Regulation of MAPK phosphatase expression in the prostate cancer cell line LNCaP - Possible role in apoptosis

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List of abbreviations

AP-1	Activator protein-1
Apaf-1	Apoptotic-protease-activating factor-1
AR	Androgen receptor
ARE	Androgen response element
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bcl-2	B-cell lymphoma-2
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CD	Common docking domain
CDK	Cyclin-dependent kinase
DBD	DNA-binding domain
DD	Death domain
DED	Death effector domain
DHT	5α-dihydrotestosterone
DISC	Death inducing signaling complex
DUSP	Dual specificity phosphatase
ED	Glutamate-aspartate site
EDCs	Endocrine disrupting chemicals
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTP	Guanosine triphosphate
IAP	Inhibitor of apoptosis proteins
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
LBD	Ligand-binding domain
MAPK	Mitogen-activated protein kinase
MKB	MAP kinase binding domain
MKP	MAP kinase Phosphatase
NKX3.1	Homeodomain-containing transcription
PBS	Phosphate-buffered saline
PEST	Pro-Glu-Ser-Thr sequence
PIN	Prostatic intraepithelial neoplasia
PKC	Protein kinase C
PSA	Prostate Specific Antigen
PTEN	Phosphatase and tensin homolog deleted on
PTP	Protein tyrosine phosphatase
RT-PCR	Reverse-Transcriptase Polymerase Chain
SAPK	Stress-activated protein kinase
siRNA	Small interfering RNA
TBP	TATA binding protein
TG	Thapsigargin
TNF-R	Tumour necrosis factor receptor
TPA	12-O-tetradecanoyl-13-phorbol-acetate
TRE	TPA-response element
TUNEL	TdT-mediated dUTP nick end labeling
UV	Ultraviolet
VHR	VH1-related

General introduction

Apoptosis

Cell birth and cell death are tightly regulated in time and space, both during the development of an organism and at maturity. With the exception of the passive dying of cells in necrosis, cell death is 'programmed', serving a variety of processes like the sculpturing of the embryo, the maintenance of tissue homeostasis, the termination of immune responses, and the restriction of infections. Programmed cell death is often equated with apoptosis [1]. Many diseases, including cancer, autoimmunity and degenerative disorders, could be caused by disturbed regulation of apoptosis [2]. The morphological changes that can be seen in apoptotic cells are cell shrinkage, loss of contact with neighboring cells, chromatin condensation, blebbing of the plasma membrane, and fragmentation into compact membrane-enclosed structures called apoptotic bodies [3, 4]. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. In contrast, necrosis is characterized by rapid swelling of cells that have been exposed to stresses such as chemical or mechanical injury, or environmental changes that lead to irreversible damage. Eventually, these cells burst and spill their contents into the extracellular space causing a strong inflammatory response in the surrounding tissue [5].

Apoptosis is an intriguing process, involving many different factors. There are four major functional groups of molecules involved in triggering and affecting the apoptotic process. These are members of the tumor necrosis factor receptor (TNF-R) family, the cysteine-dependent aspartate-specific proteases (caspases), the adaptor proteins that control the activation of initiator caspases, and members of the B-cell lymphoma 2 (Bcl-2) family of proteins [6]. The apoptosis signaling pathways can be divided in the extrinsic and intrinsic pathways. The extrinsic pathway is receptor-mediated, whereas the intrinsic pathway is mitochondria-mediated. Both pathways converge on a common machinery of cell destruction that is activated by the caspase family (Fig. 1) [6, 7].

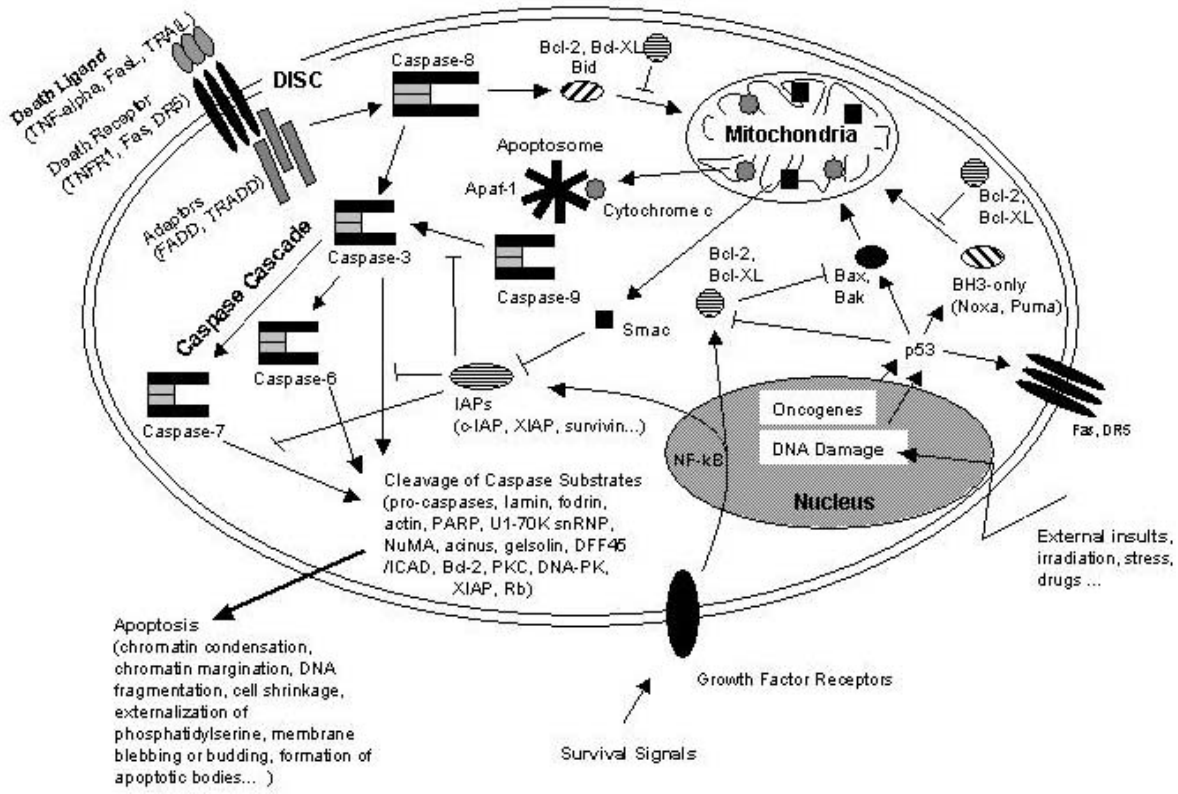

Figure 1. Schematic representation of some major apoptotic signaling pathways*. Apoptosis can be induced in response to various signals from inside and outside of the cell, e.g. by ligation of death receptors or by cellular stress triggered by oncogenes, irradiation, or drugs. Signals emanating from death receptors activate the death inducing signaling complex (DISC) which mediates activation of the initiator caspase-8. Caspase-8 activates the caspase cascade which eventually leads to apoptosis. Mitochondrial apoptotic signaling includes the release of cytochrome c from the mitochondrial intermembrane space to the cytosol where it contributes to the formation of the apoptosome, a complex of cytochrome c and the adaptor protein Apaf-1 which forms in the presence of dATP. The apoptosome activates caspase-9 which then activates the caspase cascade which leads to the characteristic morphological and biochemical features of apoptosis. Figure from Gewies 2003 [1].*

A group of proteins important for initiating the apoptotic process are members of the TNF-R family including TNFR-1, Fas/CD95, and TRAIL receptors. Depending on the cell type and type of signal, these receptors can trigger proliferation, survival, differentiation, or cell death [8]. These receptors are activated by a group of structurally related ligands that belong to the TNF-ligand family; upon binding the receptor-ligand complexes oligomerize and recruit intracellular adaptor proteins. The members of the TNF-R family are characterized by having a death domain (DD) in their intracellular region by which

they bind adaptor proteins such as FADD and TRADD [9]. Interactions between DDs in members of the TNF-R family and that of the adaptor proteins is required for the efficient recruitment of the caspases and allows caspase aggregation and activation. Caspase recruitment and aggregation are themselves mediated by death effector domains (DED) or by caspase recruitment domains (CARD) [10].

The caspase family includes proteins that are the central initiators and executors of apoptosis and at least 14 members have been identified in mammals [6, 11]. Caspases are synthesized as inactive zymogens, called procaspases, which carry a prodomain at their N-terminus followed by a large and a small subunit that are sometimes separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed resulting in a small and a large subunit. A heterotetramer consisting of two small and two large subunits then forms an active caspase. The apoptotic caspases can be divided into a group of initiator caspases including procaspases-2, -8, -9, and -10, and into a group of effector caspases including procaspases-3, -6, and -7 [12]. Recruitment and activation of the initiator caspases is achieved by binding to adaptor proteins, in contrast to the effector caspases that are unable to interact with the adaptor proteins and that are instead activated by upstream active caspases [6].

A final group of important proteins that control apoptosis is the Bcl-2 family. Members of this family are divided into two groups, a prosurvival group including Bcl-2, Bcl-xL, A1, and Mcl-1, and a proapoptotic group including Bax, Bak, Bid, Bad, Bim, Bik, Bmf, HRK, Puma, and Noxa [2, 13]. There is no agreement on how the Bcl-2 family controls apoptosis. There are two proposed models: one proposing that Bcl-2 members directly control caspase activation, whereas the other claiming that they mainly act by guarding mitochondrial integrity [2]. The first model speculates that Bcl-2 might control the activation of several initiator caspases that act upstream or independently of the mitochondria. These caspases, in turn, process proteins that activate Bax and Bak that produce damage to organelles resulting in amplification of the proteolytic cascade. The second model claims that in a viable cell, the proapoptotic Bcl-2 family members such as Bax and Bak are antagonized by antiapoptotic members such as Bcl-2. In response to an apoptotic stimulus, proapoptotic members are activated by transcriptional up-regulation (Bax, Noxa), subcellular relocalization (Bim, Bmf), dephosphorylation (Bad), or proteolysis (Bid). Activated proapoptotic members then inactivate antiapoptotic members

and subsequently Bax and Bak insert into the mitochondrial membrane where they contribute to the release of cytochrome c [2, 14, 15]. Cytochrome c release is specific for mitochondrial apoptotic signaling as will be described in the next section.

As mentioned above, the apoptotic signaling pathways are divided into extrinsic and intrinsic pathways. The extrinsic pathway involves the TNF receptor family which upon activation recruits adaptor proteins such as FADD or TRADD through their DDs. The adaptor proteins recruit procaspase-8 through the DEDs or CARDs and together, the receptors, the adaptor proteins and the procaspases form a death inducing signaling complex (DISC). The local concentration of several procaspase-8 molecules leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death (Fig. 1) [1, 6].

The second pathway that leads to apoptosis is the intrinsic pathway. This pathway is activated in response to cell death signals originating from inside the cell and is mediated by mitochondria that release cytochrome c from the intermembrane space. Apoptotic signaling from inside the cell can be initiated by anticancer drugs, DNA damaging agents, kinase inhibitors, hypoxia, growth factor withdrawal, and UV and ionizing radiation [16]. In the cytosol cytochrome c and the adaptor protein Apaf-1 form a so-called apoptosome in the presence of dATP [17]. The apoptosome activates caspase-9 which in turn activates the caspase cascade by activating caspase-3 (Fig. 1) [18]. The resulting activation of the caspase cascade can be inhibited by inhibitors of apoptosis proteins (IAPs) which can be up-regulated in response to survival signals [19, 20]. Crosstalk between the extrinsic and intrinsic pathway is mediated by Bid, a proapoptotic Bcl-2 family member. Caspase-8 mediated cleavage of Bid increases its activity and results in translocation to the mitochondria where it acts in concert with Bax and Bak to induce cytochrome c release, thereby activating the intrinsic pathway [19].

Apoptosis and cancer

For developing a tumor, tumor cells have to avoid apoptosis that is induced not only by unregulated oncogene expression, but also by limited supplies of growth factors, oxygen, or nutrients [21]. For tumors to become invasive and metastatic the cancer cells must, besides avoiding apoptosis, also generate their own growth signals, become insensitive to anti-growth signals, be able to replicate in a limitless manner and be able to induce and sustain angiogenesis [22]. Several genes that code for proteins involved in the regulation of apoptosis have been shown to be mutated or to exhibit altered expression in tumor cells. The tumor suppressor gene p53 is the most commonly mutated gene in human cancers. p53 is involved in regulation of gene transcription, DNA synthesis, DNA repair, senescence, apoptosis, and is a key regulator of the cell cycle, controlling the transition from the G1 phase to the S phase [23, 24]. Under conditions conducive to DNA damage, p53 can either induce apoptosis or arrest the cell cycle for DNA repair. Mutated p53 has a prolonged half-life, leading to accumulation of the abnormal protein not able to induce apoptosis [25]. Also important is Bcl-2 which often is over-expressed in cancer cells. Increased expression of this antiapoptotic protein is associated with decreased susceptibility of many tumor cells to undergo apoptosis in response to anticancer treatments [6]. Thus the down-regulation of apoptosis seems to be important for malignant transformation.

