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Science is what you know.
Philosophy is what you don't know.

Bertrand Russell

ABSTRACT

Cancer is a widespread disease. In spite of all research resulting in new improved therapies, there are still many tumors that cannot be cured. To oppose that, also a preventive research approach is important with goals such as decreasing the human exposure to carcinogens. An early step in that process could be to find biomarkers of exposure and to develop mechanistically informative test systems. The aim of this study was to characterize Mdm2 phosphorylations in order to investigate their potential applications in preventive research.

p53 is a tumor suppressor protein that mediates cell cycle stop and apoptosis in response to cellular stress such as DNA damage. There is a strong coupling between p53 and cancer since it is mutated or inactivated in the majority of tumors. Mdm2 is a key regulator of p53 that induces proteasomal degradation of p53, through ubiquitination. p53 induces transcription of *Mdm2*, and together p53 and Mdm2 form an autoregulatory feedback loop. The activity of Mdm2 is regulated by phosphorylations. It has been shown that phosphorylation of Mdm2 at Ser166 promotes degradation of p53 and that Akt mediates this phosphorylation. Thus TCDD, which has a tumor promoting effect, induces phosphorylation of Mdm2 at Ser166 and thereby attenuates p53 response to DNA damage. Mdm2 is also phosphorylated by ATM at Ser395, a residue within the 2A10 epitope, upon DNA damage. This phosphorylation is important for DNA damage induced p53 stabilization. Also other proteins such as DNA-PK, c-Abl and ATR are involved in Mdm2 phosphorylation and thereby also have indirect effects on p53.

We demonstrate that Mdm2 phosphorylation at Ser166 is mediated via MEK-ERK, and not by Akt, in hepatocytes. We also show that inhibition of Akt activation induces phosphorylation of Mdm2 at Ser166 via MEK-ERK in hepatocytes. This results in downstream effects such as attenuated p53 and p21 responses to DNA damage. Our data suggest Akt as a negative regulator of Mdm2 in these cells and propose a hepatocyte specific regulation of Mdm2 phosphorylation at Ser166.

We also find that low concentrations of genotoxic compounds such as mitomycin C, etoposide, 5-fluorouracil and benzo[*a*]pyrene induce phosphorylation of Mdm2 at the 2A10 epitope. No p53 accumulation was observed at these low doses. Low concentrations of BP also induce Mdm2 2A10 phosphorylation in human lymphoblasts. These data indicate that Mdm2 could be used as a sensitive marker for certain types of genotoxicity and a potential use of Mdm2 as a marker for human exposure of genotoxic agents. The possibility to use Mdm2 as a biological marker was further strengthened as we identified two different patterns of proteins associated to chromatin induced by polycyclic aromatic hydrocarbons, where a loss of Mdm2 binding to chromatin seemed to indicate severe and non-repairable DNA damage.

In conclusion, this study show a liver specific regulation of Mdm2 phosphorylation at Ser166 and that Mdm2 phosphorylation at the 2A10 epitope could be used as a tool in detecting and characterizing certain types of genotoxicity.

LIST OF PUBLICATIONS

I. Malmlöf, M., Roudier, E., Högberg, J. and Stenius, U. (2007) MEK-ERK-mediated phosphorylation of Mdm2 at Ser-166 in hepatocytes Mdm2 is activated in response to inhibited Akt signaling. Journal of Biological Chemistry, 282: 2288-2296.

II. Malmlöf, M., Pääjärvi, G., Högberg, J. and Stenius, U. Mdm2 as a sensitive and mechanistically informative marker for genotoxicity induced by benzo[*a*]pyrene and other DNA damaging compounds. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
γH2AX	H2AX phosphorylated at Ser139
ATM	ataxia-telangiectasia mutated
ATR	ATM- and Rad3-related
BP	benzo[*a*]pyrene
CYP	cytochrome P450
DBP	dibenzo[*a,l*]pyrene
DEN	diethylnitrosamine
DNA	deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase
DSB	double strand break
E3	ubiquitin protein isopeptide ligase
ERK	extracellular signal-regulated kinase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
Hdm2	human double minute 2
IR	ionizing radiation
JNK	c-Jun amino-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	mitogen-activated protein kinase/ERK kinase
Mdm2	murine double minute 2
MAPKAP	MAPK-activated protein
MK2	MAPK-activated protein kinase 2
MNR	Mre11/Nbs1/Rad50
mTOR	molecular target of rapamycin
PDK1	phosphoinositide-dependent kinase 1
PI3K	phosphatidyl inositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PVDF	polyvinylidene fluoride
S6K	S6 kinases
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interference RNA
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TSC	tuberous sclerosis protein complex
UV	ultraviolet

1. GENERAL BACKGROUND

1.1 Introduction

In spite of the development of improved therapies there are still tumors that cannot be cured. To further minimize cancer development and death, a decrease of human exposure to tumor promoting and genotoxic substances is desirable. This could be achieved by identifying biomarkers of exposure and thereby develop mechanistically informative test systems.

