V for 45-60 minutes. Then the DNA bands were observed under the UV-light and they were photographed by using appropriate camera.

5. RESULTS

5.1. Transfection Preparations

5.1.1. Transformation of Plasmids

pcDNA3-flagMEKK1Δ, DN (-/-) pcDNA3-flagMEKK1Δ (K432M) (Lee et al. 1998) and SRα3HA−MEKK3 DN (-/-) SRα3HA−MEKK3 (K→M) (Yang et al. 2000) were provided kindly from Prof. Tom Maniatis from Harvard Medical School and Prof Bing Su from University of Texas MD Anderson Cancer Center. These plasmids were used in transformation of E.coli to amplify their amounts. Results can be seen in figure 5.1.

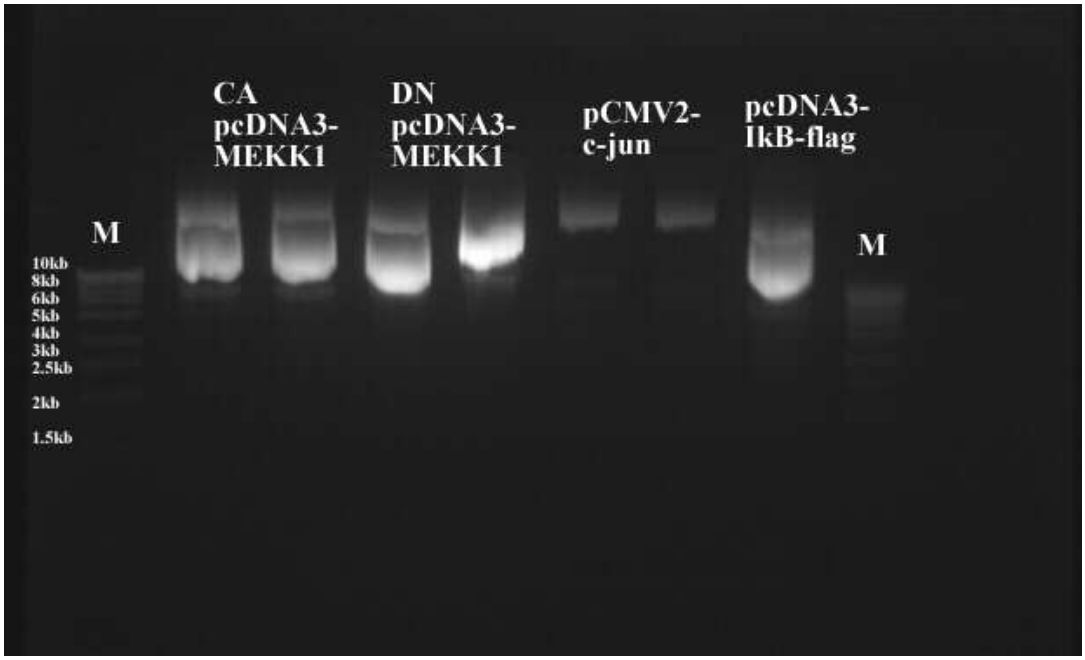


Figure 5.1: CA pcDNA3-flagMEKK1Δ, and DN (-/-) pcDNA3-flagMEKK1Δ (K432M) Miniprep Results (markers are Mass Ruler High RangeDNA, Fermentas)

Both plasmids were loaded in duplicates from different miniprep vials. Also, control data for the untransformed bacteria is available and can be seen in the following figure (figure 5.2).

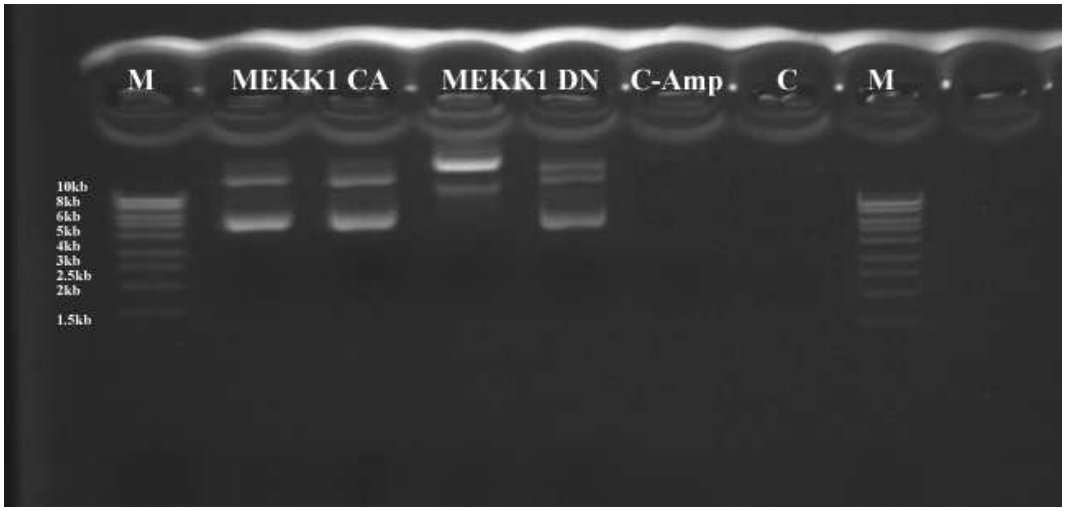


Figure 5.2: CA pcDNA3-flagMEKK1Δ, and DN (-/-) pcDNA3-flagMEKK1Δ (K432M) Miniprep results with untransformed control cells (markers are Mass Ruler High RangeDNA)

Also control cells (untransformed) were grown with ampicillin and without ampicillin in the LB medium. Being placed on the pcDNA3 plasmid (see appendix), Ampicillin provided selection for transformants so that there was no growth in the untransformed cells plated onto LB-ampicillin media. Though no growth was observed in the ampicillin containing medium, we performed miniprep of the plasmids to these controls and no plasmid bands were observed as expected.

5.1.2. Control of Plasmids with Restriction Enzyme Digestion

Both MEKK1 and MEKK3 plasmids were transformed by the use of selective media (LB ampicillin for each case) so that only transformed cells were able to survive. To verify that the plasmids we transformed were the correct ones, we digested CA and DN forms of MEKK1 plasmids with EcoRI at 37°C. Though a single band is observable, close examination revealed that EcoRI has cut our plasmids from two different places leading to two fragments, one of which was too small and other was readily observable from the gel photograph under UV light. We expected a single cut from the EcoRI digestion in pcDNA3 plasmid; however the sequence information of MEKK1 construct suggests that there is another EcoRI site in the construct which is in the close proximity of MCS site of the plasmid (see below, figure 5.2).

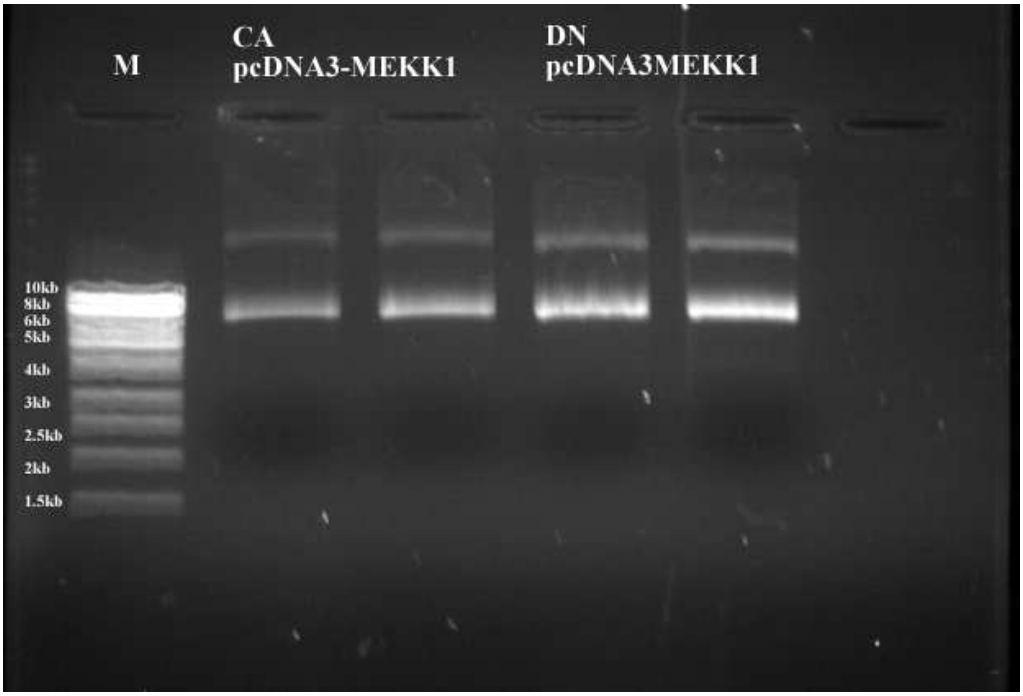


Figure 5.3: EcoRI digestion of CA pcDNA3-flagMEKK1Δ, and DN (-/-) pcDNA3-flagMEKK1Δ (K432M)

Since we considered that a single restriction digestion may be misleading we repeated the procedure with two different enzymes, ClaI and SalI. ClaI gave a single cut as expected from pcDNA3 sequence but SalI gave two cuts as opposed to single restriction site in the pcDNA3 but construct sequence was found to contain additional SalI site in both constructs (figure 5.4). The second restriction enzyme digestions were carried only by the use of CA MEKK1 since the two constructs differ by only one amino acid which had no effect on the selected restriction enzymes. Undigested control plasmids were forgotten to be loaded but you can see undigested plasmids on the figure 5.1 and 5.2. As further evidence to digestion, you can observe that the digested plasmids do not contain any open circular and supercoiled forms as opposed to undigested ones in the previous figures. The process was not repeated with MEKK3 constructs since the sequence data for these plasmids were missing. However, the constructs will be checked via sequencing as soon as possible.