The prostate

The prostate is a gland of the male reproductive system and produces some of the fluid for the semen and may facilitate sperm motility [26]. Normally the prostate is quite small, nearly the same size of a chestnut. It is located in front of the rectum, just below the bladder and wraps around the urethra. The prostate is made up of at least three distinct cell types including secretory epithelial cells, basal cells and neuroendocrine cells that can be distinguished by their morphological characteristics, functional significance, and relevance for carcinogenesis. The epithelial cells are androgen-dependent for growth and secrete PSA. The basal cell layer is not dependent on androgens and is believed to contain stem cells. A stroma that includes fibroblasts, smooth muscle, nerves and lymphatic cells surrounds the gland [26].

Prostate cancer

Prostate cancer is the sixth most common cancer in the world and the third leading cause of death in men [27]. It has become a major public health problem, being prevalent in Scandinavian countries and with the highest incidence and mortality rates in African-Americans. In contrast, prostate cancer is relatively uncommon in Asian populations [28]. In the United States the age-adjusted incidence rate is 173.8 cases per 100,000 men from 1998 to 2002. The age-adjusted death rate in this time period was 30.3 per 100,000 men [29]. Norway has an incidence rate of prostate cancer with 77 cases per 100,000 men in average from 1997 to 2002. This is in contrast to 24.7 cases per 100,000 men in the time period 1953 to 1957 [30]. The increase that is observed in prostate cancer incidence might be due to early detection methods including increased screening of prostate-specific antigen (PSA) levels rather than true differences in underlying risk [31].

Epidemiologic studies have provided a great amount of information regarding risk of prostate cancer. Risk factors can be classified as endogenous or exogenous although some factors belong to both groups. Endogenous risk factors include family history, hormones, race, aging, and oxidative stress [31]. Results from multiple studies provide evidence for aggregation of prostate cancer in families. Segregation analyses support the existence of high-risk alleles for prostate cancer and twin studies have estimated that a substantial fraction of more than 40% of the disease has a genetic component [28]. Abundant biological data suggest the importance of androgens in the growth and maintenance of the prostate and the fact that prostate cancer regresses after androgen ablation or anti-androgen therapy makes androgens an important risk factor for prostate cancer [32]. Differences in prostate cancer risk by race may reflect differences in diet, differences in the detection of the disease and genetic differences. As previously pointed out, incidence rates are highest among African-American men [31]. In addition, the frequency of prostate cancer increases dramatically with age, almost 90% of the cases arise after 60 years of age [30].

The exogenous risk factors for prostate cancer include diet, environmental agents, and industrial and occupational exposures [27, 31]. Dietary influences such as red meat, high fat (elevated risk), and antioxidants (lowered risk) have been implicated with prostate cancer incidence and mortality, as has the lack of fruits and vegetables in one's diet [28, 33]. Environmental agents and industrial and occupational exposures are especially

exemplified by one class of environmental agents, endocrine disrupting chemicals (EDCs). These chemicals can be defined as agents that positively or negatively alter hormone activity and ultimately lead to effects on reproduction, development, and carcinogenesis, particularly of the reproductive organs [31]. In fact, the risk of prostate cancer among Asians increases when they immigrate from Asia to North America, implicating the environment and lifestyle-related factors in causing prostate cancer as pointed out above [34].

Most prostate tumors are adenocarcinomas, sharing numerous common features with other prevalent epithelial cancers, such as breast and colon cancer [35]. At some point during the disease, androgen-dependent epithelial prostate cells progress to an androgen-independent state in which the cells become more prone to metastasise and more resistant to apoptosis. The molecular events involved in the transformation of the normal prostate to a hormone insensitive cancer is a process that is poorly understood, despite the recognition of various events during prostate cancer tumorigenesis such as the deregulation of receptors, oncogenes, and tumor-suppressor genes [27]. By the time of prostate cancer diagnosis, prostate cells may have undergone somatic mutations, gene amplifications, gene deletions, chromosomal rearrangements, and changes in DNA methylation pattern [33]. Losses of genetic material are much more common than gains or amplifications, indicating that tumor-suppressor genes, which are believed to harbour the frequently deleted regions, probably play an important role in the tumorigenesis of the prostate [36]. Somatic targets of genomic damage include homeodomain-containing transcription factor 3.1 (NKX3.1), a candidate 'gatekeeper' gene [37, 38], phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor-suppressor gene [39, 40], and the androgen receptor (AR) [33, 41-43]. Many of the chromosomal losses can be detected already in the early stages of prostate cancer, whereas gains and amplifications are mostly seen in hormone-refractory tumors [36].

Prostate development and normal prostate function require androgens, testosterone and 5α-dihydrotestosterone (DHT), which through binding to AR induce its transcriptional activity [44]. The AR belongs to the steroid receptor subfamily which is part of the nuclear receptor superfamily that is believed to be derived from a common ancestor [45]. Like other members of this family it is characterized by an N-terminal domain for transcriptional regulation, a centrally located DNA-binding domain (DBD), and a ligand-

binding domain at the C-terminal end (LBD) [44]. The AR gene is located on the X-chromosome and therefore is single-copy in males, which allows for the phenotypic manifestation of mutations [44]. The effect of androgen on cell death occurs predominantly by interference with caspase activation and the inhibition of caspase cleavage in both the extrinsic and intrinsic cell death pathways [35, 46]. In the prostate, androgenic action is determined not only by androgen concentration but also by several other factors, such as the levels of the androgen receptor and its coactivators, the presence of growth factors, and perhaps factors yet to be identified [32].

Since the prostate is dependent on androgens for its growth and maintenance, one of the most common treatments for advanced prostate cancer is androgen-ablation therapy, involving the removal of androgens and estrogens by chemotherapeutic agents or castration, which effectively results in tumor regression caused by massive apoptosis of androgen-dependent cancer cells. Unfortunately, some prostate cancer cells are androgen-independent, survive in an androgen deprived environment and so the prostate cancer relapses into a highly aggressive and metastatic state that is androgen independent [36, 47]. Defining the signaling pathways that are induced by the androgen receptor is very important since there is evidence that in advanced androgen-independent prostate cancer the androgen receptor signaling pathways may be functioning and may have a role in the progression of the disease [48]. Tumors that have become androgen-independent show an increase in androgen-dependent genes such as PSA and kallikrein-2 [48]. Furthermore, over-expression and somatic mutations of the AR gene often appear in androgen-independent cancer [44, 49, 50]. These mutations can lead to increased sensitivity of the receptor to low levels of circulating androgens and increases the receptors ability to recognize a broadened spectrum of ligands as potent agonists of AR action [41, 43]. In addition, the presence of AR gene amplification in androgen-independent prostate cancer may reflect an adaptation of the cancer cells to the low levels of circulating androgens [42].

Hormonally regulated cell death and cell proliferation are balanced to maintain the normal size of the prostate and it has been shown that 85% of the cells in the rat prostate disappear within two weeks after castration due to increased levels of apoptosis [51]. In contrast, prostatic intraepithelial neoplasia (PIN), a precursor lesion of prostate cancer, and early invasive carcinomas are characterized by a seven- to ten-fold increase in the proliferation rate, whereas advanced and metastatic prostate cancers also display an

approximately 60% decrease in apoptosis [35]. Decreased apoptosis in prostate cancer cells is characterized by apoptosis suppressing genes that code for proteins such as IAPs and Bcl-2 [52]. IAP family proteins represent critical regulators of apoptosis that serve as inhibitors of the caspase family. Alteration in the expression of several IAP family proteins occur commonly and often simultaneously in prostate cancers and is suggested to occur early in the pathogenesis of this disease [53]. Antiapoptotic Bcl-2 has been found to be expressed in clinical samples of androgen-dependent and –independent prostate tumors. Over-expression of Bcl-2 prevents cells from initiating the process of apoptosis in response to a number of different stimuli and the expression of this protein has been associated with androgen-independent progression, treatment resistance, and is commonly associated with a poor prognosis in prostate cancer [47, 54].

To date no curative treatment is found for androgen-independent prostate cancer. In fact, many of the antiproliferative chemotherapeutic agents only lead to cancer cell death if the cells are proliferating. Cancer cells that are not proliferating at the time of treatment will not be affected. It is known that more than 90% of prostatic cancer cells in an individual patient are not proliferating and thus are resistant to normal cytotoxic therapy [20, 47]. Much work is done to improve the therapeutic arsenal in treatment of advanced prostate carcinoma and hopefully this will help to improve the duration and quality of life of prostate cancer patients.

Mitogen-activated protein kinases (MAPKs)

Nearly every cellular process is regulated at least to some degree by protein phosphorylation. In particular, signal transduction pathways employ protein phosphorylation and dephosphorylation as a mechanism to transmit information to different cellular compartments to elicit distinct physiological responses. Target proteins are phosphorylated at specific sites by protein kinases and dephosphorylated at the same sites by protein phosphatases. One of the best-studied groups of protein kinases is that of the mitogen-activated protein kinases (MAPKs). MAPKs are important mediators of signal transduction and play a key role in the regulation of many cellular processes [55, 56]. The MAPKs are serine/threonine kinases that are phosphorylated and activated on a Thr-X-Tyr motif by diverse stimuli such as cytokines, growth factors, and environmental stress. The MAPK cascades are composed of three protein kinases acting in series. A MAPK is

activated by a MAPK kinase (MAPKK) which in turn is activated by a MAPKK kinase (MAPKKK). In order to increase the variation of possible responses to various types of stimuli, a diverse range of MAPKKKs activate a smaller amount of MAPKKs which then in turn activate a few MAPKs. In addition, some MAPKKKs and MAPKKs are not specific for a particular substrate, contributing to a complex web of crosstalk reactions between the cascades of kinases [57].

The three major and best studied MAPK pathways result in the activation of extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 MAPKs (Fig. 2). These MAPKs are expressed in a wide variety of tissues and the cellular response of these pathways depends on the nature of the stimulus and the duration of each kind of signal [55]. The ERK pathway is mainly involved in growth, differentiation, and development while JNK and p38 MAPK pathways are involved mainly in inflammation, apoptosis, growth, and differentiation [57].

Since there is a staggering number of MAPK substrates and diverse biological processes that they regulate, it raises the question of how MAPK substrate specificity is determined and regulated. In addition to dephosphorylation by MAPK phosphatases, as will be discussed later, there are mainly two mechanisms involved, the docking interaction and the scaffolding. MAPKs utilize the common docking (CD) domain for interactions with MAPKKs, substrates, and phosphatases and another site, the glutamate-aspartate (ED) site, could determine the docking specificity towards the substrates [58]. In addition, MAPK interacting proteins have a so-called D domain and removal of this domain was shown to prevent recognition of the cognate MAPK [58]. The other mechanism involves scaffolding proteins that interact with several components of the MAPK cascades to tether both enzymes and substrates specifically to achieve accurate signal transduction. They do not contain any intrinsic enzymatic activity but possess a structure that enables them to recruit different factors of a specific pathway simultaneously [59]. Several scaffold proteins such as KSR and MP-1 are known to function as ERK scaffolds whereas for example the JNK-interacting protein (JIP) family includes members that are scaffolds for JNK and p38 MAPK [60]. The different MAPKs will be discussed in more detail in the next sections.

ERK

The ERK (p44 ERK1/p42 ERK2) pathway is an evolutionary conserved pathway that is involved in the control of many fundamental cellular processes that include cell proliferation, survival, differentiation, apoptosis, motility, and metabolism [55, 61]. ERK1 and ERK2 are activated by the dual specificity MAPK kinases MEK1 and MEK2, which are activated by Ras GTPases and Raf. Following activation, ERKs translocate to the nucleus and phosphorylate a variety of substrates such as the transcription factors c-Myc, Elk-1, and Ets-1 (Fig. 2) [57]. The ERK pathway is known to control growth and survival of a broad spectrum of human tumors. In fact, many tumors have activating mutations in Ras GTPases and Raf that result in activation of the ERK signaling cascade [62].