1.2 p53 and Mdm2

1.2.1 p53

p53 is a tumor suppressor protein that is named after its molecular weight. It manifests its tumor suppressor function by mediating cell cycle stop and apoptosis in response to cellular stress e.g. DNA damage, hypoxia, chromosomal aberrations and telomere shortening (Oren 2003). There is a strong coupling between p53 and cancer since it is mutated or inactivated in the majority of tumors (Horn and Vousden 2007), which indicates that functioning p53 is a very important defense against cancer development.

p53 is a transcription factor that induces transcription of a lot of genes, for example *p21*, *GADD45* and *Bax*. These genes give rise to expression of proteins that are important for induction of cell cycle stop, DNA repair and apoptosis (Levine 1997). p53 also induces transcription of *Mdm2*, an oncogene that gives rise to the protein that is a negative key regulator of p53 (Michael and Oren 2003).

Phosphorylation of p53

p53 consists of 393 amino acids which constitutes four domains; the amino-terminal domain, containing the transactivation domain to which Mdm2 binds, the DNA-binding domain, the tetramerization domain and the regulatory domain. Posttranslational modifications of p53, like phosphorylations, play an important role in p53 activation and stabilization (Römer et al 2006). Phosphorylation of p53 at the amino-terminal domain prevents Mdm2 binding which in turn results in p53 accumulation. p53 is phosphorylated at Ser15 as a result of DNA damage. This Ser15 phosphorylation of p53 is followed by other amino-terminal p53 phosphorylations at residues like Ser9, Thr18, and Ser20, and in particular the last two phosphorylated residues affect Mdm2 binding and thereby p53 stability (Meek 2004).

Upon severe DNA damage p53 is phosphorylated at Ser46. This phosphorylation is important for activation of a gene called p53-regulated Apoptosis-Inducing Protein 1 and thereby indicates apoptosis (Oda et al 2000). It has also been shown that a phosphorylation of p53 at Ser46 shifts the p53 promoter selection from *Mdm2* promoter which attenuates apoptosis to *PTEN* promoter that instead amplifies apoptosis (Mayo et al 2005).

1.2.2 Mdm2

Murine double minute 2 (Mdm2) was first identified as one of three genes (*Mdm1, 2* and *3*) that was responsible for the spontaneous transformation of a murine (mouse) cell line, BALB/c 3T3-DM. The genes were located on chromatin bodies called double minutes (Iwakuma and Lozano 2003). The human form of this protein is sometimes called Hdm2.

The Mdm2 protein consists of 491 amino acids that from the amino terminal constitute functional domains such as p53 binding domain, nuclear localization sequence, nuclear export sequence, acidic domain, zinc finger, RING finger and nucleolar localization sequence (Meek and Knippschild 2003). Many domains are conserved through evolution from zebra fish to human (Freedman et al 1999). The RING finger domain is a motif that is common for E3 ubiquitin ligases, a family of proteins to which Mdm2 belongs. Ubiquitination involves several steps and the reaction chain starts with and E1 enzyme that binds and activates ubiquitin, which is a 76 amino acid long protein. The E2 protein receives the ubiquitin from E1 and transfers it to the E3 protein. The E3 ubiquitin ligase attaches ubiquitin covalently to the substrate, an event that targets the substrate to degradation. Mdm2 ubiquitinates p53 and is thereby a major negative regulator of p53. Mdm2 can also ubiquitinate itself (Iwakuma and Lozano 2003). It has also been shown that Mdm2 can monoubiquitinate p53, an event that does not target p53 for degradation but instead affects its cellular localization. Monoubiquitination of proteins is a way, besides for example phosphorylation, to affect activity and stability of signaling proteins (Salmena and Pandolfi 2007).

Mdmx is a protein relative to Mdm2, which have most of its homology to Mdm2 in the amino terminal, where the p53 binding domain is located. Mdmx also regulates p53 negatively, mainly through interaction with p53, which inhibits the transcriptional activity of p53 (Marine et al 2007).