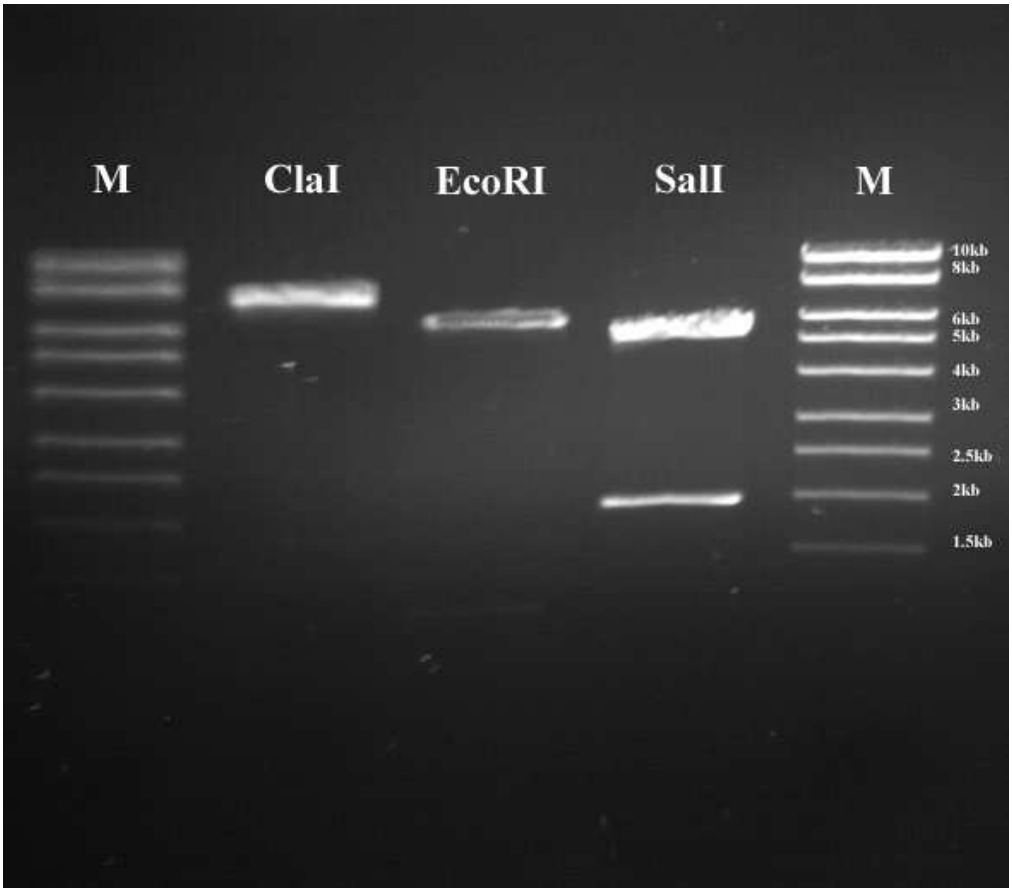


Figure 5.4: Restriction enzyme digestion of CA pcDNA3-flagMEKK1Δ

5.1.3. Midiprep of Plasmids for Preparation to Transfection

MEKK1 and MEKK3 plasmid constructs were amplified with mini and midipreps to obtain sufficient and concentrated plasmid stocks (figure 5.5). Moreover, pmaxGFP vectors were amplified via midiprep to be used as transfection efficiency control (figure 5.6).

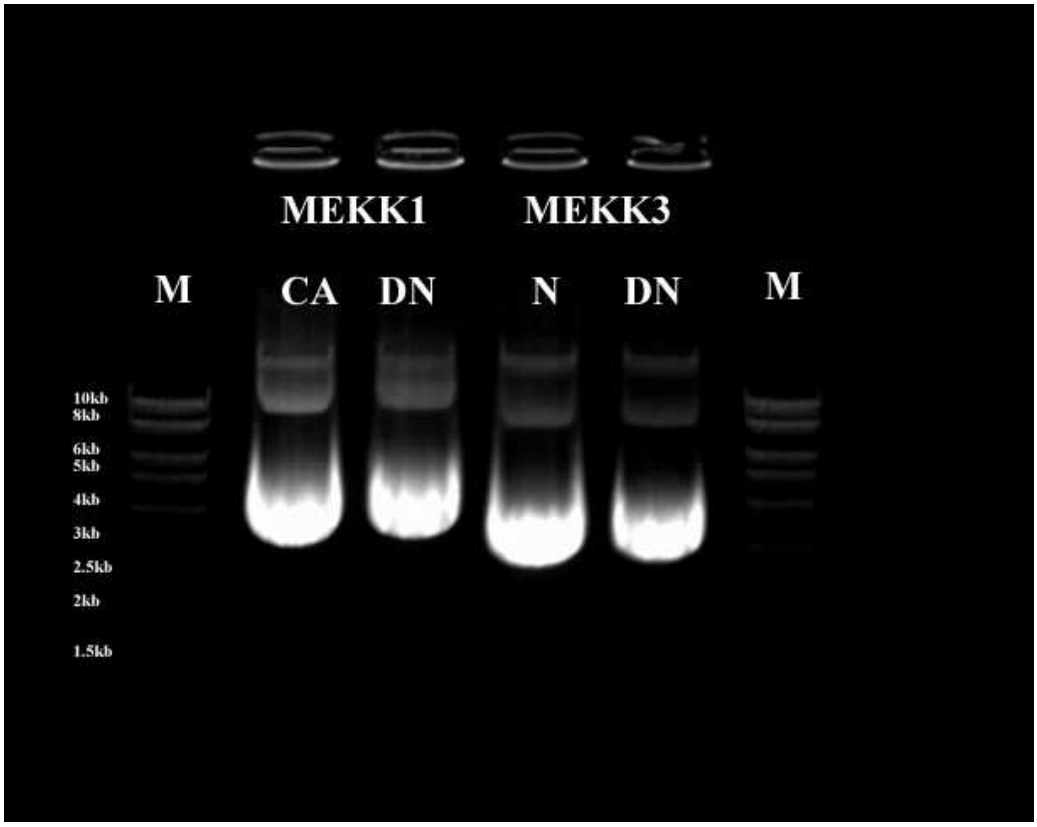


Figure 5.5: Midiprep of MEKK1 and MEKK3 plasmid constructs

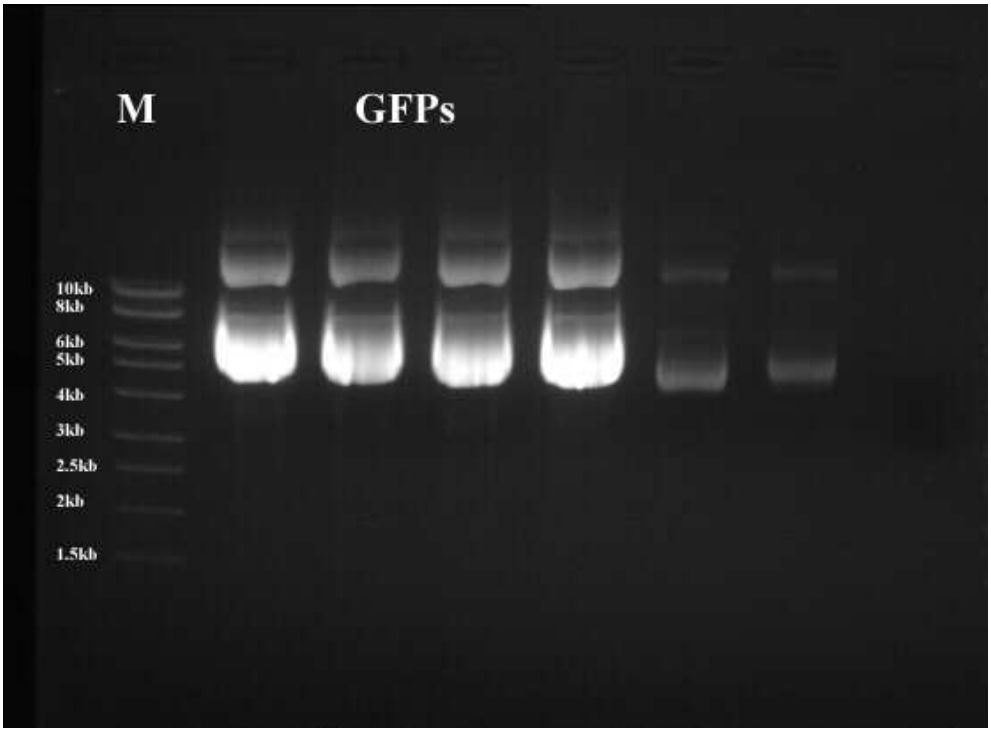


Figure 5.6: Midiprep results of pmax-GFP

5.2. MAPK Profiles for Untransfected Cells

To understand expression profiles of MAPKs to TNF induction and to verify previously obtained data, we treated cells with TNF-α prior to protein isolation with 0 to 4 or 0 to 12 hours time periods (see below) without transfecting the cells with our overexpression plasmids. From these treatments we obtained proteins ranging 2-6mg/ml and finally loaded 15-30mg of total protein to SDS-PAGE. After blotting these proteins onto PVDF membranes we probed suitable antibodies with 1/1000 to 1/10000 dilutions from their original stocks, again 1/2000 to 1/20000 dilutions were used for secondary antibodies and following profiles were obtained for JNK1 and JNK2.