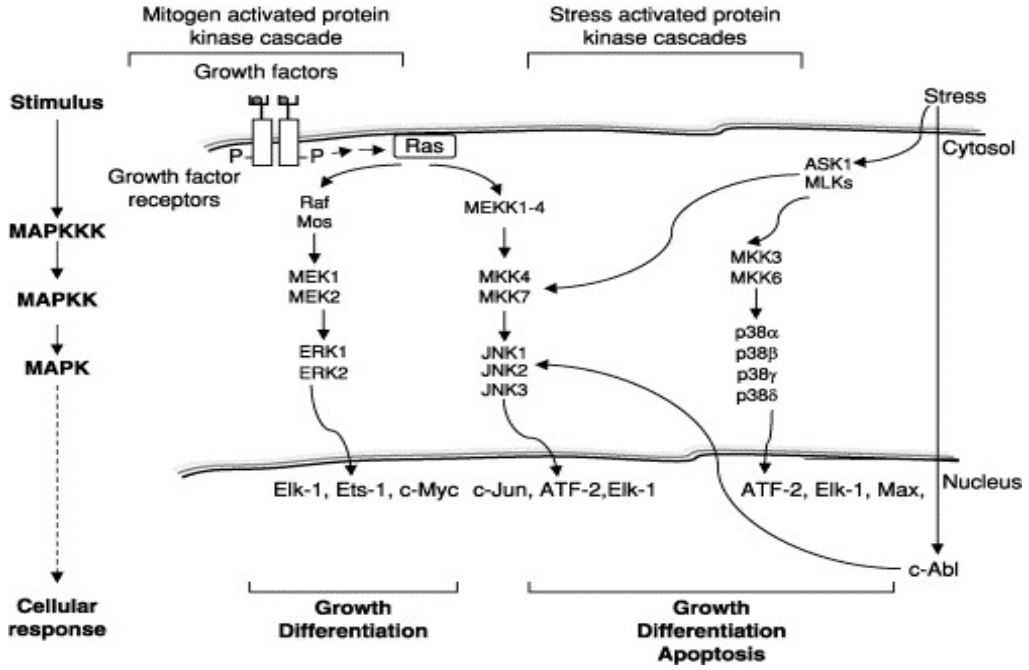

Figure 2. A simplified scheme of the MAPK signaling cascades*. Three major groups of MAP kinases, ERK, JNK, and p38 MAPK are activated by various extracellular stimuli, leading to phosphorylation and activation of different transcription factors. Figure from Viktorsson et al 2005 [6].*

p38 MAPK

Another MAPK is p38 MAPK of which four isoforms (α, β, γ, and δ) have been identified. p38 MAPK signaling pathways are activated in response to extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines, and growth factors and the

cellular responses depend on cell type as well as the stimulus [63]. Activation of p38 MAPK through either stress or cell surface receptors involves members of the Rho family of small GTPases, which can phosphorylate and activate the MAPKKKs MLKs, TAK1, and ASK1 [63]. These in turn activate MKK3 and MKK6 which directly activate p38 MAPK. The p38 MAPKs control the function of kinases, phosphatases, or transcription factors such as ATF-2, MEF2, and Elk-1 (Fig. 2) [57].

JNK

The final major MAPK pathway results in the activation of JNK that has been implicated in apoptosis, oncogenic transformation, inflammation, development, and differentiation, as mentioned above [55]. In mammals, there are three JNK genes, JNK1, JNK2, and JNK3, each located on a different chromosome [64]. The JNK1 and JNK2 genes are expressed ubiquitously, in contrast, the JNK3 gene has a more limited pattern of expression and is largely restricted to brain, heart, and testis. The three genes are alternatively spliced to create ten isoforms (Fig. 3) [65-67]. Similar to p38 MAPK, JNKs are activated by the Rho family of small GTPases, including Rho, Rac, and Cdc42. These proteins in turn activate MAPKKKs such as MEKK1-4 which then activate MKK4 or 7 that are immediate upstream MAPKK of JNK. MKK4 and 7 are also activated by ASK-1, TAK-1 and MLKs (Fig. 2) [6, 67].

As the name suggests, the JNKs have been characterized by their ability to associate with and phosphorylate regulatory sites in the N-terminus of the transcription factor c-Jun [68]. Phosphorylated c-Jun forms homodimers or heterodimers with active c-Fos, forming a so-called activator protein-1 (AP-1) that has the ability to recognize specific DNA sequences known as TPA-responsive elements (TRE), which are found in the regulatory regions of a variety of genes, including cell-cycle related and AP-1 genes themselves [69, 70]. AP-1 activity can be regulated by dimer composition, transcription, post-translational modification, and interactions with other proteins [71-74]. In addition to c-Jun, JNK can also activate additional transcription factors such as ATF-2, Elk-1, and p53 (Fig. 2) [57, 67, 75, 76]. Furthermore, JNK phosphorylates proteins that are not transcription factors such as the anti-apoptotic Bcl-2 and Bcl-xL [77, 78].

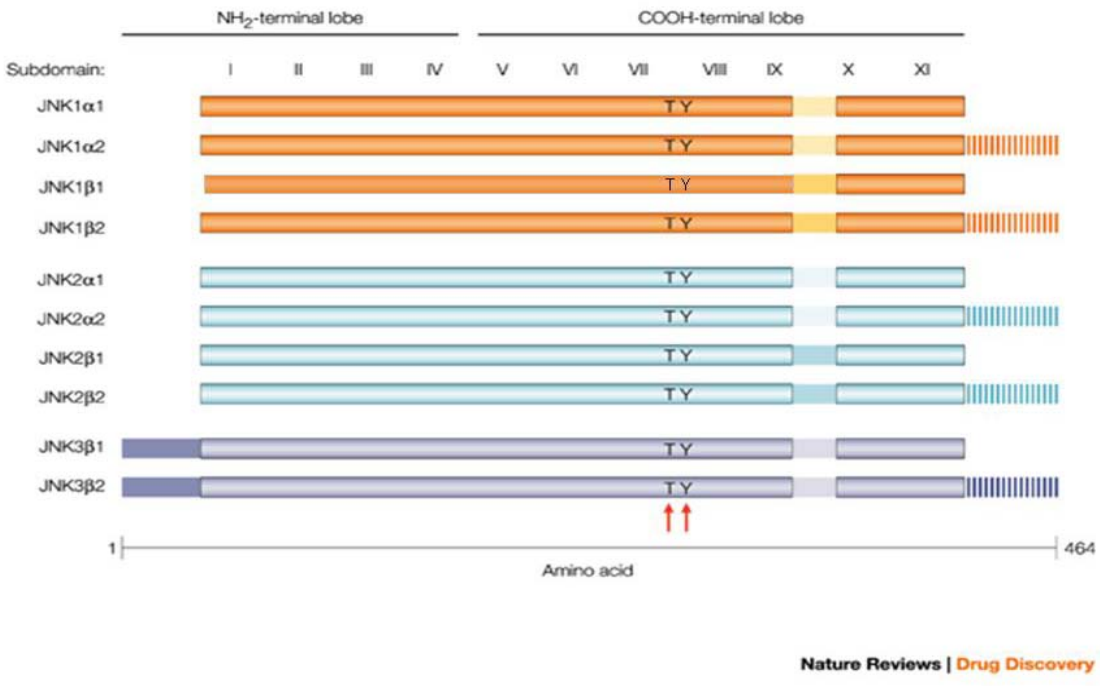

Figure 3. Structural features of the JNK proteins*. JNK protein kinases comprise 11 protein kinase subdomains (indicated as I-XI). The protein activation loop is located between domains VII and VIII and contain the threonine (T) and tyrosine (Y) residues that are phosphorylated for kinase activation. JNK1, JNK2, and JNK3 (in orange, light and dark blue, respectively) produce ten different isoforms by alternative splicing. The differences are indicated by the shaded regions. Two key alternative splicing sites are shown. The first between subdomain IX and X which results in altered substrate specificity, the second at the C-terminus which results in a difference in length. Reproduced with permission from Nature Reviews Drug Discovery Manning et al 2003 Macmillan Magazines Ltd [66].*

Studies on the roles of JNK1, JNK2, and JNK3 showed that mice with compound mutations in JNK1 plus JNK3 or JNK2 plus JNK3 are viable [79]. In contrast, compound mutations of the ubiquitously expressed JNK1 and JNK2 genes causes early embryonic death associated with decreased apoptosis in the hindbrain and increased apoptosis in the forebrain [79-81]. In addition, embryonic murine fibroblasts, disrupted for JNK1 and JNK2, were shown to be protected against UV-induced apoptosis [82]. Involvement of JNK3 in the death process was supported through studies with $JNK3^{-/-}$ mice that manifested severe defects in the apoptotic response in the hippocampus [83]. To summarize, gene disruption studies have demonstrated a role for JNK in the different cellular processes mentioned before.

It has been proposed that JNK activation triggers apoptosis in response to many types of stress, including UV and γ-irradiation, osmotic stress, toxins, heat shock, anti-cancer drugs,

inflammatory cytokines, and several other stimuli [57, 66]. Induction of apoptosis might be due to transcription-dependent signaling (leading to secretion of death-ligands) or by transcription-independent signaling (leading to cytochrome c release from mitochondria) [69]. A strong activator of JNK is UV irradiation [19]. UV-induced JNK activation leads to increased transcription of c-Jun and c-Fos mediated through phosphorylation of transcription factors c-Jun, ATF-2 and Elk-1 and results in a large increase in activator protein-1 (AP-1) activity [84]. The activation of JNK was shown to be independent of damage to nuclear DNA, instead it was dependent on activation of various cell surface receptors, possibly through damage to cell membranes [84].

Regarding prostate cancer, JNK is strongly activated and linked to cell death under stressful stimuli including cytotoxic drugs [85, 86]. Two inducers of JNK in the androgen-responsive prostate cancer cell line LNCaP are 12-O-tetradecanoyl-13-phorbol-acetate (TPA) and thapsigargin (TG). TPA has been shown to induce apoptosis through up-regulation of protein kinase C (PKC)-δ or -α and activation of JNK [87]. The downstream signals of PKC that mediate TPA-induced apoptosis in LNCaP cells are currently unclear. TG is a potent inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase pump which results in a rapid increase in intracellular calcium ion [88]. Both TPA and TG have been reported to induce apoptosis in LNCaP cells whereas TPA does not induce apoptosis in the androgen-independent prostate cancer cell lines PC3 and DU145. In LNCaP cells apoptosis induced by these two agents was correlated with activation of JNK and specific inhibition of JNK reduced both TPA- and TG-induced apoptosis [89, 90]. Recently it was shown that androgens decrease TPA- and TG-induced cell death of prostate cancer cells and this is mediated through the down-regulation of JNK activation [91]. JNK and the other MAPKs can be inactivated by mitogen-activated protein kinase phosphatases (MKPs) that specifically dephosphorylate active MAPKs at phosphorylated tyrosine and threonine residues. This group of phosphatases will be discussed in the next section.

MAPK phosphatases (MKPs)

Dephosphorylation of the MAPKs is vital for their control and is achieved by removal of phosphate groups from either the threonine residue or the tyrosine residue, or both. Phosphatases that are required for inactivation of the MAPK pathways are the serine/threonine phosphatases, the tyrosine phosphatases, and the dual specificity phosphatases (DUSPs) that dephosphorylate both serine/threonine and tyrosine. The various phosphatases are able to dephosphorylate components of the MAPK pathways at different stages of the pathways [92, 93]. The DUSPs have been recognized as key players for inactivating MAPKs and have been designated mitogen-activated protein kinase phosphatases (MKPs) [94]. MKP-1 was the first MAPK phosphatase discovered in 1991 [95]. To date there are fourteen known dual specificity phosphatases acting directly on the MAPKs (Table 1). As shown in Table 1, the MKPs have multiple names which make it difficult to distinguish them from each other. They are characterized by different subcellular localization (nuclear, cytosolic, or both), MAPK affinity, and structure and can be divided in groups according to these features. Furthermore, the great diversity among the MKPs and their MAPK affinity indicate an unexpected complexity for the negative regulation of MAPK signaling [96].

Two domains, a catalytic and a non-catalytic domain, are specific for the MKPs. The carboxyl terminal of each phosphatase contains a catalytic domain. This domain consists of a highly conserved signature motif -HCXXXXXR- that has significant sequence homology to the VH-1 gene, coding for a phosphatase in vaccinia virus that was discovered in 1991 [94]. The cysteine and arginine residues within this signature motif located in the active site loop and an additional highly conserved aspartate residue are essential for the dephosphorylation process [97]. The second domain is non-catalytic and is found in the amino-terminal. This domain contains two short regions of sequence homology with the catalytic domain of the cdc25 phosphatase which plays an important role in cell cycle regulation by removing inhibitory phosphates from tyrosine and threonine residues of cyclin-dependent kinases (CDKs) [97, 98].

Table 1. Overview over the different MKPs/DUSPs. *This table summarizes the different features of the MKPs/DUSPs grouped by means of their structure. The human names, the mouse orthologue names, subcellular localization, MAPK affinity, and accession number are shown. The newly discovered phosphatase MKP-8 is not included in this Table [99]. The Table is modified from Farooq et al [94].*

	Human	**Mouse orthologue**	**Subcellular localization**	**MAPK specificity**	**Accession No**
Type I	DUSP-3/VHR		Nuclear	ERK » JNK ~ p38	NM_004090
	DUSP-22/DSP2			P38 ~ JNK » ERK	NM_020185
	DUSP-14	MKP-6		ERK ~ JNK » p38	NM_007026
Type II	DUSP-1/hVH1	MKP-1	Nuclear	P38 ~ JNK » ERK	NM_004417
	DUSP-4/hVH2	MKP-2	Nuclear	ERK ~ JNK ~ p38	NM_001394 NM_057158
	DUSP-5/hVH3		Nuclear	-	NM_004419
	DUSP-6/PYST1	MKP-3	Cytosolic	ERK » JNK ~ p38	NM_001946 NM_022652
	DUSP-7/PYST2	MKP-X	Cytosolic	-	NM_001947
	DUSP-9	MKP-4	Nuclear/ cytosolic	ERK ~ JNK ~ p38	NM_001395
	DUSP-2/PAC1		Nuclear	ERK » p38 ~ JNK	NM_004418
Type III	DUSP-10	MKP-5	Nuclear/ cytosolic	P38 ~ JNK > ERK	NM_007207 NM_144728
Type IV	DUSP-16	MKP-7	Cytosolic	JNK ~ p38 » ERK	NM_030640
	DUSP-8/hVH5	M3/6	Nuclear/ cytosolic	JNK ~ p38 » ERK	NM_006167

Farooq *et al* group the MKPs by means of their structure and this divides them into four different groups (Fig. 4 and Table 1) [94].