Phosphorylation of Mdm2

Mdm2 can be phosphorylated at several residues and these phosphorylations regulate the activity of Mdm2 in different ways (Meek and Knippschild 2003). One of the phosphorylations in focus is the Ser166 phosphorylation of Mdm2. It has been shown that this phosphorylation enhances the E3 ligase activity by Mdm2 and thereby promotes proteasomal degradation of p53. For example TCDD has been shown to induce phosphorylation of Mdm2 at Ser166 and to attenuate the p53 response to DNA damaging agents (Pääjärvi et al 2005).

The phosphorylation of Mdm2 at Ser166 has been shown to be mediated by Akt (Zhou et al 2001, Mayo and Donner 2001, Gottlieb et al 2002). It has also been shown that the Akt mediated Ser166 phosphorylation of Mdm2 inhibits Mdm2 self-ubiquitination, resulting in an increased Mdm2 level and thereby a down-regulation of p53 (Feng et al 2004). Besides Akt, some other proteins can mediate Ser166 phosphorylation of Mdm2 (figure 1). The other proteins are ZIP kinase (Burch et al 2004), MAPKAP kinase 2 (MK2) (Weber et al 2005), mTOR (Pääjärvi et al 2005) and p70S6K1 (Fang et al 2006).

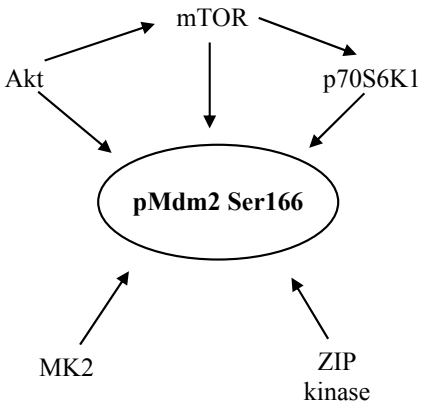

Figure 1. Proteins that have been shown to be able to mediate phosphorylation of Mdm2 at Ser166.

Other Mdm2 phosphorylations of importance are found at the two 2A10 epitopes of Mdm2. Amino acids 255-266 and 389-400 constitute the 2A10 epitope (Balass et al 2002). Of special interest is the Ser395 phosphorylation within the 2A10 epitope, which is mediated by ATM, a kinase that is rapidly activated in response to DNA damage. This Ser395 phosphorylation plays an important role in DNA damage induced p53 stabilization (Khosravi et al 1999, Maya et al 2006).

There are also other residues of Mdm2 that can be phosphorylated. Besides Akt and ATM proteins such as DNA-PK, c-Abl and ATR are involved in these different Mdm2 phosphorylations which all have indirect effects on p53 such as increased p53 transactivation, decreased p53 degradation or inhibited export of p53 from the nucleus to the cytoplasm (Meek and Knippschild 2003, Shinozaki et al 2003).

1.2.3 Mdm2 regulation of p53

Normally, p53 and Mdm2 physically interacts with each other. The amino terminal of Mdm2 forms a hydrophobic pocket where the transactivation domain of p53 binds. This binding inhibits the transcriptional activity of p53. Phosphorylation of p53 at Ser15, Thr18 and Ser20 have all been suggested to affect the binding between p53 and Mdm2, although the individually contribution of these phosphorylations to the disruption of the p53/Mdm2 complex is not unanimously defined. Anyhow, the disruption of the p53/Mdm2 complex may result in accumulation of active p53 in the cell (Moll and Petrenko 2003, Michael and Oren 2003).

As p53 induces transcription of *Mdm2* and since Mdm2 targets p53 for proteasomal degradation through ubiquitination, they together form an autoregulatory feedback loop (figure 2). In this manner Mdm2 keeps the levels of p53 low in unstressed cells. An interruption of this loop leads to p53 accumulation (Michael and Oren, 2003). For example ARF has been shown to affect this loop and induce p53 accumulation by inhibiting the E3 ligase function of Mdm2 (Gallagher et al 2006).

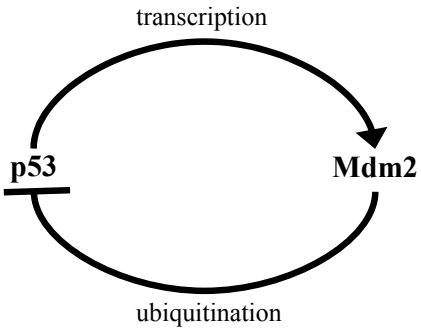

Figure 2. The autoregulatory feedback loop involving p53 and Mdm2.