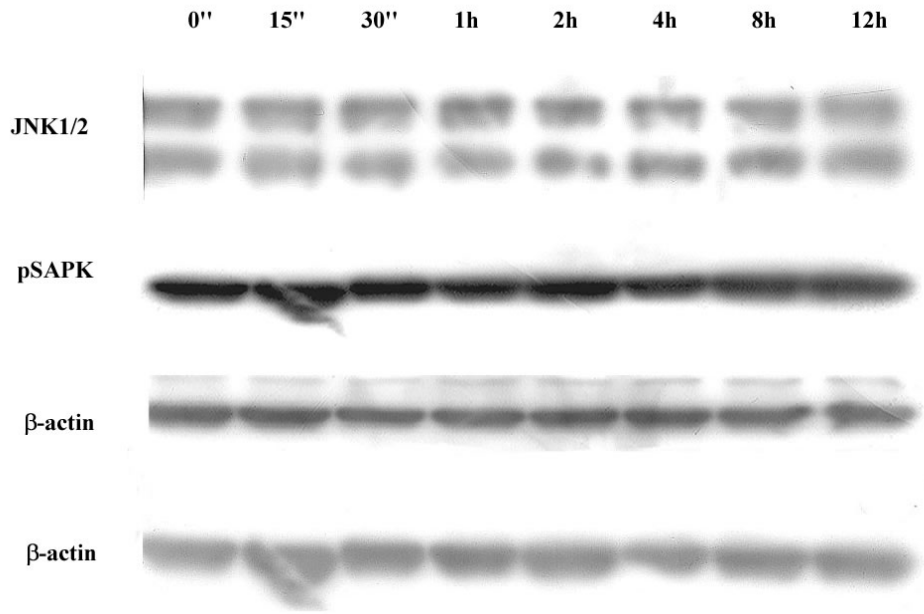


Figure 5.7: JNK and pJNK profiles upon TNF-α treatment (0-12 hours)

As described in previous studies, JNK was observed to be active throughout our time scale (0-4 hours but 4-12 hours shown first time) while some of the blots made us think a slight increase in pJNK concentrations upon TNF-α treatment but this was not

the case in each blot as it is obvious in figure 5.7. Actually, two distinct bands were observable for JNK1 (p46) and JNK2 (p54). Our antibodies for JNK1 and pJNK have identified the both JNK types while anti-pSAPK specifically bonded to p46 subunit (anti-pJNK also recognized total JNK, data not shown).

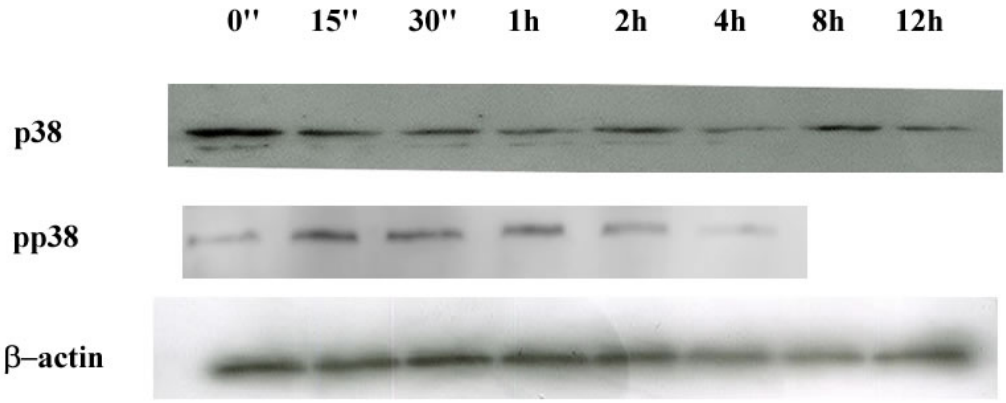


Figure 5.8: p38 and pp38 profiles in response to TNF-α treatment (0-12 hours)

Similarly we observed a slight activation in pp38 upon TNF-α treatment while p38 decreased somewhat till 15 minutes and remained the same onwards. Furthermore, p38 was found to be active at basal level for our cells as can be seen in control sample (figure 5.8). Here we obtained pp38 data from later experiment and we have abandoned 4-12 hour scans since there were no significant changes after 4 hours (will be explained in discussions). p38 phosphorylation at basal level was expected to be observed but it was still activated upon TNF-α induction which suggests that p38 is upregulated via TNF-α. Therefore, equal loading accounts only for p38 while pp38 loading was found be correct but the quality of the ECL film was not appropriate to include here.

On the other hand, ERK and ERK2 were also observed to be active (figure 5.9) as their phosphor counterparts were found in all time scale. However, there was a slight increase in the phosphorylation was evitable after 1 hour. Again we could only have a pERK profile for 0-4 hours as opposed to JNK1&2 profiles we shown above (figure 5.7).

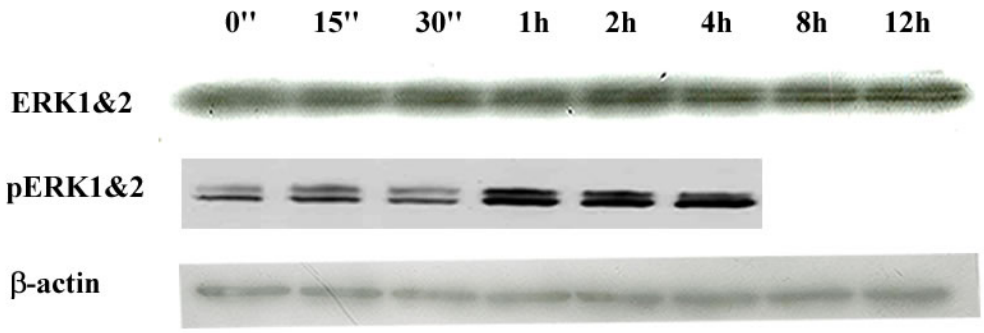


Figure 5.9: ERK and pERK profiles in response to TNF-α treatment (0-12 hours)

5.3. NF-κB Activation in Untransfected Cells via TNF-α Treatment

We have also looked for IκB response due to the TNF-α treatment to understand NF-κB behavior. IκB would be found to be associated with NF-κB if we have tried to probe it in the total proteins. For this reason, we differentially isolated another group of TNF-treated cells and we separated cytosolic and nuclear proteins. We used cytosolic part to probe IκB subunits while we put nuclear fraction aside for NF-κB activation studies. Although we could analyze NF-κB activation via EMSA, we observed that IκB subunits were phosphorylated and degraded. For this purpose, we have shown IκBα phosphorylation with anti-IκBα antibody but we didn't have anti-IκBβ and we have only demonstrated its degradation over time (figure 5.10). Eventually, IκB complex phosphorylation and degradation is a good indicator of NF-κB activation since we have already discussed events concerning the IκB phosphorylation in the chapter 2.

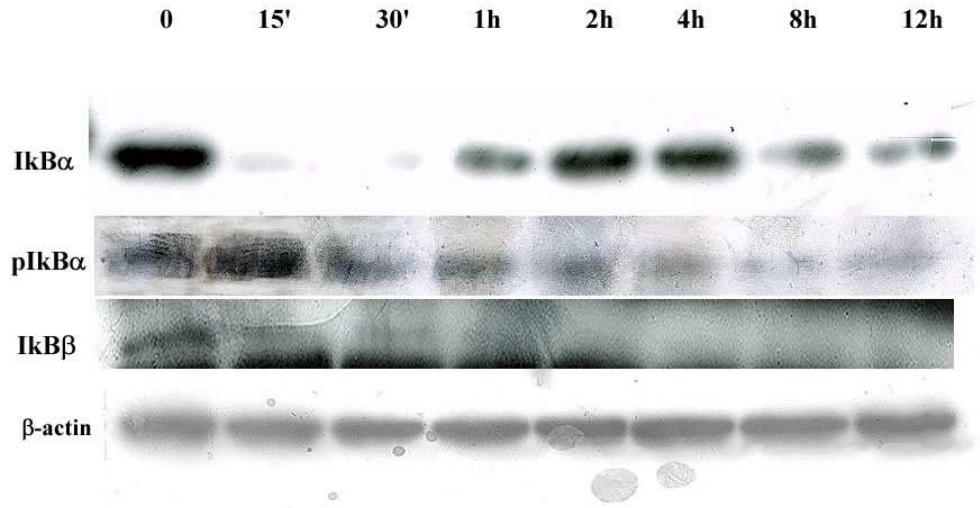


Figure 5.10: IκB-α and IκB-β degradation due to TNF-α treatment over time (0-12 hours)

5.4. Transfection of HeLa Cells with MEKK1 and MEKK3

HeLa cells were transfected with both MEKK1 and MEKK3 constructs. According to immunoblots carried with anti-flag antibodies, we have shown that our cells were successfully transfected with DN MEKK1 and CA MEKK1 (data not shown). However, we couldn't observe any binding in anti-HA probing of MEKK3 constructs although pmax-GFP transfected cells were observed to fluorescence under the ultraviolet light of fluorescent microscope with 30-50% efficiency (data not shown but it will be explained in the discussions). For this reason and we have already seen anti-flag probing in MEKK1 construct we assumed that also this transfection should have been successful but it should be further verified with another technique such as immunoprecipitation.