- Type I phosphatases are approximately 200 amino acid residues in length and contain only a catalytic domain characterized by the phosphatase signature sequence –HCXXXXXR-. Members of this group are VHR [100-104], DUSP22 [105], and MKP-6 [106].

- Type II phosphatases are 300-400 amino acid residues in length and contain a catalytic and a MAPK binding domain. Members of this group are MKP-1 [107-110], DUSP2 [110, 111], MKP-2 [110], VH3 [112], MKP-3 [113-115], MKP-X [116], and MKP-4 [117].

- Type III phosphatases have the catalytic domain, the MAPK binding domain, and in addition an N-terminal domain of unknown function. The only known member of this group is MKP-5 [118, 119].

- Type IV phosphatases are 600-700 amino acids residues in length, contain the catalytic and MAPK binding domain and also a proline (P)-glutamate (E)-serine (S)-threonine (T) rich C-terminal. The function of the latter region, also called PEST, remains to be elucidated although, in analogy with other PEST-like sequence containing proteins, it has been suggested that it may be involved in rapid degradation of the type IV MKPs through ubiquitin-mediated proteolysis [120]. This may provide an important regulatory mechanism for phosphatases in this group. Members of this group are MKP-7 [121] and DUSP8 [122].

Despite the knowledge about the domains of MKP family members, little is known about their atomic structure. To date, the atomic structures of the catalytic domain of VHR [123], MKP-3 [114], and DUSP-2 [111] have been determined. The conformation and positioning of key residues in both the catalytic domain and the so-called general acid loop are essential for their enzymatic activity [94]. Crystal structure studies also demonstrate that the dynamic interaction between the MAPK binding domain (MKB) and the catalytic domain is directly coupled to a MAPK-induced conformational change of the phosphatase catalytic site, which is required for optimal enzymatic activity [111]. Many of the MKPs exhibit catalytic activity only on binding to their substrates which then induces the

conformational change. This holds for MKP-1, MKP-2, MKP-3, MKP-X, and MKP-4, but is not the case for VHR [94] and VH3 which already are in an optimal conformation for catalysis [112].

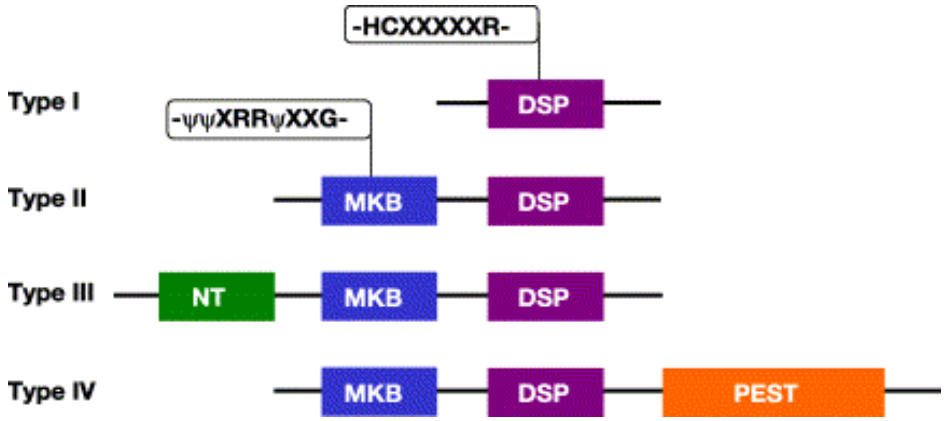

Figure 4. Subgrouping of the MKP-family according to their structure. *Members and description of the groups are pointed out in the text. DSP: catalytic domain, MKB: MAPK binding domain, NT: N-terminal domain, and PEST: proline (P)-glutamate (E)-serine (S)-threonine (T) rich C-terminal. Figure from Farooq et al 2004.*

Aim of the present study

Previously it was shown that both TPA and TG induce apoptosis in androgen-responsive LNCaP cells. In contrast, TPA did not induce apoptosis in the androgen-independent prostate cancer cell lines PC3 and DU145. Apoptosis was correlated with activation of JNK and specific inhibition of JNK reduced both TPA- and TG-induced apoptosis [89]. Further research showed that androgens decrease TPA-and TG-induced cell death of prostate cancer cells and that this correlates with the down-regulation of JNK activation [91]. In addition, UV-induced JNK activation was also reduced in androgen treated LNCaP cells. In this last case, the effect of androgens was dependent on RNA synthesis and occurred in a dose- and time-dependent manner [91]. The mechanism as to how androgens down-regulate JNK activation is unknown. The goal of this study was to investigate the possible role of MKPs in androgen-induced inhibition of JNK activation and their subsequent involvement in androgen-mediated protection of LNCaP cells from TPA- or TG-induced apoptosis.

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Manuscript

Regulation of MAPK phosphatase expression in the prostate cancer cell line LNCaP - Possible role in apoptosis

Summary

Androgen ablation during the initial stages of prostate cancer causes regression of the tumor due to an increase in apoptosis and reduced cellular proliferation. However, some prostate cancer cells survive in the androgen deprived environment and prostate cancer invariably progresses to an androgen-independent state for poorly understood reasons. Two different agents, 12-O-tetradecanoylphorbol-13-acetate (TPA) and thapsigargin (TG) activate c-Jun N-terminal kinase (JNK) and induce apoptosis in the androgen-responsive prostate cancer cell line LNCaP. Previous results from our laboratory show that androgen treatment of LNCaP cells protects them from TPA- and TG-induced apoptosis due to down-regulation of JNK activation. Ultraviolet light (UV)-induced JNK activity was also inhibited by androgens in LNCaP cells. Gene expression was required for this inhibition and ATP depletion experiments indicated an increase in phosphatase activity. This suggested a possible role for the MAPK phosphatases (MKPs) in inactivating JNK during inhibition of apoptosis in LNCaP cells; this is consistent with other data from our laboratory that showed an up-regulation of MKP-1 in cells treated with apoptosis inducing agent TPA and R1881, synthetic androgen. In this study, we have investigated the possible regulation of MKPs in UV, TPA, or TG treated cells in the presence or absence of R1881 and the data show that some MKPs are up-regulated in the presence of R1881 and TPA or TG. All tested MKPs were significantly down-regulated at the mRNA level in cells exposed to UV irradiation; R1881 did not appreciably affect MKP expression that is inhibited by UV. Analysis of TPA- and TG-induced apoptosis in LNCaP cells ectopically expressing VHR wild-type or a catalytically inactive mutant indicated that VHR interferes with apoptosis. In summary, these data indicate a role for the MKPs in the down-regulation of JNK activation in LNCaP cells and may be at least part of the mechanism as to how androgens inhibit JNK activation.

Introduction

Apoptosis is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. It is necessary for sculpting embryos, maintaining tissue homeostasis, terminating immune responses, and restricting the process of infections. This process involves many factors that are all tightly regulated and deregulation may cause severe damage leading to many diseases, including cancer, autoimmunity, and degenerative disorders [1]. Cells that are undergoing apoptosis typically display several morphological changes including cell shrinkage, loss of contact with neighbouring cells, chromatin condensation, blebbing of the plasma membrane, and fragmentation into apoptotic bodies [2, 3].

Signaling pathways that have been implicated in the regulation of apoptosis are the mitogen-activated protein kinase (MAPK) pathways. The MAPK cascades play an important role in transduction of environmental stimuli to the transcriptional machinery in the nucleus by virtue of their ability to phosphorylate and regulate the activity of various transcription factors [4-6]. The MAPK cascades are composed of three protein kinases acting in series activating one another by phosphorylation. A MAPK is activated by a MAPK kinase (MAPKK) which in turn is activated by a MAPKK kinase (MAPKKK) [4, 7]. In mammals, there are three main MAPKs, extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK. The ERK pathway is typically stimulated by growth-related signals and is mainly involved in growth, differentiation, and development. p38 MAPK and JNK are activated by growth factors, pro-inflammatory cytokines, and cellular stress and their activation has been implicated in apoptosis, as well as in oncogenic transformation, inflammation, development, and differentiation [8, 9]. JNK protein kinases are encoded by three genes, JNK1, JNK2, and JNK3 that are alternatively spliced to create ten isoforms [10]. The JNK1 and JNK2 genes are expressed ubiquitously whereas the JNK3 gene has a more limited pattern of expression and is largely restricted to brain, heart, and testis [11]. The cell type, magnitude, and duration of the stimulus determine the biological effect of JNK signaling and leads to phosphorylation and activation of several downstream effectors [12-15].

The MAPK pathways can be inactivated by tyrosine phosphatases (PTPs), serine/threonine phosphatases, and dual specificity phosphatases/MAPK phosphatases (DUSPs/MKPs). These phosphatase families are implicated as central regulators of mitogenic and other signaling pathways mediated by the MAPK pathways [16]. The MKPs, in contrast to the two other phosphatase families, specifically dephosphorylate the MAPKs at phosphorylated threonine/serine and tyrosine residues located in the activation loop of the MAPKs. To date there are fourteen known MKPs that are known to have different subcellular localization (nuclear, cytosolic, or both), MAPK affinity, and structure. In fact, this indicates great complexity for the negative regulation of MAPK signaling [17]. MKP-1/DUSP1 was the first MKP to be discovered in 1991 and was shown to have significant sequence homology to the VH-1 gene coding for a phosphatase in vaccinia virus [18]. One MKP of interest in this study is VHR/DUSP3 which was shown to effectively dephosphorylate both ERK and JNK [19, 20]. VHR (VH1-related) was first discovered in 1992 by Ishibashi *et al*. This protein had sequence homology to the VH1 gene in vaccinia virus and it was shown to be a phosphatase with dual specificity based upon its ability to hydrolyze phosphoserine from casein as well as phosphotyrosine from a number of tyrosine-phosphorylated growth factor receptors [21]. The cysteine (Cys^{124}) situated in the catalytic domain is essential for catalysis and mutation of Cys^{124} to a serine completely abrogates the enzymatic function of VHR, indicating the importance of this amino acid [22, 23].

The androgen receptor (AR) belongs to the nuclear receptor superfamily [24]. Like other members of this family it is characterized by an N-terminal domain for transcriptional regulation, a centrally located DNA-binding domain, and a ligand-binding domain at the C-terminal end [25]. AR binds androgens which play an important role in regulating growth, differentiation and cell death responses in the normal and cancerous prostate [26]. During the initial stages of prostate cancer an increased rate of apoptosis and a decreased rate of cell growth is observed after androgen depletion and results in regression of the tumor [27]. However, prostate tumors include androgen-independent prostate cancer cells which survive in an androgen depleted environment and so the disease progresses to an androgen-independent state. Many different adaptive survival factors are found in androgen-independent cells such as amplification of the AR gene, hypersensitivity of the AR to androgens, AR mutations that allow non-specific ligand binding, deregulated

expression of coactivators that may enhance AR-dependent gene transcription, and up-regulation of survival genes such as anti-apoptotic Bcl-2 [28-30]. To date the molecular mechanisms of transition from androgen-dependence to androgen-independence remain poorly understood and little is known about the link between androgens and the apoptosis signaling pathways in prostate cancer.

Previously, 12-O-tetradecanoylphorbol-13-acetate (TPA) and thapsigargin (TG) were shown to induce apoptosis through a mechanism that requires JNK activation in the androgen-responsive prostate cancer cell line LNCaP [31]. JNK activation in response to TPA and TG shows a biphasic activation profile with activation of JNK at 30 minutes of treatment which is reduced to basal levels by 6 hours, followed by a second sustained JNK activation that correlates with an increase in apoptosis. Highly significant levels of apoptosis were observed at 12 and 24 hours of TPA treatment, and at 36 and 48 hours of TG treatment. The effect of TPA on apoptosis was not observed in androgen-independent cell lines (PC-3, DU-145 and HeLa), indicating a different role of TPA on androgen-responsive and androgen-independent cell lines [31]. Therefore, additional studies to unravel the molecular mechanisms of JNK signaling and apoptosis in prostate cancer cells are needed. Previous results in our laboratory showed that androgens protect LNCaP cells from TPA- and TG-induced apoptosis and that this response is mediated by the down-regulation of JNK activation. In addition, androgens inhibited UV irradiation-induced JNK activation and this response is dose- and time- dependent, dependent on AR, and requires *de novo* gene transcription [32]. The cells treated with androgens had a faster rate of JNK dephosphorylation than vehicle treated cells and this suggested that phosphatases may, at least in part, mediate inhibition of JNK by androgens [32]. Furthermore, the phosphatase MKP-1 was up-regulated in TPA and R1881 treated LNCaP cells compared to cells treated with TPA alone. It was therefore of interest to further investigate the expression and regulation of the MKPs in LNCaP cells treated with TPA, TG, or UV irradiation in the presence or absence of R1881 and to assess if they could play an essential role in reducing JNK activation and therefore apoptosis.