1.3 Cellular division and survival signaling pathways

Signaling pathways mediating main messages such as cell division or survival can differ between cell types as well as between organs. Signaling pathways also often have a potential to affect each other in complex cross-talks, making it impossible to see a pathway as a consecutive reaction chain.

1.3.1 PI3K-Akt pathway

The PI3K-Akt pathway is a major survival pathway in cells. An imbalance in this survival signaling could result in cell transformation and eventually in cancer.

Extra-cellular stimuli, for example growth factors, activate Akt. These stimuli initiate the signaling by recruiting PI3K to phosphotyrosine receptors on the inner side of the plasma membrane. There PI3K phosphorylates PIP_2, generating PIP_3 which in turn recruits some proteins like Akt and PDK1 to the plasma membrane. PDK1 phosphorylates Akt at Thr308. To be fully activated, Akt also needs to be phosphorylated at Ser473. The kinase responsible for this phosphorylation is not clearly determined but has been called PDK2. There are different studies, suggesting different proteins as potent PDK2 candidates, such as mTOR/Rictor complex, protein kinase C, Akt itself and integrin-linked kinase. Activated Akt then affects several downstream proteins. These Akt mediated effects, no matter if it is inhibition of proteins involved in apoptosis like Bad and caspase 9, or initiation of cell cycle proteins like cyclin D1, do ultimately result in cell survival (Dillon et al 2007).

Akt is over-expressed in several tumors and the PI3K-Akt signaling pathway is an attractive target for drug development (Bellacosa et al 2005), with the goal to inhibit activation of this survival pathway in cancer cells. That may result in apoptosis as well as an enhanced efficacy of chemotherapy. There are already registered drugs that affect upstream targets of PI3K and ongoing clinical trials concerning both upstream and downstream targets of PI3K (Vara 2004).

Akt has been shown to phosphorylate Mdm2 at Ser166 in many cell types (Feng et al 2004, Gottlieb et al 2002, Mayo and Donner 2001, Zhou et al 2001). Also Ser186 of Mdm2 has been identified as a target for Akt mediated phosphorylation (Mayo and Donner 2001, Zhou et

al 2001, Ogawara et al 2002). However, especially the Ser166 phosphorylation of Mdm2 has been pointed out as an activation event that induces ubiquitination and thereby degradation of p53. In some studies it has also been shown that the Ser166 phosphorylation of Mdm2 results in translocation of Mdm2 from the cytoplasm to the nucleus (Mayo and Donner 2001, Zhou et al 2001).

mTOR

mTOR is known as a downstream target of Akt. Recent studies revealed that mTOR forms two complexes, with Raptor and Rictor respectively. It is the Raptor complex that is found downstream of Akt. For activation of the Raptor/mTOR complex, the TSC complex needs to be inactivated. Akt inactivates the TSC complex by phosphorylating it (Tsang et al 2007). There are also studies showing that Akt can phosphorylate mTOR directly at Ser2448. However the functional significance of this phosphorylation is not fully elucidated (Nave et al 1999). It is also the Raptor/mTOR complex that is inhibited by rapamycin, the inhibitor that has given mTOR its name. The Rictor/mTOR complex on the other hand has been identified as PDK2, which means being a kinase that phosphorylates Akt at Ser473. mTOR is important for cell proliferation. A downstream target of mTOR is S6K1 which upon activation increases protein synthesis (Tsang et al 2007). Figure 3 shows a schematic picture of the Akt and mTOR signaling.

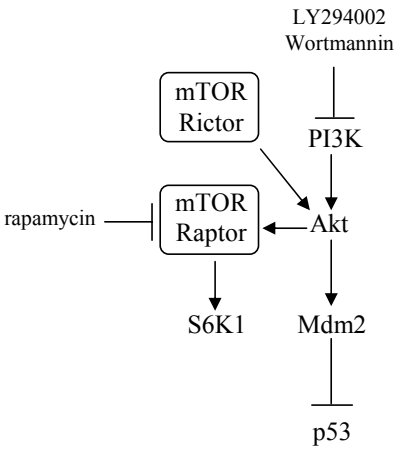

Figure 3. Akt and mTOR signaling. Inhibitors are also shown.

1.3.2 MAPK pathways

There are principally three different MAPK families that each consist of three evolutionary conserved kinases, MAPKKK, MAPKK and MAPK. Theses families are often called as their MAPK; ERK, p38 or JNK (figure 4). These MAPK are all activated by their MAPKK by dual phosphorylation. The ERK pathway is foremost activated by mitogens while the p38 and JNK pathways mainly are activated by stress (Roux and Blenis 2004).