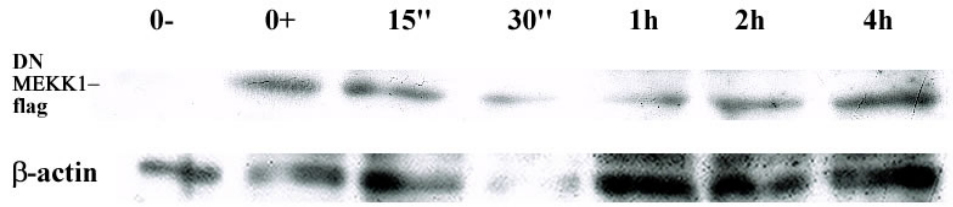


Figure 5.11: DN MEKK1-flag overexpression

Once we verified the overexpression of MEKK1 constructs, we further investigated its effects on MAPK activities under TNF-α treatment. Continuously active MEKK1 had no significant effect on JNK phosphorylation and we obtained similar results to untransfected control cells with TNF-α treatment (figure 5.12). When we looked for JNK activation in dominant negative MEKK1 overexpression; however, we couldn't observed any active JNK1 or 2 except for untransfected control. This suggested that in the absence of MEKK1, JNK cannot be phosphorylated or activated. Therefore, we have confirmed that MEKK1 is involved in JNK activation under TNF-α signaling and may be crucial for NF-κB activity. We obtained similar results for normal MEKK3 overexpression (expected to act like CA MEKK3 form) in terms of JNK activation but its role couldn't be verified without omitting MEKK3 by the use DN MEKK3 construct, which couldn't be transfected yet.

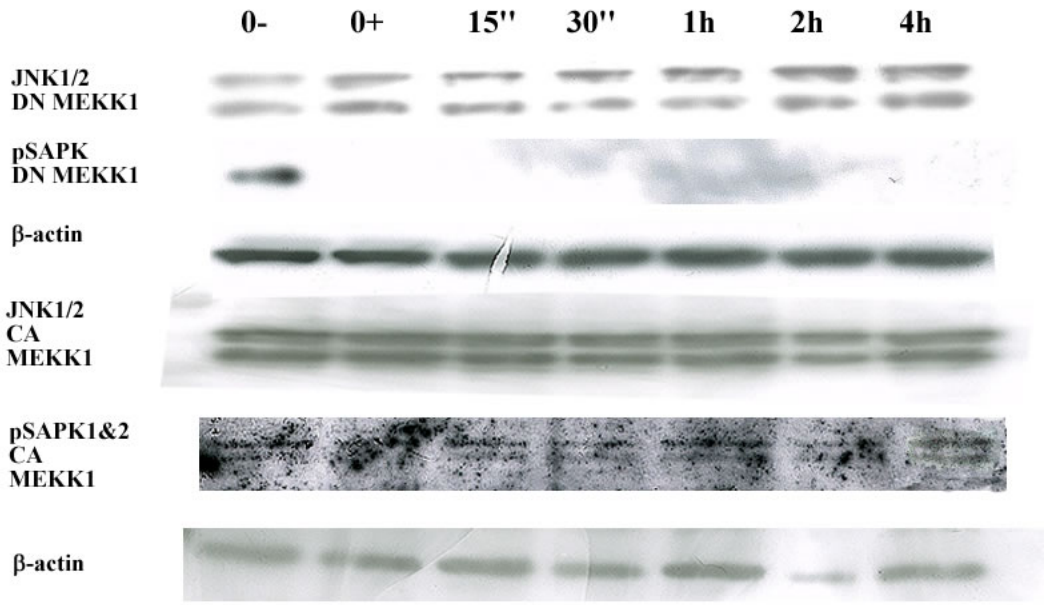


Figure 5.12: Effects of MEKK1 overexpression on JNK activation

6. DISCUSSIONS

Cytokine signaling is one of the most important issues in signal transduction since they are involved in diverse array of signaling pathways in which outer signals may induce different responses in the cell, tissue or in the organism. Understanding cytokine signaling is possible by identifying correct relation of the systems with the effector systems that are involved in cellular responses. For these reasons, understanding cytokine signaling is one of the greatest challenges in medical sciences, especially in immunology and cancer molecular biology. Although many aspects of the most studied cytokine signaling systems like TNF-α have been discovered, there still exist many interactions to be solved. Though the role of the TNF-α is well characterized, we have to clarify the downstream events that lead to cell survival or death due to TNF-α.

Of course the two pathways are not simultaneously activated in any case. The cell should choose its way to NF-κB activation or to apoptosis via caspase cascade. The important moderators were characterized and we have already mentioned about them in the previous sections. However, there is still a controversy in finding the principal moderator of the TNF-α signaling in the downstream. In some cell types, MEKK3 was claimed to involve in RIP reinforcement to TFRP and by the interaction of this couple with TRAF2 IκB activation was observed. This was evident by the studies involving gene knock-out mouse embryos missing MEKK3 were dying due to excessive apoptosis. This is possibly due to absence of TNF-α induced NF-κB activation and strongly suggests us MEKK3 is the principal activator. However, in the ultraviolet

induced apoptosis MEKK3 was found to have no role while MEKK1 or NIK are good candidates for this job. In other studies, NIK or MEKK1 was suggested that IκB activator and hence the moderator of NF-κB activation. This was supported by the IκB phosphorylation profiles in MEKK overexpressed cells. Moreover, recent studies have shown that MEKK3 was principally involved in the downstream of IL-1 and toll-like receptors and it coordinate NF-κB activation in the downstream of these elements.

All of the above discussions led us to explore principal element under TNF-α signaling. First of all we know that MEKK1 is activated upon TNF- induced cleavage of 195kDa protein into 91kDa of an active MEKK1 which further affects the downstream MAP kinases. Here most probably activation of JNK and p38 should be important since we have already known that these MAP kinases are activated via cytokines rather than mitogens, as in the case of ERKs. JNK is known to be regulated by NIK but MEKK1 was thought be involved in its activation. For example, in overexpression studies it may happen that overexpressed MAP3K can replace the function of other proteins in the same family. This was explained by the structural and functional similarity of these proteins by being in the same family. Furthermore, overexpressed proteins are found in vast amounts in which they can easily violate affinity barriers that keep them away from nonspecific functions at low concentrations. For these reasons, MEKK3-TNF-α interactions supporters [Yang et al., 2000] has claimed that this is the case in "MEKK1 involvement" in TNF-α signaling and they also claimed that this problem can be solved by developing genetic in vivo systems. To some extend they were reasonable, but we need strong evidence from protein data to prove the exact interaction. Although they have also shown MEKK3-mediated RIP reinforcement, as we have mentioned above, there exist other studies strongly in the favor of MEKK1 involvement. Since the same group [Huang et al. 2004] has shown the role of MEKK3 in IL-1 signaling, we had to decide which interaction is correct for our model system or both are involved in the overall picture.

Finally, we have decided to explore function of both MAP3 kinases under TNF-α signaling to attribute their roles in the HeLa cells. We have chosen HeLa cells since they have been studied extensively and we had a fair knowledge of events involved in these cells. Moreover, HeLa cells are not normally sensitive to TNF-α induction for short exposure. However, elongated treatments may lead to apoptotic induction. Solving

this problem was an important challenge in developing therapeutic solutions to treat ovarian cancer. For example, our laboratory has already shown that aspirin sensitizes HeLa cells towards TNF-α induced apoptosis via inhibition of the NF-κB activation pathway. Finding similar interactions will provide us with dispensable tools in the development of specific drugs or chemotherapeutic agents directly targeted to the control mechanism that gives decisions about cellular fate. Since, MAP3 kinases are strong candidates of such interactions, they deserve to be studied and their exact roles should be associated to complete overall picture in not only TNF-α signaling but also related cytokines in the picture. This is obvious when we remember the complex picture of the NF-κB signaling we mentioned in figure 2.8.