Materials and Methods

Materials

pcDNA3-VHR, pcDNA3-VHR-C124S, and anti-VHR rabbit polyclonal antibody were generous gifts from John M Denu. RPMI 1640, penicillin/streptomycin and L-glutamine were purchased from BioWhittaker-Cambrex. R1881, 12-O-tetradecanoylphorbol 13-acetate (TPA), thapsigargin (TG), fetal calf serum (FCS), oligo dT, Triton X-100, HePes, NaCl, EDTA, $MgCl_2$, dithiothreiol (DTT), β-glycerophosphate, sodiumortovanadate, phenylmethylsulphonyl-fluoride (PMSF), leupeptin, dimethyl sulfoxide (DMSO), Tween 20, bovine serum albumin (BSA), ampicillin, anti-α-Tubulin mouse monoclonal antibody, horseradish peroxidase-conjugated anti-rabbit IgG antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody, and DAPI were obtained from Sigma-Aldrich. TRIzol® Reagent, Superscript II reverse transcriptase, Alexa fluor 594-linked goat anti-rabbit IgG, and Alexa fluor 488-linked goat anti-rabbit IgG were purchased from Invitrogen. Lightcycler® Faststart DNA Master SYBR green, *In Situ* cell death detection kit, and FuGene 6 were from Roche Diagnostics. ECL Western Blotting Analysis System was from Amersham Pharmacia Biotech. Bradford solution, Precision Plus Protein dual protein standard, and PVDF membrane were obtained from Bio-Rad. JETstar midi-prep kit was obtained from Genomed. Paraformaldehyde was purchased from Electron Microscopy Sciences. Anti-ph-JNK rabbit polyclonal antibody and anti-total JNK rabbit antibody were purchased from Cell Signaling.

Cell culture

The human prostate cancer cell line LNCaP was purchased from the American Type Culture collection (Rockville, MD). The passage number for LNCaP cells was always between 15 and 30. Cells were routinely kept in a humidified 5% CO_2 and 95% air incubator at 37ºC in RPMI 1640 containing 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. Medium was changed every second day. For experiments cells were plated in RPMI 1640 containing 10% FCS at a density of 1.6×10^5 cells per well in six-well plates or 9×10^5 cells per 10 cm dish. Cells were serum starved for two days in medium containing 2% charcoal treated (CT)-FCS to deplete the cells from androgens and to lower the basal level of JNK. The cells were starved further for 10 hours in medium provided with 0.5% CT-FCS. For the UV experiments the cells were treated with synthetic androgen, R1881 [10^{-7}M], or ethanol

vehicle and at the indicated time points the cells were subjected to UV irradiation (2 J/cm^2 for 5 seconds). After further incubation at 37ºC for 30 minutes the cells were collected by scraping the wells with phosphate-buffered saline (PBS). For the experiments involving 12-O-tetradecanoylphorbol 13-acetate (TPA) and thapsigargin (TG) treatment, cells were cultured as above. After starvation, cells were either left untreated or treated with R1881 [10^{-7}M] for 40 hours before adding TPA (5 ng/ml) or TG (100 nM). TPA stock solution was 5 µg/ml in ethanol and TG stock solution was 100 µM in DMSO. The cells were then collected after 6h, 12h, 24h, 36h and 48h as indicated. Ethanol or DMSO was used as vehicle.

Real-time RT-PCR

Total RNA was extracted from cell pellets with TRIzol® Reagent according to the manufacturer's instructions. RNA concentrations and purity were measured with a Lambda25 spectrophotometer (Perkin Elmer Life Sciences) at 260nm and 280nm. mRNA (2-5µg) was reverse transcribed using Superscript II reverse transcriptase and oligo dT. PCR amplification was performed on a lightcycler (Roche) using Lightcycler® Faststart DNA Master SYBR green as a DNA-specific binding dye and for continuous fluorescence monitoring. PCR conditions included an initial denaturation step at 95ºC for 10 minutes followed by 45 cycles of 95ºC denaturation for 10 seconds, annealing for 5 seconds (for specific annealing temperature for each primer pair see Table 1) and 72ºC extension for 20 seconds. Analysis was carried out in lightcycler capillaries with 10 µl reaction volumes containing 0.25 µl SYBR green, 2-4 mM $MgCl_2$, 0.5 µM of each primer and 2 µl of cDNA template. Template dilution, $MgCl_2$ concentrations and temperature conditions were optimized for each primer pair. Primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and each primer pair bound to an exon flanking a large intron to avoid amplification of genomic DNA. Melting curve analysis was done after each PCR reaction and the level of expression for each mRNA was determined using the crossing points given by the Lightcycler computer software. For each sample the ratio between the relative amounts of the target gene and housekeeping gene was calculated. Amplified DNA was run on a 1% agarose gel to verify the product size.

Table 1. The sequence of the primers used and the reaction conditions

Name	Sense	Antisense	Template dilution	$MgCl_2$ concentration	Annealing temperature
DUSP-3/ VHR	cgtctgtggctcaggacatc	cattgagctggcagagttgg	1:2	2 mM	66 ˚C
DUSP-5/ VH3	acaggccagcttatgaccag	gagaccatgctcctcctctg	1:2	2 mM	62 ˚C
DUSP-6/	gctgctgctcaagaagctca	aattgggggtgacgttcaag	1:2	2 mM	62 ˚C
DUSP-7/ MKP-	ctgtccagatcctgccctac	ccccatgaagttgaagttgg	1:2	3 mM	63 ˚C
DUSP-9/	cagatcctgcccaacctcta	actgccccatgaagttgaag	1:2	2 mM	63 ˚C
DUSP10 /MKP5	cttgcccttcctgttccttg	agcaactgccccatgaagtt		4mM	64 ˚C
DUSP14 /MKP6	cgccgatttgatttgtatcc	ttggggccagttgaaattag	1:10	3 mM	63 ˚C
DUSP16 /MKP7	gctctgctggaaagtggaac	ggctgagaaatgcaggtag g		2 mM	63 ˚C
DUSP22 / DSP2	agcggattcaccatctcaaa	cagcaccagcctaaactctg	1:10	2 mM	63 ˚C
TBP	gaatataatcccaagcggtttg	acttcatcacagctcccc	1:10	3 mM	62 ˚C
GAPDH	ggcctccaaggagtaagacc	aggggtctacatggcaact g	1:2	2,9 mM	60 ˚C

Western blot analysis

Whole cell extracts were obtained by resuspending the cell pellets in 100 µl lysis buffer containing 0.1% Triton X-100, 20 mM HePes (pH 7.7), 300 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 20 mM β-Glycerophosphate, 0.1 mM sodiumortovanadate, 0.5 mM PMSF, and 2 µg/ml leupeptin. After rotating for two hours at 4ºC, the suspension was centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected. All protein concentrations were determined utilising the Bio-Rad protein assay. Equal amounts of protein (100 or 200 µg) were separated on 12% SDS-page gels. A Precision Plus Protein dual protein standard was used as molecular weight marker. Proteins were transferred to a PVDF membrane activated in methanol. The blotted membrane was blocked in 5% nonfat

dry milk in Tris-buffered saline (TBS) containing 0.1% Tween for one hour followed by incubation with primary antibody in TBS-Tween containing 5% bovine serum albumin (BSA) for 14-16 hours at 4ºC. Antibodies used were anti-VHR rabbit polyclonal antibody (1:5000 dilution), anti-ph-JNK rabbit polyclonal antibody (1:500 dilution), anti-total JNK rabbit antibody (1:500 dilution) and anti-α-Tubulin mouse monoclonal antibody (1:5000 dilution). The membranes were incubated with secondary antibodies in 5% nonfat dry milk dissolved in TBS-Tween for 1 hour at room temperature. Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:10000 dilution) or horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000 dilution). ECL Western Blotting Analysis System was utilized for detection of the immunoreactive bands according to the manufacturer's instructions.

Bacterial expression and purification of plasmids

Electroporation was used to transform pcDNA3-VHR and pcDNA3-VHR-C124S into electro competent bacteria (DH10B). The electroporation procedure was carried out at 1.3 Volt (200 Ω) and bacteria were allowed to grow for one hour in LB before they were plated out on agar plates containing ampicillin (100 ug/ml). Colonies containing positive clones were grown overnight and the JETstar midi-prep kit was utilized to purify the plasmids. The plasmids were then digested with the appropriate restriction enzymes to verify their identity.

TUNEL assay

LNCaP cells were grown on cover slips in six-well plates containing 1.4×10^5 cells per well. They were starved as described above and transfected with pEGFP, pcDNA3-VHR or pcDNA3-VHR-C124S in medium containing 2% CT-FCS. FuGene 6 was used as transfection reagent according to the manufacturer's protocol. The FuGene:DNA ratio was optimized and kept at 3:1. The cells were treated with TPA (5ng/ml) or TG (100nM) for the indicated times and fixed for 20 min in 4% paraformaldehyde followed by 10 min in cold methanol at -20ºC. After 2 washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cover slips were blocked by incubation with 5% BSA in PBS-Triton X-100 0.1% for one hour at room temperature and after 3 washes with PBS the cells were incubated with primary antibody, anti-VHR rabbit antibody (dilution 1:1000), in 3% BSA in PBS or in 3% BSA in PBS without antibody as a negative control, overnight at 4ºC. Secondary antibodies Alexa fluor 594 or 488-linked goat anti-rabbit IgG were left on

the cells for one hour at room temperature. To detect apoptosis, an *In Situ* cell death detection kit (TUNEL technology) was used according to the manufacturer's instructions. Either *In Situ* cell death detection kit-Fluorescein or *In Situ* cell death detection kit-TMR Red was utilized to detect apoptosis in the cells. DNA strand breaks that typically occur with programmed cell death are by the aid of Terminal deoxynucleotidyl transferase (TdT) being labelled with fluorescein labelled nucleotides to the free 3'-OH DNA ends (TUNEL-reaction). To visualize cell nuclei, cells were counterstained with DAPI (1:1000 dilution). Fluorescence was observed using an Axioplan2 imaging microscope (Zeiss) and pictures were taken with an AxioCam HRc camera (Zeiss). At least three areas and a minimum of 300 cells per area were counted and the number of TUNEL-positive cells was expressed per 100 of the total number of cells.

Statistics

Statistical analysis was performed using the Student's t-test. Values of $p < 0.05$ were looked upon as significant.

Results

Expression and regulation of MKPs in TPA or TG treated LNCaP cells

Previously it was shown that androgens reduce TPA- or TG-induced apoptosis in LNCaP cells through down-regulation of JNK activation. Furthermore, androgens inhibited UV irradiation-induced JNK activation and this response occurred in a dose- and time-dependent manner, requires *de novo* gene transcription, and indicated an increase in phosphatase activity [32]. In addition, earlier work indicated that MKP-1 is up-regulated in TPA and R1881 treated LNCaP cells in contrast to cells treated with TPA alone [32-34]. For these reasons, the expression and regulation of members of the MKP family that specifically dephosphorylate MAPKs was investigated. To investigate this, the mRNA expression level for several MKPs was determined by real-time RT-PCR. LNCaP cells were treated with synthetic androgen R1881 for 40 hours to optimally block JNK activation before TPA or TG was added. Cells were harvested at the indicated times, total RNA was extracted from the cells, and cDNA was made and used for real-time PCR. Differential mRNA expression patterns were obtained as summarized in Table 2.

Table 2. Regulation of different MKPs at mRNA level in response to TPA+R1881 or TG+R1881. *LNCaP cells were treated with R1881 [10^{-7}M] and TPA or TG as described in Materials and Methods. The mRNA expression levels of the different MKPs (relative to TBP expression) were measured by real-time RT-PCR. The results presented illustrate the average mRNA expression level of three independent experiments in duplicate and are presented as +: up-regulation, -: down-regulation, NC: no significant regulation in TPA/TG and R1881 treated cells compared to TPA/TG treated cells.*

Name	TPA+R1881	TG+R1881
DUSP-3/ VHR	+	+
DUSP-5/ VH3	+	+
DUSP-6/ MKP3	-	NC
DUSP-7/ MKP-X	-	NC
DUSP-9 /MKP4	-	NC
DUSP-14/ MKP6	+	NC
DUSP-22/ DSP2	NC	-

As mentioned above, TPA and TG activate JNK in a biphasic manner, with an increase of JNK activity at half an hour of treatment, followed by a reduction to basal levels and a second activation at 6 hours of treatment. The mRNA expression of the MKPs was investigated in short and long time courses of TPA and TG treatment including half an hour and 6 hour treatments. The MKPs with the most interesting up-regulation in TPA and R1881 treated cells (although the level of up-regulation was different) are MKP-6, VHR, and VH3 (Fig 1). In addition, as noted above, MKP-1 was previously shown to be up-regulated in TPA and R1881 treated cells (data not shown).

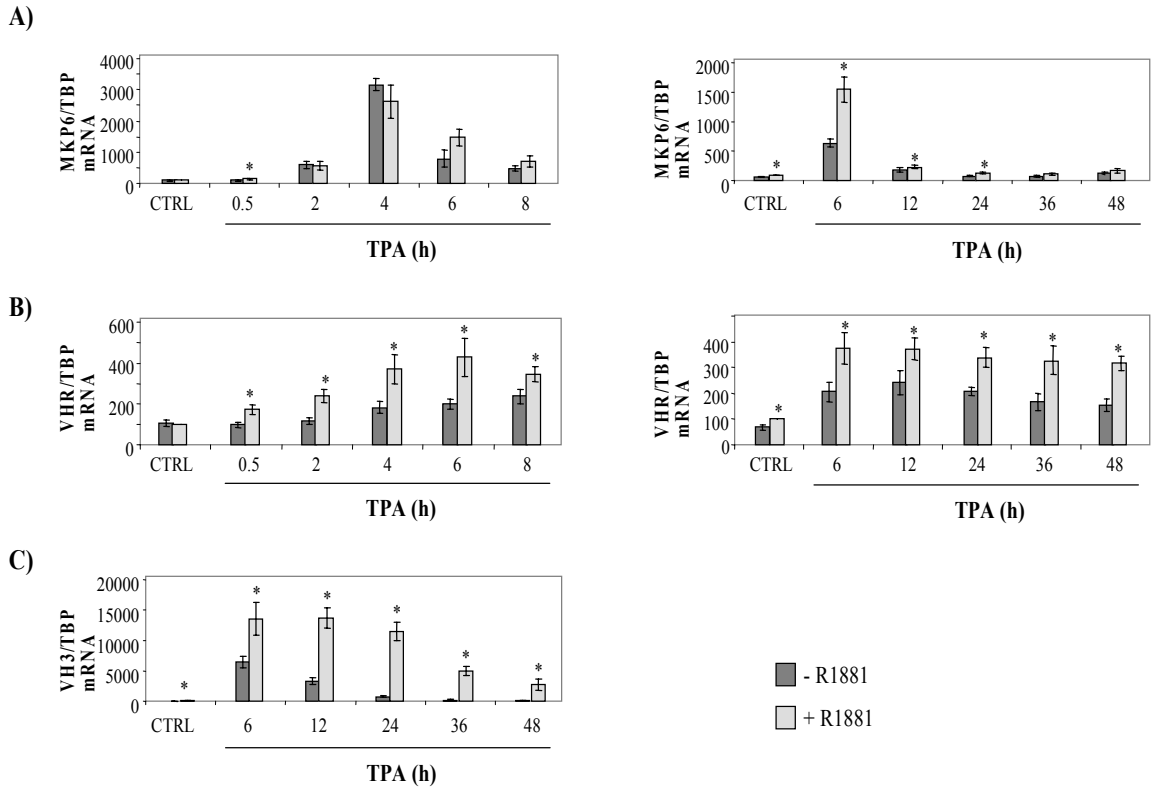


Figure 1. Up-regulation of MKP-6, VHR, and VH3 in LNCaP cells treated with R1881 and TPA. *LNCaP cells were grown and treated with TPA and R1881 for the indicated times as pointed out in Materials and Methods. CTRL cells were incubated with R1881 or vehicle for 8 and 48 hours in the short and long time courses, respectively. The expression level of MKP-6 (A), VHR (B), and VH3 (C) mRNA (relative to TBP mRNA expression) were measured by real-time RT-PCR. Values represent the mean ± SE of three independent experiments performed in duplicate. * $p<0.05$ indicate significant difference between TPA and R1881 treated cells versus TPA treated cells.*

As observed in Figure 1A, the expression level of MKP-6 mRNA has a significant 1.5 fold increase at half an hour of TPA and R1881 treatment in contrast to TPA treated LNCaP cells. In addition, a significant three fold increase at 6 hours of TPA and R1881 treatment compared to TPA treated cells was observed in both time courses (Fig. 1A). After 6 hours the expression level is lower but still a significant increase is observed in 12 and 24 hour treated cells with TPA and R1881. In addition, it is significantly up-regulated in R1881 treated control cells compared to vehicle treated control cells in the long time course of treatment (Fig. 1A). VHR is another MKP that was significantly up-regulated. It is up-regulated approximately two fold in all the time points of TPA and R1881 treatment compared to TPA treated cells in both the short and long time courses of treatment (Fig. 1B). As for MKP-6, VHR is up-regulated in R1881 treated control cells versus vehicle treated control cells in the long time courses of treatment (Fig. 1B). The final MKP that showed a significant up-regulation is VH3. The expression of this phosphatase was only assessed in a long time course of TPA treatment and is significantly up-regulated approximately three fold from 6 to 48 hours with TPA and R1881 treatment (Fig. 1C). In addition, it is up-regulated two fold in R1881 treated control cells compared to vehicle treated control cells.

Figure 2 shows the expression and regulation of VHR and VH3 mRNA in TG treated LNCaP cells. VHR is significantly up-regulated at 6 to 12 hours of TG and R1881 treatment compared to TG treated cells in short and long time courses of treatment and is significantly down-regulated at 48 hours of treatment. Furthermore, VHR is significantly up-regulated in R1881 treated control cells compared to vehicle treated control cells in the long time courses of treatment (Fig. 2A). The expression of VH3 was only assessed in long time courses of TG treatment. It is significantly up-regulated approximately two fold at 6 hours and three fold at 48 hours of TG and R1881 treatment. In addition, it is up-regulated three fold in the R1881 treated control cells in contrast to vehicle treated control cells (Fig. 2B). The results from these experiments indicate that certain MKPs are up-regulated in R1881 and TPA or TG treated LNCaP cells and they may have essential roles in the down-regulation of phosphorylated JNK.

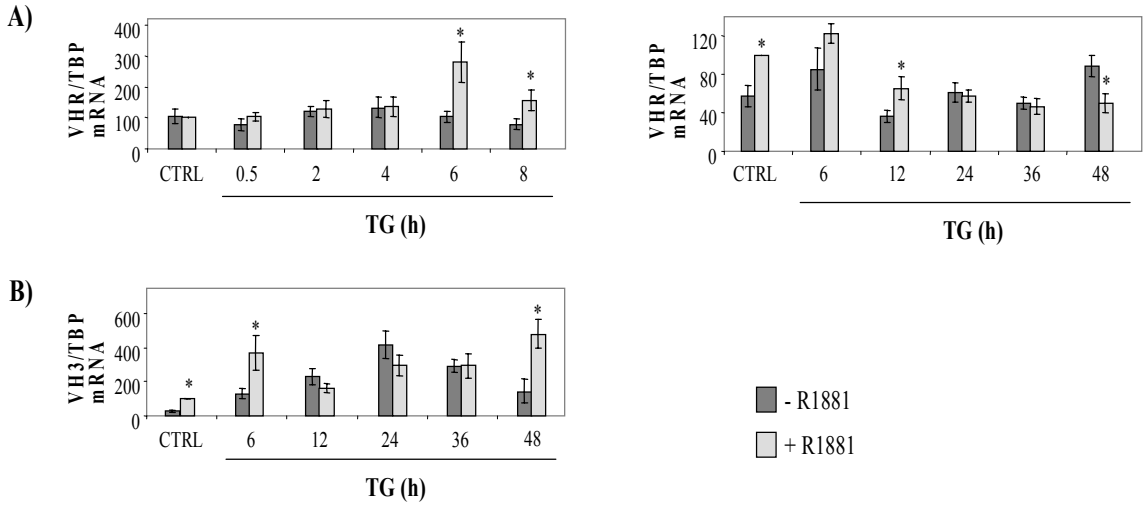


Figure 2. Up-regulation of VHR and VH3 in LNCaP cells treated with TG and R1881. *LNCaP cells were grown and treated with TG and R1881 for the indicated times as pointed out in Materials and Methods. CTRL cells were incubated with R1881 or vehicle for 8 and 48 hours in the short and long time courses, respectively. The expression level of VHR (A) and VH3 (B) mRNA (relative to TBP mRNA expression) were measured by real-time RT-PCR. Values represent the mean ± SE of three independent experiments performed in duplicate. * $p<0.05$ indicate significant difference between TG and R1881 treated cells versus TG treated cells.*

VHR is up-regulated at the protein level in TPA and TG treated LNCaP cells

VHR was chosen for further studies because of its significant up-regulation in both TPA and TG treated cells in the presence of R1881. In addition, this phosphatase has been shown to effectively dephosphorylate JNK [19]. To assess whether the regulation of VHR at the protein level is the same as the previously observed regulation at the mRNA level, whole cell extracts from TPA and TG treated cells with or without R1881 were examined by Western blot analysis using a VHR antibody. The result is shown in Figure 3. The increase of protein expression in cells treated with TG and R1881 compared to TG treated cells (Fig. 3A and B) gives a good indication of the expression of this phosphatase at protein level, although this result is not supporting the data for some of the time points of treatment obtained above for VHR mRNA (Fig. 2A). An up-regulation at protein level was also observed in LNCaP cells treated with TPA and R1881 in contrast to TPA treated cells only (Fig. 3C and D) and corresponds with the result of VHR expression at mRNA level

(Fig. 1B) where it is significantly up-regulated. However, VHR is highly up-regulated in R1881 treated control cells compared to vehicle treated control cells, indicating an effect of R1881 alone on VHR. Thus, the results indicate an increase in VHR protein expression in TPA- or TG-induced LNCaP cells treated with R1881 although the experiments need to be repeated to verify this result.

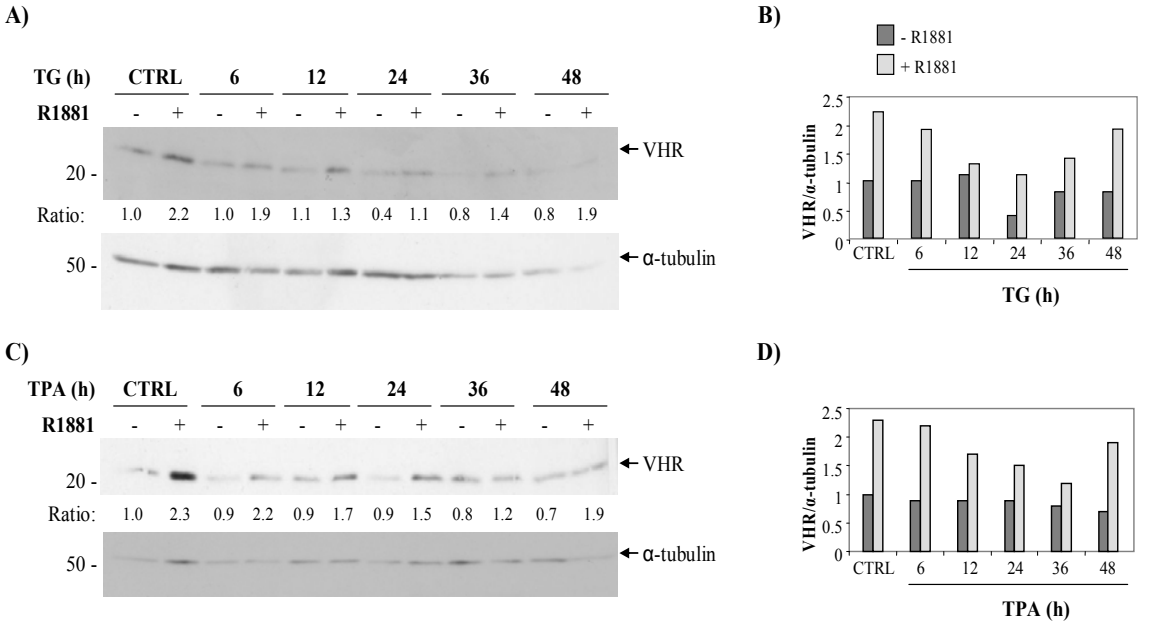


Figure 3. VHR protein expression is up-regulated after treatment with R1881 and TPA or TG. *LNCaP cells were grown, starved, and incubated with R1881 [10^{-7}M] for 40 hours. Thereafter TPA (5ng/ml) or TG (100 nM) was added and cells were harvested at the indicated times. CTRL cells were incubated with R1881 or vehicle for 48 hours. Whole cell extracts were obtained and 200 µg of protein was used for western blot analysis. Immunoreactive VHR protein levels after TG treatment (A) or TPA treatment (C) were quantified, normalized to the intensity of the immunoreactive α-tubulin bands and expressed relative to the vehicle-treated controls. The VHR/α-tubulin ratios are plotted in a bar-graph showing the result for TG in B) and for TPA in D). The results represent one experiment.*

Expression and regulation of the different MKPs in LNCaP cells irradiated with UV-light

UV irradiation is known to be another activator of JNK [35, 36]. Previously it was shown that androgens down-regulate UV-induced JNK activation and that this effect on JNK was dependent on the time of androgen exposure. Androgen treatment shorter than 12 hours gave no significant decrease in JNK activation, whereas treatments up to 48 hours nearly blocked all activation [32]. To assess the role of the MKPs in reducing JNK phosphorylation, LNCaP cells were treated with R1881 for the indicated times and exposed to UV irradiation. Real-time RT-PCR was utilized to investigate the level of mRNA expression of the MKPs. The data obtained for MKP-6 and VHR are shown in Figure 4A and B, respectively. The mRNA expression of the MKPs was surprisingly low compared to the controls and no significant up-regulation was observed in cells treated with R1881 compared to vehicle treated cells.