Before starting to our overexpression studies we wanted to verify results in the literature in the MAPK activation due to the TNF-α induction. We realized that usually elongated effects of the TNF-α induction was not studied in the literature and we decided to extend our studies up to 12 hour exposure. However, we have seen no obvious change after 4 hours and thereafter we only looked for 0-4 hour profiles for the remaining studies. For this reason, some profiles have 0-12 while other have 0-4 hours scans of MAPK activity profiles. First of all we know that JNK was active slightly in the HeLa cells in the basal level as shown in previous studies and verified by our findings (figure 5.7 and 5.12). This seems to be reasonable since we have already mentioned that JNK and p38 are activated upon cytokine response and involved in the activation of various downstream events leading to cell survival. TNF-α induction has an important activator role in other cell types but HeLa cells are already transformed and they need to survive even in the presence of such death signals. Here the role of the ERKs was very different and if we have omitted the cell growth to synchronize their growth by replacing media with serum free (0.2% FCS), we couldn't able to see any ERK activity due to inhibition of growth. For this reason, we haven't synchronized the cells and we could also observed ERK activation via phosphorylation (figure 5.9). Therefore, we obtained activity profiles of all MAPKs we concerned due to TNF-α induction. The next step was to compare these results with MAP3 kinase overexpressed activity profiles of them.

We have also looked for NF-κB activation in response to TNF-α induction. We have obtained reasonable data for IκBα and IκBβ degradation over time. This can be considered as a good proof of NF-κB activation but it should be also verified by EMSA

or immunoblotting of NF-κB subunits from nuclear protein fraction. Although we intended to show this activity, we didn't have p50 and p65 antibodies in our stocks. We want to confirm this phenomenon in our further studies as additional proof. Nevertheless, we couldn't able study NF-κB activation in response to overexpression of MAP3 kinases due to time management problems. For this reason, the fate of cell survival in MEKK activation or deactivation remains to be solved in our further investigations.

Once we had an overall picture of the MAPK profiles in response to TNF-α treatment, the next step was to determine the changes in the overexpression of MEKK1 and MEKK3. We first started with amplification of our plasmids with midipreps and we have verified the identity of MEKK1 construct as we mentioned in results section. However, MEKK3 was remaining to be verified since we have not got the plasmid construct map. For that reason, we have to verify its identity probably by sequencing and since MEKK3 construct were full constructs expected to give a protein of 80kDa (on the contrary, MEKK1 constructs only contains 36kDa C-terminal active site). Another way of confirming MEKK3 construct was to express the MEKK3 with HA tag but we couldn't observe HA tag in our trials. Probably, we will immunoprecipitate cell extracts with anti-HA antibody in excess amount to verify both overexpression and the identity of the plasmid in the next step. Normal MEKK3 overexpression yielded similar data with MEKK1 as we described in figure 5.12. Therefore, it makes us to think that MEKK3 is involved under TNF-α signaling but more solid evidence is required to prove this. DN MEKK3 overexpression is still ongoing and we have no data available yet.

On the other hand, we obtained meaningful data from MEKK1 overexpression in comparison to previously claimed functions. Accordingly, the removal of MEKK1 activity due to DN MEKK1 overexpression leads to a cease in JNK phosphorylation. We could only observe an active JNK in the case of untransfected control cells (figure 5.12) while in all of the transfectants no JNK activation via phosphorylation was evident. Since we have confirmed the overexpression of DN and CA MEKK1 with anti-flag antibody, MEKK1 have a crucial role under TNF-α signaling. In its presence JNK is activated while its removal causes the inhibition of this activity. Therefore, MEKK1 is a moderator of TNF-α signaling over the MAPKs. However, its effect on other

MAPKs should also be associated along with its consequences on NF-κB activation, which remains to be investigated.

To sum up, the results we obtained will only gave us clues about the involvement of MEKKs in cytokine signaling. We further investigate their roles with other systems to correctly associate their roles to individual systems. However, we won't be finished if we do so. We will need additional information from in vivo animal systems to completely associate the roles of each moderator and effector in the overall picture, since in vitro cell cultures give us only a glimpse of the events in a living organism. The complete structure of the signaling mechanisms can be only accomplished by verifying our findings in the animal models and finally in the medical applications, which is our ultimate goal in developing therapeutic strategies towards cancer or related diseases.

7. CONCLUSIONS

In this study we tried to investigate MEKK1 and MEKK3 involvement in TNF-α signaling

From these studies we obtained that:

- All three MAPKs are either already active or activated in response to TNF-α induction
- Continuously active MEKK1 activates JNK phosphorylation (directly or indirectly) but the nature of this effect should be clarified.
- Dominant negative MEKK1 diminish MEKK1 activity in the HeLa cells leading to JNK inhibition as shown by the absence of pJNK in the DN MEKK1 overexpressed TNF-α induction
- Normal MEKK3 overexpression has similar effects on MAPKs as MEKK1 overexpression but the effect of its removal via DN MEKK3 overexpression is yet to be investigated

In the light of these observations we need to further explore

- Nature of MEKK1 moderated activation of MAPK mechanisms in TNF-α signaling
- Association of the MEKK1 overexpression with p38 and ERK activation and their action mechanism in addition to JNK activation we proved
- Consequences of the MEKK3 removal in TNF-α signaling
- Integration of the MEKK1 and MEKK3 studies into NF-κB activation and their co-involvement in TNF-α signaling
- Integration of the TNF-α signaling to IL-1 signaling, so that we can decide which MAP3K is involved primarily under these signaling pathways or whether they are involved at the same time
- Confirmation of these studies in vivo animal models

8. REFERENCES

1. Adams, J.M., Cory, S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; 281: 1322-1326.

2. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. Molecular Biology of the Cell. 2001 (4th edition). New York: Garland Science.

3. Arch, R.H., Gedrich, R.W., Thompson, C.B. Tumor necrosis factor receptor associated factors (TRAFs)-a family of adapter proteins that regulates life and death. Genes Dev. 1998; 12: 2821-2830.

4. Arslan, M.A., Göktuna, S.İ., Kütük, Ö., Başağa, H. Signaling mechanisms as drug targets in cancer. Advances in Molecular Medicine 2005; 1 (Suppl.): 559-565.

5. Attardi, L.D., Lowe, S.W., Brugarolas, J., Jacks, T. Transcriptional activation by p53, but not induction of p21 gene, is essential for oncogene-mediated apoptosis. EMBO Journal 1996; 15: 3693-3701.

6. Baichwal, V.R., Baeuerle, P.A. Activate NF-kappaB or die? Curr. Biol. 1997; 7: 94-96.

7. Baldwin, A.S. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. 1996; 14: 649-681.

8. Baeuerle, P.A., Baltimore, D. NF-kappa B: ten years after. Cell 1996; 87: 10-13.

9. Blow, J.J., Nurse, P. A cdc2-like protein is involved in the initation of DNA replication in Xenopus egg extracts. Cell 1990; 62: 855-862.

10. Bruemmer, D., Yin, F., Liu, J., Kiyono, T., Fleck, E, Van Herle, A.J., Law, R.E. Expression of minichromosome maintenance proteins in vascular smooth muscle cells is ERK/MAPK dependent. Experimental Cell Research 2003; 290: 28-37.

11. Chao, D.T., Korsmeyer, S.J. Bcl-2 family: regulators of cell death. Ann. Rev. Immunol. 1998; 16: 395-419.

12. Chinnaiyan, A.M., O'Rourke, K., Tewari, M., Dixit, V.M. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995; 81: 505-512.

13. Chu, Z.L., McKinsey, T.A., Liu, L., Gentry, J.J., Malim, M.H., Ballard, D.W. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc. Natl. Acad. Sci. U.S.A. 1997; 94: 10057-10062.

14. Cordon-Cardo, C. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am. J. Pathol. 1995; 147: 545–560.

15. Degterev, A. Boyce, M. Yuan, J. A decade of caspases. Oncogene 2003; 22: 8543-8567.

16. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A. Jr., Butel, J.S., Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature 1992; 356: 215-221.

17. Downward, J. Targeting Ras signaling pathways in cancer therapy. Nature Rev. Cancer 2003; 3, 11–22.

18. Draetta, G., Luca, F. Westendorf, J. Brizuela, L., Ruderman, J., Beach, D. cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell 1989; 56: 829-838.

19. Dunphy, W.G., Brizuela, L., Beach, D. The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell 1988; 54: 423-431.

20. Elion, E.A. Routing MAP kinase cascades. Science 1998; 281: 1625–1626.

21. Ellis, R.E., Yuan, J.Y., Horvitz, R.A. Mechanisms and functions of cell death. Annu. Rev. Cell Biol. 1991; 7: 663-698.

22. Evan, G. I. & Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. Nature 2001; 411, 342–348.

23. Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., Hunt, T. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 1983; 33: 389-396.

24. Fang, B., Roth, J.A. Tumor-suppressing gene therapy. Cancer Biol. Ther. 2003; 2: 115–121.

25. Farooq, A. & Zhou, M.-M. Structure and regulation of MAPK phosphatases. Cell. Signalling 2004; 16, 769–779.

26. Filomeni, G. Acquilano, K., Rotilio, G., Criolo, M.R. Reactive oxygen species-dependent c-Jun NH2-terminal kinase/c-Jun signaling cascade mediates neuroblastoma cell death induced by diallyl disulfide. Cancer Research. 2003; 63: 5940-5949.