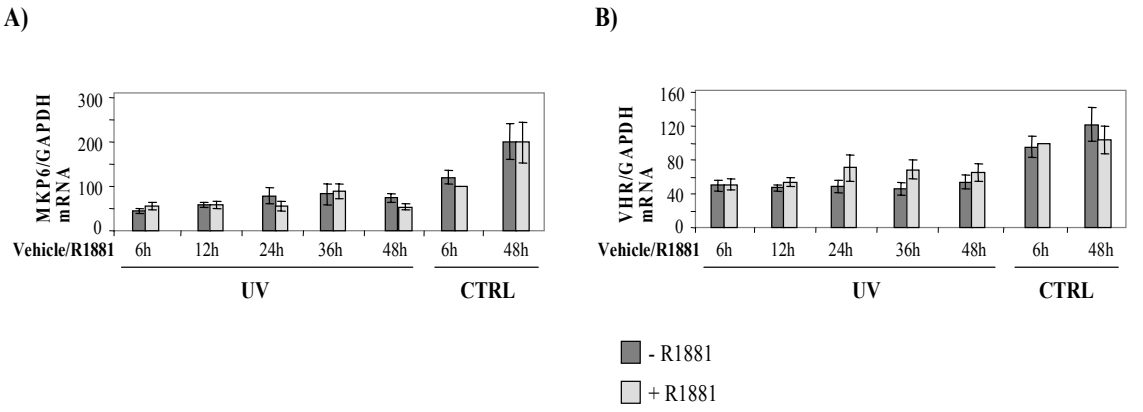


Figure 4. Reduced expression of MKP-6 and VHR in UV-irradiated LNCaP cells. *LNCaP cells were grown and treated as described in Materials and Methods. cDNA was made from isolated mRNA and the expression levels of MKP-6 and VHR (relative to GAPDH mRNA expression) were measured by real-time PCR. Real-time RT-PCR results for MKP-6 (A) and VHR (B) are shown. The values represent the average of three independent experiments in duplicate ± SE. * $p<0.05$ indicates significant difference from vehicle-treated cells.*

In addition, the mRNA expression of MKP-4, MKP-5, MKP-X, DUSP-22, and MKP-7 was investigated and approximately the same expression pattern was observed as shown for MKP-6 and VHR (data not shown). No real-time RT-PCR results were obtained for MKP-3 and VH3 possibly because of too low expression levels. In conclusion, these results indicate that the expression levels of the MKPs are low in cells irradiated with UV-light although some MKPs are slightly up-regulated in the presence of R1881. Further studies are needed to investigate the low expression levels of the MKPs in UV irradiated cells.

VHR protein expression is up-regulated after treatment with R1881 and UV irradiation

To investigate the regulation of VHR at the protein level in UV irradiated cells, whole cell extracts were obtained from LNCaP cells treated or untreated with R1881 and UV irradiated to activate JNK at the indicated times. Western blot analysis was done to assess protein expression of phosphorylated JNK, total JNK, and VHR (Fig. 5). The results verify the down-regulation of activated JNK in R1881 treated and UV irradiated cells as previously shown [32]. VHR is highly expressed in non-irradiated, R1881 treated control cells compared to vehicle treated cells. This does not correspond with the result obtained for VHR mRNA expression (Fig. 4B). In addition, the results show that VHR is slightly down-regulated at 12 hours of treatment but is up-regulated approximately 2- and 3-fold at 24 and 48 hours, respectively. In conclusion, these results show that when JNK activation is reduced by R1881 treatment in UV irradiated cells to activate JNK, VHR protein expression is up-regulated. Furthermore, this experiment will have to be repeated to verify the results.

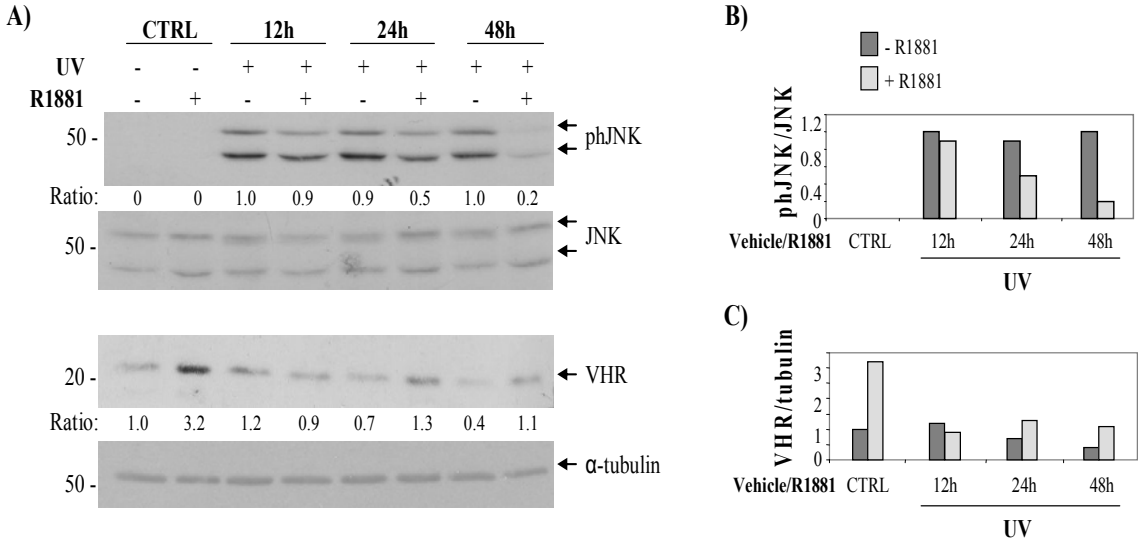


Figure 5. VHR protein expression is up-regulated after treatment with R1881 and UV irradiation*. LNCaP cells grown and treated as previously described. UV irradiation was performed at the indicated times. CTRL cells were incubated with R1881 or vehicle for 48 hours. A) Whole cell extracts were prepared and 100 μg of protein was used for western blot analysis. Immunoreactive phospho-JNK protein levels after R1881 treatment and UV irradiation were quantified and normalized to the intensity of the immunoreactive total-JNK bands. VHR protein levels were quantified and normalized to the intensity of the immunoreactive α-tubulin band. Ratios for phJNK/totJNK (B) and VHR/ α-tubulin (C) are presented in bar graphs. The results are expressed relative to the vehicle-treated controls and represent one experiment.*

Ectopic expression of VHR protects LNCaP cells from TPA- and TG-induced apoptosis

As described above, VHR was found to be up-regulated both at the mRNA and protein level in LNCaP cells treated with R1881 and TPA or TG. Up-regulation of this phosphatase in the presence of R1881 could be involved in the down-regulation of JNK activation in LNCaP cells undergoing apoptosis. In order to elucidate the role of up-regulated VHR, TUNEL assay was performed on cells transiently transfected with pEGFP, VHR wild-type, or mutant VHR-C124S. The cells were grown in the presence or absence of R1881 and apoptosis was induced with TPA or TG. At the indicated times the cells were fixed, incubated with VHR antibody, and TUNEL assay was performed. Apoptosis was investigated at two different times of treatment, 12 and 24 hours for TPA, and 36 and 48 hours for TG, respectively, according to substantial levels of cell death shown in earlier

experiments carried out in the lab [31]. Figure 6 shows the results for one of these treatments (TG 48h). Non-treated cells, either transfected with pEGFP, VHR-wt, or VHR-C124S, are undergoing less apoptosis than TG treated cells for 48 hours as the TUNEL staining indicates in the right panel. In addition, cells ectopically expressing VHR-wt are undergoing less apoptosis than cells expressing VHR-C124S as shown in the centre panel.

Quantification of the incidence of apoptosis in these treatments is shown in Figure 7. Compared to control cells, apoptosis was significantly reduced in cells ectopically expressing VHR wild-type. However, cells transfected with the dominant-negative mutant of VHR, pcDNA3-VHR-C124S, were undergoing apoptosis but not to the same extend as for non-transfected cells. In order to investigate whether the transfection procedure itself had influence on the process of apoptosis, cells were transfected with pEGFP. No significant difference in apoptosis in non-transfected and transfected cells was detected, thus confirming that the transfection procedure had no or little influence on cell death. The results are similar for cells treated either with TPA (Fig. 7A) or TG (Fig. 7B), showing a reduction in apoptosis in VHR transfected cells in contrast to VHR-C124S transfected cells. From these results it can be suggested that ectopically expressed VHR is able to reduce apoptosis in TPA or TG treated LNCaP cells to a greater extent than its catalytically inactive mutant.

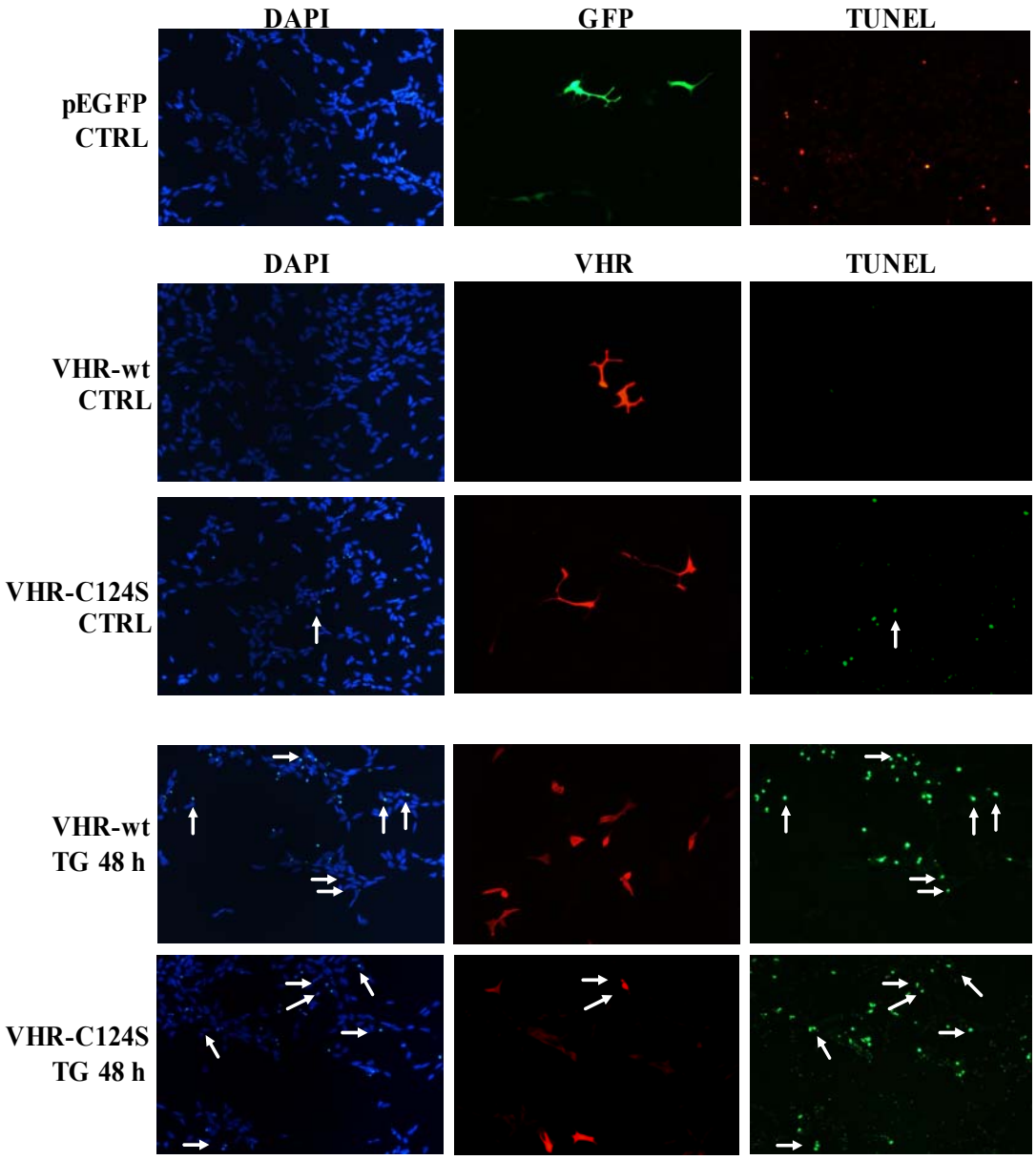
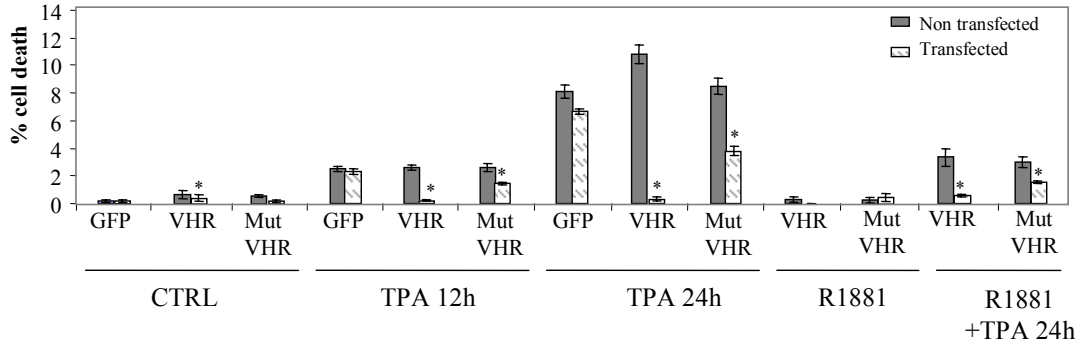


Figure 6. Ectopic expression of VHR protects LNCaP cells from TG-induced apoptosis. *LNCaP cells were cultured on cover slips and were transiently transfected with pEGFP, VHR-wt, or VHR-C124S. The cells were further grown in the presence or absence of R1881 and TG as described experimentally. After fixation, VHR transfected cells were incubated with VHR-antibody, TUNEL assay was performed, and cells were counterstained with DAPI. TUNEL positive cells appear as green or red fluorescent dots. Arrows indicate apoptotic cells. DAPI staining,VHR immunofluorescence, and TUNEL staining is shown for pEGFP-, VHR-wt-, and VHR-C124S-transfected cells that were non-treated (CTRL), or treated with TG for 48h.*

A)



B)

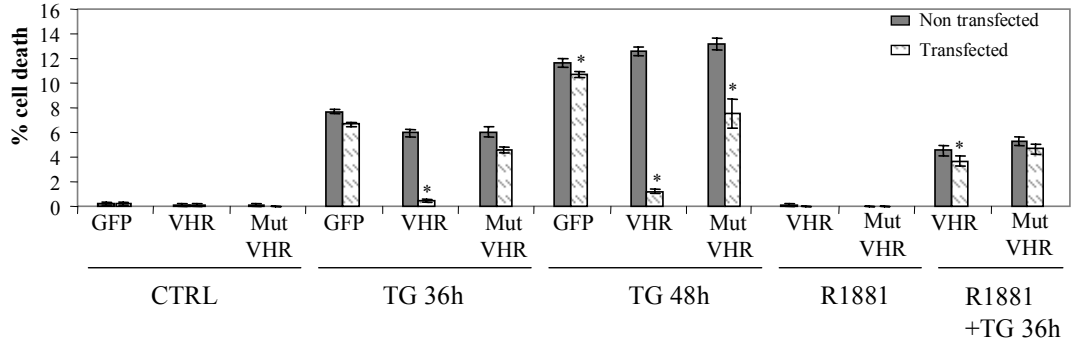


Figure 7. Ectopic expression of VHR reduces apoptosis in LNCaP cells treated with TPA or TG*. Cells were grown as above. For microscopy, at least three areas and a minimum of 300 cells per area were counted. The data show the percentage of nonviable cells after A) 12 and 24 hours of TPA treatment or B) 36 and 48 hours of TG treatment. For 12 hour treatment with TPA and 48 hour treatment with TG an average of one experiment in duplicate ± SE is shown. For 24 hour treatment with TPA and 36 hour treatment with TG an average of 2 experiments in duplicate is shown. * $p<0.05$ indicates significant difference from non-transfected cells.*

Discussion

Intracellular signal transduction pathways are key regulators of a number of cellular functions. The MAPK signaling pathways have been implicated in such diverse cellular processes as differentiation and apoptosis. To regulate the cellular responses, the timing of activation and inactivation of these kinases must be strictly regulated. This can be achieved either by regulation of activation, i.e. phosphorylation, or regulation of inactivation, i.e. dephosphorylation.

The data presented here show that members of the MKP family may have an important role in the inhibition of the JNK pathway in TPA or TG treated LNCaP cells. We have found that three different phosphatases, MKP-6, VHR, and VH3, are significantly up-regulated at the mRNA level in cells treated with R1881 and TPA compared to cells treated with TPA alone. In addition, VHR and VH3 were shown to be up-regulated in R1881 and TG treated cells compared to cells treated with TG alone. Consistent with up-regulated VHR mRNA, VHR was also up-regulated at the protein level. However, R1881 alone could influence MKP expression since VHR mRNA and protein was up-regulated in R1881 treated control cells. Cells, including control cells, in TPA and TG experiments are pre-treated with R1881 for 40 hours whereas cells in the UV experiments are not. Control cells treated with R1881 for 40+48 hours showed an up-regulation of MKP-6, VHR, and VH3 compared to vehicle treated control cells. In contrast, control cells treated with R1881 for 40+8 hours did not give an increase in MKP-6 and VHR mRNA. The R1881 treated control cells (6 and 48 hours) in the UV experiments show no up-regulation of MKP-6 and VHR compared to vehicle treated control cells. This suggests that R1881 can influence mRNA expression of these MKPs in longer treatments (~88 hours) and indicates that a role of R1881 alone on the expression of the MKPs needs to be investigated.

MKP-6, VHR, and VH3 have different affinities for the MAPKs. MKP-6 was shown to effectively dephosphorylate ERK and JNK, and to a lesser extent p38 MAPK [37]. VHR has been shown to dephosphorylate all three MAPKs but it has the greatest specificity for ERK [17, 19, 20]. In contrast to VHR, little is known about the specificity of VH3 towards the MAPKs but recently one study showed that it effectively binds ERK, and to a lesser degree JNK and p38 MAPK [38].

Several studies show that MKP-1 mRNA is over-expressed in high-grade PIN, compared to normal prostate and that the proportion of apoptosis is significantly lower in PIN lesions expressing MKP-1. Furthermore, JNK1 enzymatic activity was shown to be inversely related to MKP-1 levels [33, 34, 39]. The fact that many MKPs are regulated in R1881 and TPA or TG treated LNCaP cells, presented here, indicate that these MKPs may inhibit JNK activation and thereby block apoptosis. Additional studies are required to assess this possibility.

The TUNEL assay in this study suggests that ectopically expressed VHR reduces TPA- and TG-induced apoptosis compared to non-transfected cells, possibly through inactivating JNK. In contrast, mutant VHR-C124S transfected cells were undergoing more apoptosis compared to VHR transfected cells but here as well there was less apoptosis than in non-transfected cells. Although cells ectopically expressing the catalytically inactive mutant of VHR show a decrease in apoptosis compared to non-transfected cells, VHR wild-type transfected cells still have a greater reduction in apoptosis than the mutant VHR transfected cells. VHR has previously been demonstrated to down-regulate the JNK signaling pathway at the level of JNK dephosphorylation but nothing is known about the physiological effects of inactivation of JNK by VHR [19]. Recently MKP-1 was demonstrated to promote cell survival by attenuating stress-responsive MAPK-mediated apoptosis and suggested participation of other MKPs in this process [40]. Although the first MKP was discovered in 1991, not much is known about the mechanism and structural basis for recognition of phosphorylated MAPKs by MKPs but it is clearly an important level of MAPK signaling pathway regulation. Further experiments are needed to verify if the reduction of apoptosis in VHR transfected LNCaP cells is due to a decrease of activated JNK; nevertheless, these data indicate that ectopic expression of VHR reduces apoptosis.

One of the findings of this study was that in response to UV irradiation all MKPs tested were down-regulated at the mRNA level. It is known that UV irradiation works by activating upstream kinase pathways which finally phosphorylate the MAPKs [36]. The down-regulation of MKPs by UV indicates that inhibition of phosphatases may be equally important in achieving MAPK activation. It is conceivable that MKPs are down-regulated in a coordinate manner, e.g. by the deployment of common transcriptional repressors or

inhibition of common transcriptional activators. Further work is needed to elucidate these possibilities.

Since the expression of the MKPs is lower in UV irradiated cells than in TPA and TG treated cells, this might suggest that the MKPs possibly do not have a role in reducing UV-induced JNK activation in R1881 treated cells. Recently it was shown that MKP-1 is down-regulated in UV irradiated (1 J/m^2) transcription-coupled repair deficient fibroblasts and this correlated with an increase in JNK activity [41]. This suggests that UV irradiation can repress MKP-1 and possibly several other MKPs but further research will be needed to assess the role of the MKPs in UV-irradiated LNCaP cells.

Since the effect of androgen treatment on UV-induced JNK activity was shown to be dependent of new gene expression, it will be important to investigate the promoters of the different MKPs to assess if they have androgen response elements (AREs) to allow binding of ligand-bound AR. MKP-1 does not have an ARE in its promoter and therefore increased transcription of this gene is unlikely to be due to binding of ligand-bound AR [42]. In our laboratory MKP-1 was previously shown to be up-regulated in cells treated with TPA or TG in the presence of R1881 and thus should be caused by other mechanisms. Another member of the nuclear receptor superfamily, glucocorticoid receptor, has been indicated to inhibit MAPK cascades to regulate inflammatory processes and MKP-1 was shown to have an important role in the down-regulation of glucocorticoid activated MAPKs in mast cells [43]. MKP-1 has a glucocorticoid response element in its promoter and its expression is induced by binding of ligand-bound glucocorticoid receptor to this element [43].

Knock down of MKP-6, VHR, and VH3 with siRNA should make it possible to observe if the effect of androgens on JNK is blocked. It is, however, possible that other MKPs get a different regulation in order to bypass the inhibited function of the MKP that is knocked down. Since the MKPs have overlapping substrate specificity displayed by members of the various subgroups, it seems likely that there will be a certain degree of redundancy [18].

There are many other phosphatases than included in this study which may be involved in modulating JNK activity. For example, in the DUSP family, there is a poorly characterized atypical group of DUSPs that lack specific MAPK targeting motifs. The so-called DUSP13, DUSP19-21, and the recently discovered DUSP15/VHY belong to this group. The physiological functions of most of these phosphatases are unknown and most of

them have not yet been characterized experimentally [44]. Studies on DUSP19 (also called SKRP1) in Cos-7 cells co-expressing JNK2 and SKRP1 have shown that it interacts with its physiological substrate JNK2 through binding MKK7, a MAPKK upstream of JNK, and inhibits activation of JNK2 induced with TG but not UV irradiation [45].

Also the action of the threonine/serine phosphatases might explain the down-regulation of JNK activation in LNCaP cells treated with R1881 and TPA or TG. They consist of four major groups (PP1, PP2A, PP2B and PP2C) in eukaryotes and several distinct isoforms exist for these phosphatases [46]. PP2Cα (a PP2C gene product) and PP2A expression was shown to strongly inhibit the activation of JNK. PP2Cα was able to inhibit activation of the JNK pathway at the MAPKK level and PP2A was shown to directly inhibit JNK activity at the MAPK level [47, 48]. It will therefore be of great interest to investigate the regulation of at least these two serine/threonine phosphatases in LNCaP cells with TPA and TG induced JNK activation. To date, the third group of phosphatases, the PTPs, have not been shown to inactivate JNK.

Many different signaling pathways are involved in death responses, indicating that no simple explanation can be found for the down-regulation of JNK activation, and the crosstalk between androgen signaling and the MAPK pathway. Recently, activation of androgen receptor by androgen was shown to induce AP-1 activity and to change AP-1 factor DNA-binding, and may contribute to androgen-induced changes in prostate cancer cell growth [49]. Furthermore, JNK activation of c-Jun was shown to result in the inhibition of MKP expression and is a way for c-Jun to control the efficiency of MAPK signaling [50]. To conclude, our study shows that several MKPs might have a role in reducing TPA- and TG-induced JNK activation in androgen treated LNCaP cells. Furthermore, ectopically expressed VHR was shown to abolish TPA- and TG-induced apoptosis, most likely due to its ability to dephosphorylate and inactivate JNK. The findings in this study support the hypothesis that MKP inactivation may be a valid strategy to sensitize prostate cancer cell death in combination with conventional cancer chemotherapeutic strategies. However, further investigations are required to elucidate the specific functions of the MKPs in androgen-mediated down-regulation of JNK activation and inhibition of apoptosis in prostate cancer.

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