27. Gautier, J., Norbury, C., Lohka, M., Nurse, P. Mahler, J. Purified maturation-promoting factor contains the product of a Xenopus homologue of the fission yeast cell cycle control gene cdc2. Cell 1988; 54: 433-439.

28. Ghoda, L., Lin, X., Greene, W.C. The 90-kDa ribosomal S6 kinase (pp90rsk) phosphorylates the N-terminal regulatory domain of IkappaBalpha and stimulates its degradation in vitro. J. Biol. Chem. 1997; 272: 21281–21288.

29. Ghosh, S., May, M.J., Kopp, E.B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev Immunol 1998; 16: 225-260.

30. Gilmore, TD. The Rel/NF-kappaB signal transduction pathway: introduction. Oncogene 1999; 18: 6842-6844.

31. Gutkind, J.S. Signaling networks and cell cycle control: the molecular basis of cancer and other diseases. 2000. Totowa: Humana Press.

32. Guttridge, D.C., Albenese, C., Reuther, J.Y., Pestell, R.G., Baldwin, Jr. A.S. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell Biol. 1999; 19:5785-5799.

33. Hahn, W. C. & Weinberg, R. A. Modelling the molecular circuitry of cancer. Nature Rev. Cancer. 2002; 5: 331-341.

34. Han, H., Landreneau, R.J., Santucci, T.S., Tung, M.Y., Macherey, R.S., Shackney, S.E., Raab, S.S., Sturgis, C.D., Silverman, J.F. Prognostic value of immunohistochemical expressions of p53, HER-2/neu, and bcl-2 in stage I non-small-cell lung cancer. Hum. Pathol. 2002; 33: 105–110.

35. Hansen, R., Oren, M. p53; from inductive signal to cellular effect. Curr. Opin. Genet. Dev. 1997; 7: 46–51.

36. Harbour, J.W., Dean, D.C. The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Development 2000; 14: 2393-2409.

37. Hartwell, L.H., Kastan, M.B. Cell cycle control and cancer. Science 1994; 266: 1821–1828.

38. Hartwell, L.H., Weinert, T.A. Checkpoints: Controls that ensure the order of cell cycle events. Science 1989; 246: 622-628.

39. Haupt, Y., Maya, R., Kazaz, A., Oren, M. Mdm2 promotes the rapid degradation of p53. Nature 1997; 387: 296–299.

40. Hayles, J., Fisher, D., Woollard, A., Nurse, P. Temporal order of S phase and mitosis in fission yeast is determined by the state of the $p34^{cdc2}$ mitotic B cyclin complex. Cell 1994; 78: 813-822.

41. Herr, C.J. Mammalian G1 cyclins. Cell 1993; 73: 1059-1065.

42. Hsu, H., Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 1995; 81: 495-504.

43. Hsu, H., Shu, H.B., Pan, M.G., Goeddel, D.V. TRADDeTRAF2 and TRADDeFADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 1996; 84: 299-308.

44. Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z., Su B. Differential regulation of interleukin 1 receptor and toll-like signaling by MEKK3. Nature Immunology 2004; 5: 98-103.

45. Huang, S., Pettaway, C.A., Uehara, H., Bucana, C.D., Fidler, I.J. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene 2001; 20: 4188-4197.

46. Huang, S., Robinson, J.B., DeGuzman, A., Bucana, C.D., Fidler, I.J. Blockade of nuclear factor-kB signaling inhibits angiogenesis and tumorigenicity of ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer. Res. 2000; 60: 5334-5339.

47. Iggo, R., Gatter, K., Bartek, J., Lane, D., Harris, A.L. Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 1990; 335: 675-679.

48. Ishikawa, Y., Kusaka, E., Enokido, Y., Ikeuchi, T., Hatanaka, H. Regulation of

Bax translocation through phosphorylation at Ser-70 of Bcl-2 by MAP kinase in NO-induced neuronal apoptosis. Mol. Cell. Neurosci. 2003; 24: 451-459.

49. Johnson, G. L. Lapadat, R. Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science 2002, 298: 1911-1912.

50. Johnston, G.C., Pringle, J.R., Hartwell, L.H. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. Exp. Cell Research 1977; 105: 79-98.

51. Kosako, H., Gotoh, Y., Matsuda, S. Xenopus MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. EMBO J. 1992; 11: 2903–2908.

52. Kütük, O., Başağa H. Aspirin inhibits TNFa- and IL-1-induced NF-κB activation and sensitizes HeLa cells to apoptosis. Cytokine 2004; 25: 229-237.

53. Lee, K.H., Hyun, M.S., Kim, J.R. Growth factor-dependent activation of the MAPK pathway in human pancreatic cancer: MEK/ERK and p38 MAP kinase interaction in uPA synthesis. Clin. Exp. Metastasis 2003; 20: 499-505.

54. Lee, F. S., Peters, R.T., Dang, L. C., Maniatis, T. MEKK1 activates both IκB kinase alpha and IκB kinase beta. Proc. Natl. Acad. Sci. 1998; 95: 9319-9324.

55. Lee, F. S., Hagler, J., Chen, Z. J., Maniatis, T. Activation of IκBa complex by MEKK1, a kinase of JNK pathway. Cell 1997; 88: 213-222.

56. Levine, A.J. p53, the cellular gatekeeper for growth and division. Cell 1997; 88: 323-331.

57. Lewin, B. Genes VII. 2000. North Yorkshire; Oxford University Press.

58. Li, H., Yuan, J. Deciphering the pathways of life and death. Curr. Opin. Cell Biol. 1999; 11: 261-266.

59. Liu, Z.G., Hsu, H., Goeddel, D.V., Karin, M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 1996; 87: 565-576.

60. Masui, Y., Markert, C.L. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. of Exp. Zoology 1971; 177: 129-146.

61. Matheny, S.A., Chen, C., Kortum, R.L., Razidlo, G.L., Lewis, R.E., White, M.A. Ras regulates assembly of mitogenic signaling complexes through the effector protein IMP. Nature 2004; 427, 256–260.

62. Mendoza, F. J., Henson, E. S., Gibson, S. B. MEKK1-induced apoptosis is mediated by Smac/Diablo release from the mitochondria. Biochemical and Biophysical Research Communications 2005; 331; 1089-1098.

63. Miyamoto, S., Verma, I.M. NF-kappaB/Rel/IkappaB story. Adv Cancer Res 1995; 66: 255-292.

64. Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C. & Blenis, J. Molecular interpretation of ERK signal duration by immediate early gene products. Nature Cell Biol. 2002; 4: 556–564.

65. Murray, A.W., Hunt, T. The Cell Cycle: An Introduction. 1993. New York; WH Freeman.

66. Nair, A., Venkatraman, M., Maliekal, T.T., Nair, B., Karunagaran, D. NF-κB is constitutively activated in high- grade squamous intraepithelial lesions and squamous cell carcinomas of the human uterine cervix. Oncogene 2003; 22: 50-58.

67. Nakano, H. Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita H., Okumura, K. Differential regulation of IκB kinase a and b by two upstream kinases, NF-κB inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc. Natl. Acad. Sci. 1998; 95: 3537-3542.

68. Nurse, P., Thuriaux, P., Nasmyth, K. Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. Mol. Gen. Genet. 1976; 146: 167-178.

69. Nurse, P. Ordering S phase and M phase in the cell cycle. Cell 1994; 79: 547-550.

70. Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6853-6866.

71. Rao, P.N., Johnson, R.T. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature 1970; 225: 159-164.

72. Rayet, B., Ge'linas, C. Aberrant rel/nfkb genes and activity in human cancer. Oncogene 1999; 18: 6938-6947.

73. Reed, S.I. The role of p34 kinases in G1 to S phase transition. Ann. Rev. Cell Biol. 1992; 61: 441-470.

74. Reinmuth, N., Mesters, R. M., Bieker, R., Hoffknecht, P., Berdel, W., Thomas, M. Signal transduction pathways as novel therapy targets in lung cancer. Lung Cancer 2004; 45 Suppl. 2; 177–186.

75. Riabowol, K., Draetta, G., Brizuela, L. Vandre, D., Beach, D. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. Cell 1989; 57: 393-401.

76. Schulte, G. and Fredholm, B. B. Signaling from adenosine receptors to mitogen-activated protein kinases. Cellular Signaling. 2003; 15: 813-827.

77. Sebolt-Leopold, J.S., Herrera, R. Targeting the Mitogen Activated Protein Kinase Cascade to Treat Cancer. Nature Reviews Cancer. 2004; 5: 937-947.

78. Shapiro, P. S. & Ahn, N. G. Feedback regulation of Raf-1 and mitogen-activated protein kinase (MAP) kinase kinases 1 and 2 by MAP kinase phosphatase-1 (MKP-1). J. Biol. Chem. 1998; 273, 1788–1793.

79. Sherr, C. J. G2 phase progression: cycling on cue. Cell 1994; 79: 551-555.

80. Sherr, C.J., Roberts, J.M. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes and Development 1995; 9: 1149-1163.

81. Siebenlist, U., Fransozo, G., Brown, K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol 1994; 10: 405-455.

82. Simanis, V., Nurse, P. The cell cycle control gene cdc2 of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell 1986; 45: 261-268.

83. Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, F., Re, F., Mantovani, A., Shanebeck, K., Grabstein, K.H., Dower, S.K.. Interleukin 1 signaling occurs exclusively via the type I receptor. Proc. Natl. Acad. Sci. 1993; 90: 6155-6159.

84. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., Leder, P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987; 49: 465-475.

85. Stewart, T.A., Pattengale, P.K., Leder, P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 1984; 38: 627-637.

86. Suda, T., Takahashi, T., Golstein, P., Nagata, S. Molecular cloning and the expression of the Fas ligand, a novel member of the TNF family. Cell 1993; 76: 1169-1178.

87. Ting, A.T., Pimentel-Muinos, F.X., Seed, B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J. 1996; 15: 6189-6196.

88. Tzivion, G., Luo, Z., Avruch, J. A dimeric 15-5-5 protein is an essential cofactor for Raf kinase activity. Nature 1998; 394: 88–92.

89. Upstate Cell Signaling Solutions Site Retrieved on April 15, 2005 from World Wide Web http://www.upstate.com/img/pathways/apoptosis_2005.jpg

90. U.S. National Library of Science GHR Site Retrieved on May 22, 2005 from World Wide Web http://ghr.nlm.nih.gov/ghr/picture/apoptosis_macrophage

91. Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., Verma, I.M. Suppression of TNF-αlpha-induced apoptosis by NF-kappaB. Science 1996; 274: 787-789.

92. Vogelstein, B., Lane, D.P., Levine, A.J. Surfing the p53 network. Nature 2000; 408: 307-310.

93. Waetzig, V., Herdegen, T. MEKK1 controls neurite regrowth after experimental injury by balancing ERK1/2 and JNK2 signaling. Molecular and Cellular Neuroscience 2005;30:67-78.

94. Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A., Boldin, M.P. TNF receptor and Fas signaling mechanisms. Annu Rev. Immunol. 1999; 17: 331-367.

95. Wang, Y., Waldron, R.T., Dhaka, A., Patel, A. Riley, M.M., Rozengurt E., Colicelli, J. The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. Mol. Cell. Biol. 2002; 22, 916–926.

96. Wang, C.Y., Mayo, M.W., Korneluk, R., Goeddel, D.V., Baldwin, Jr. A.S. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998; 281: 1680-1683.

97. Wang, C.Y., Mayo, M.W., Baldwin, Jr. A.S. TNF-α and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science 1996; 274: 784-787.

98. Weinberg, R.A. The retinoblastoma protein and the cell cycle control. Cell 1995; 81: 323-330.

99. Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., Su, B. The essential role of MEKK3 in TNF-induced NF-κB activation. Nature Immunology 2000; 2: 620-624.

100. Yeung, K., Janosch, P., McFerran, B., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W. Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein. Mol. Cell. Biol. 2000; 20: 3079–3085.

101. Zachariae, W., Nasmyth, K. Whose end is destruction: cell division and anaphase-promoting complex. Genes Development 1999; 13: 2039-2058.

102. Zandi, E., Rothwarf, D. M., Delhasse, M., Hayakawa, M., Karin, M. The IκB kinase complex (IκK) contains two kinase subunits, IκBα and IκBβ, necessary for IκB phosphorylation and NF-κB activation. Cell 1997; 91: 243-252.

9. APPENDIXES

APPENDIX A

CHEMICALS

Name of Chemical	Supplier Company	Catalog Number
Acetic Acid	Riedel-de Haén, Germany	27225
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
	Biorad Inc., USA	161-0158
Agarose low EO	Applichem, Germany	A2114
Ammonium persulfate	Carlo-Erba, Italy	420627
Ampicillin	Sigma, Germany	A9518
Chloroform	Merck, Germany	102431
Distilled water, sterile, MilliQ Filtered	Millipore, France	

EDTA (Ethylenediamine tetraacetic acid)	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa^{R}, USA	0167
HCl	Merck, Germany	100314
Isopropanol	Riedel-de Haén, Germany	24137
Kanamycin	Sigma, Germany	K4000.102
KCl	Fluka, Switzerland	60129
KH_2PO_5	Riedel-de Haén, Germany	04243
KOH	Riedel-de Haén, Germany	06005
Liquid nitrogen	Karbogaz, Turkey	
Luria Agar (Miller's LB Agar)	Sigma,Germany	L-3147
Luria Broth (Lennox L Broth)	Sigma, Germany	L-3022
2-Mercaptoethanol	Aldrich Chemical Company, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
NaCl	Riedel-de Haén, Germany	13423

$NaO_2C_2H_5.5H_2O$	Riedel-de Haén, Germany	25022
NaOH	Merck, Germany	106462
$NaPO_5H_2$	Riedel-de Haén, Germany	04269
Sodium Dodecyl Sulphate	Sigma, Germany	L-4390
TEMED	Sigma, Germany	T-7029
Triton® X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Tween® 20	Merck, Germany	822184

APPENDIX B

MOLECULAR BIOLOGY KITS

Name of Kit	Supplier Company	Catalog Number
ECL Advance Western Blotting Detection Kit	Amersham Biosciences Sweden	RPN2135
Quiaprep® Spin Miniprep Kit (250)	Qiagen, Germany	27106
QIAGEN® Plasmid Midi Kit (100)	Qiagen, Germany	12145

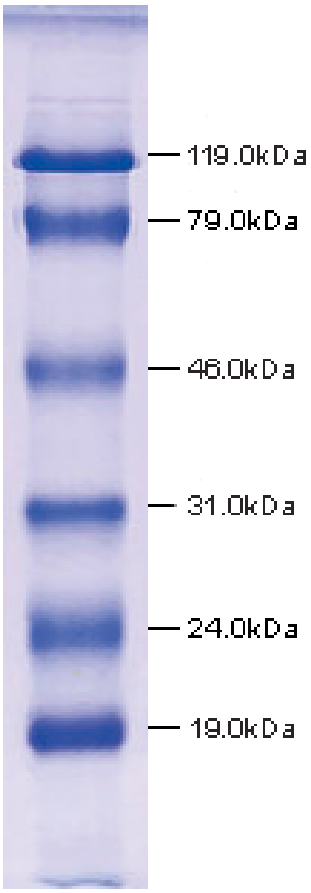
APPENDIX C

OTHER MATERIALS

NAME	Supplier Company	Catalog Number
*Eco*RI	Fermentas, Germany	#ER0271
Glass beads 0.5 mm d	Biospec Products,Inc, US.	11079105
Hybond-P membrane (PVDF)	Amersham Biosciences Sweden	RPN2020F
Hyperfilm ECL	Amersham Biosicences Sweden	RPN2103K
Mass Ruler High RangeDNA Ladder, Mix	Fermentas, Germany	#SM0393
Prestained Protein MW Marker	Fermentas, Germany	#SM0441
Full Range Rainbow MW Marker	Amersham Pharmacia Sweden	RPN800
RNAse	Qiagen, Germany	
Tumor Necrosis Factor-alpha(human)	Sigma	T6674

TOP10	Invitrogen, Germany
TOP10F’	Invitrogen, Germany

Prestained Protein Molecular Weight Marker



Full Range Rainbow MW Marker

Figure 8.1: Protein molecular weight markers on 12% SDS-PAGE

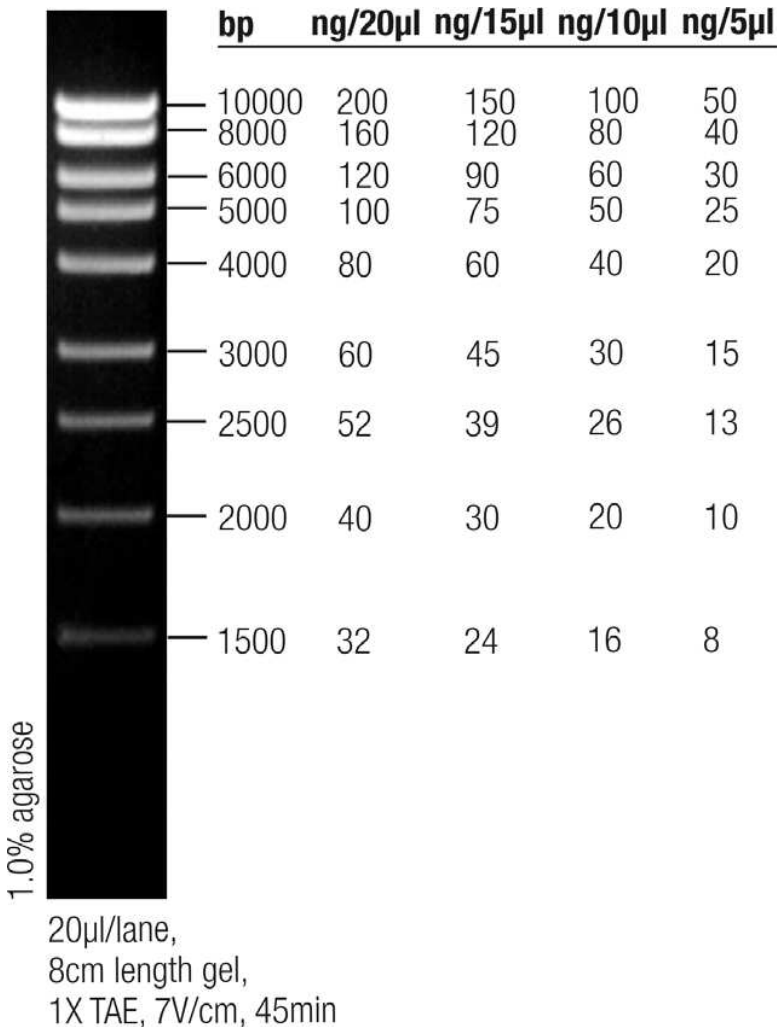


Figure 8.2: MassRuler High Range DNA Ladder, ready-to-use

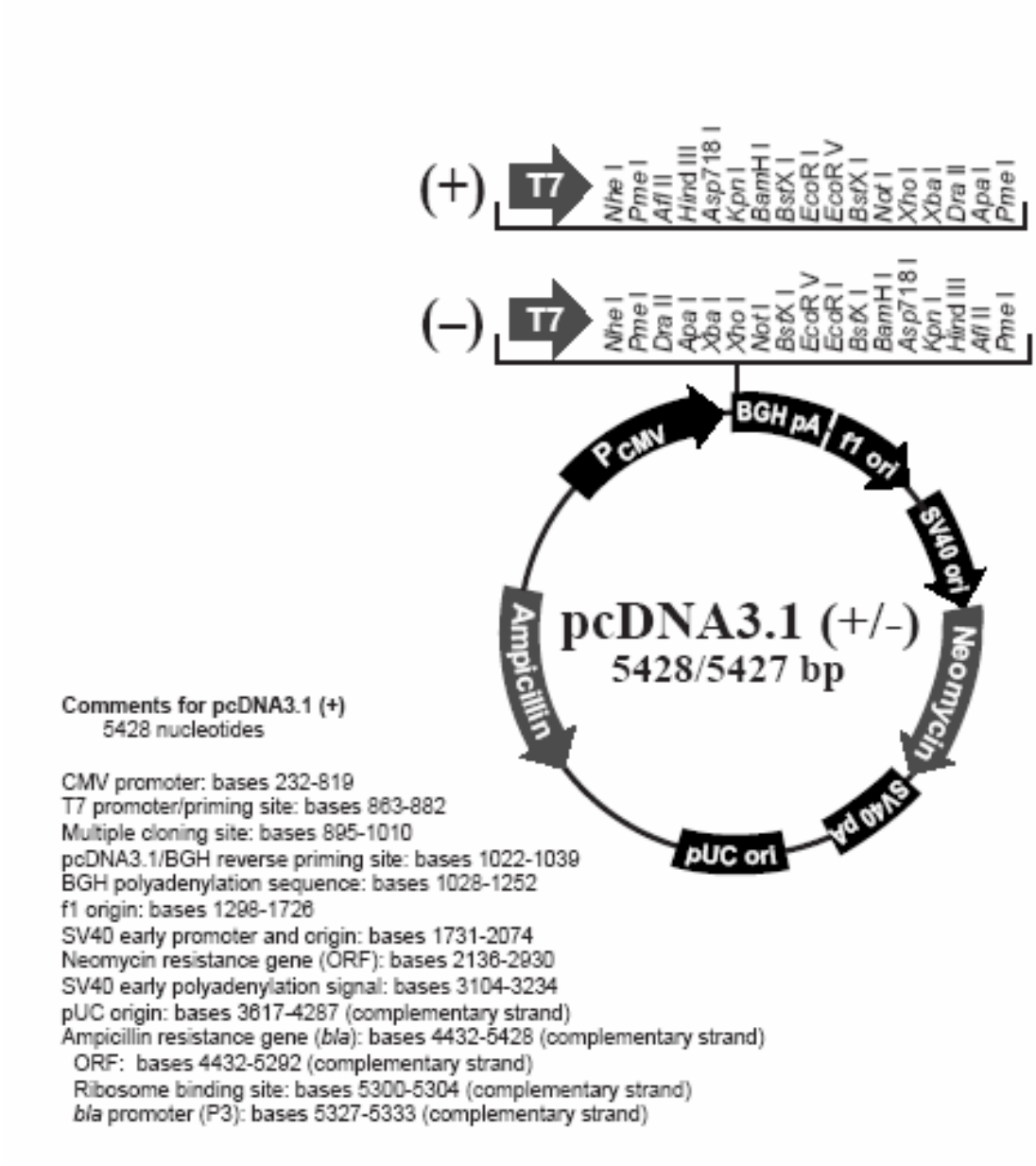


Figure 8.3: pcDNA3.1 plasmid vector. Our pcDNA3flagMEKK1D and pcDNA3-flagMEKK3 constructs were prepared by insertion of C-terminal 321 residues of MEKK1 gene [Lee et al. 1997] while dominant negatives were produced by the mutation of lysine 432 to methionine by site directed mutagenesis (figure is adopted from Invitrogen brochure).

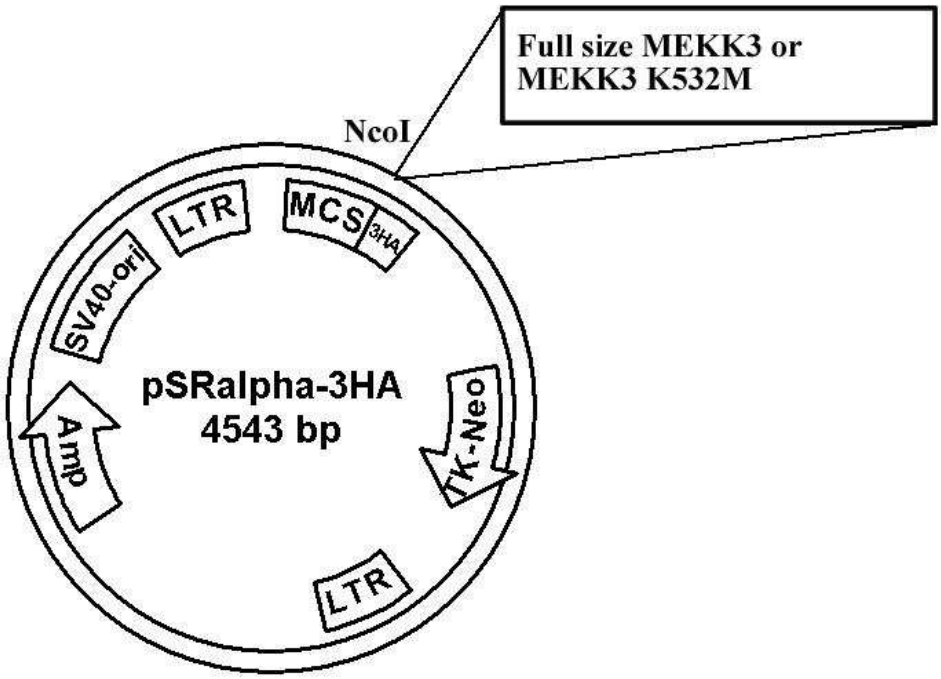


Figure 8.4: pSRa3HA -MEKK3 plasmids. They were constructed by cleaving the vector from NcoI site and inserting the full size MEKK3 PCR products, either normal or K to M mutated via SDM [Yang et al. 2000]**.**

APPENDIX D

LIST OF EQUIPMENTS

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA
Automatic Pipette:	HIRSHMANN LABORGERATE Pipettus®-Akku (Germany)
Balance:	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Blot Module	X Cell II ™ Blot Module, Novex, USA
Centrifuges:	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod., Heraeus Multifuge 5L, GERMANY
	Hitachi, Sorvall RC5C Plus, USA
	Hitachi, Sorvall Discovery 100 SE, USA
CO_2 Incubator:	BINDER® Incubator (Germany)
Deepfreezers:	-70^{o} C, Kendro Lab. Prod., Heraeus Hfu586 Basic, GERMANY

	-20^{o} C, Bosch, TÜRKİYE
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	X Cell SureLock ™ Electrophoresis Cell, Novex USA
	X Cell II SureLock™ Electrophoresis Cell, Invitrogen USA
Gel Documentation:	UVITEC, UVIdoc Gel Documentation System, UK
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Model 600, GERMANY
Laminar Flow:	Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Mettler Toledo, Volumate, USA
	Eppendorf, Germany
Microplate Reader:	BIORAD Model 680
Microscopes:	OLYMPUS CK30 (Japan) OLYMPUS IX70 (Japan) OLYMPUS BX60 (Japan)

Microwave Oven:	Bosch, Turkey
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 500, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4° C, Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
	C25HC Incubator shaker New Brunswick Scientific, USA
Spectrophotometer:	Schimadzu, UV-1208, JAPAN
	Schimadzu, UV-3150, JAPAN
	Secoman, Anthelie Advanced, ITALY
Speed Vacuum:	Savant, Speed Vac® Plus Sc100A, USA
	Savant, Refrigerated Vapor Trap RVT 400, USA
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Vortex Mixers:	VELP SCIENTIFICA (Italy)
Water bath:	Huber, Polystat cc1, GERMANY
Culture Flasks, Multiwell Plates, Falcon Tubes and Sterile Pipettes:	TPP (Europe) or Falcon (USA)