Raf-MEK-ERK pathway

This pathway regulates cell proliferation, differentiation, survival and migration (Galabova-Kovacs et al 2006). Different extra-cellular signals such as growth factors, cytokines or

mitogens initiate this complicated pathway by binding to their appropriate receptors. Upon receptor binding a complex of Shc/Grb2/SOS is activated, which in turn affect the G-protein Ras. Shc/Grb2/SOS stimulation of inactive GDP binding Ras results in an exchange of GDP to GTP, resulting in a conformational change and thereby activation of Ras (McCubrey et al 2006). Active Ras then recruits Raf-1 to the membrane. An interaction between Ras and Raf-1 as well as between Raf-1 and membrane components seems to be required for Raf-1 activation. Another key step for Raf-1 activation is dephosphorylation of the inhibitory Ser259 phosphorylation. First after this dephosphorylation, Raf-1 is further activated by phosphorylations, for example at Ser338 (Dhillon et al 2007). Raf activates MEK1 and MEK2 which in turn phosphorylates and thereby activates ERK1 and ERK2. ERK has several downstream targets such as transcription factors and proteins involved in cell cycle regulation (McCubrey et al 2006).

Inhibiting the Raf-MEK-ERK pathway has been of interest for therapeutic reasons as this pathway is up-regulated in some tumors. An inhibition of this pathway is expected to decrease unwanted cell proliferation. There are Raf and MEK inhibitors in clinical trial (Kohno and Pouyssegur 2006).

Akt-Raf cross-talk

An Akt-Raf cross-talk, where Akt inhibits the Raf-MEK-ERK pathway, has been demonstrated in different cell types (Zimmermann and Moelling 1999, Rommel et al 1999) (figure 4). One mechanistic explanation for this cross-talk is that Akt phosphorylates Raf at Ser259, an event that inhibits the Raf-MEK-ERK pathway. The PI3K inhibitor LY294002 that inhibits Akt activation was shown to increase Raf and ERK activities. It has been suggested that the Akt-Raf cross-talk may switch the cellular response from growth arrest to proliferation (Zimmermann and Moelling 1999). A study of myoblast differentiation shows that the cross-regulation between the PI3K-Akt pathway and the Raf-MEK-ERK pathway depends on the differentiation stage of the cell (Rommel et al 1999).

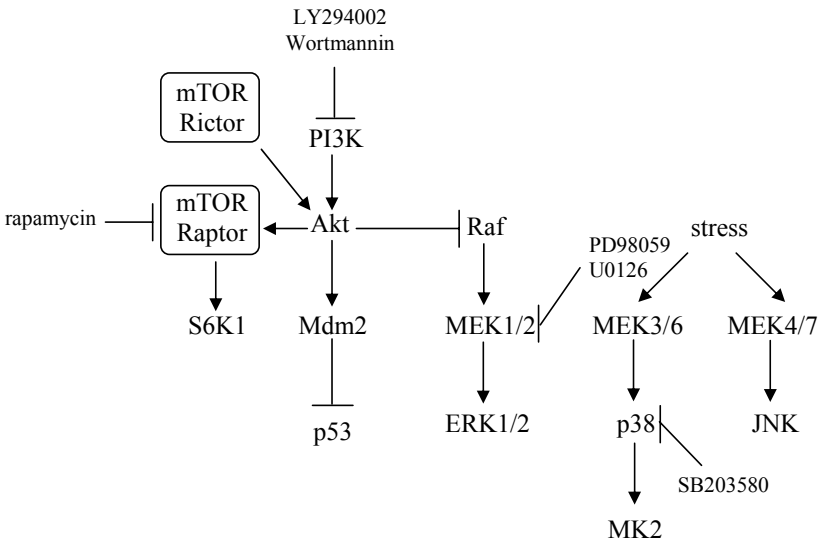

Figure 4. Akt and MAPK signaling, and the cross-talk in between. Inhibitors are also shown.

p38

The p38 pathway is activated upon environmental stress such as oxidative stress, UV radiation and hypoxia as well as by inflammatory cytokines like tumor necrosis factor alpha. p38 is thought to play an important role for normal inflammatory and immune responses even though the mechanisms are not fully understood. The upstream MAPKKs of p38 are MEK3 and MEK6. Activated p38 phosphorylates proteins among which p53 and MK2 are found (Roux and Blenis 2004). It has been shown that MK2 can phosphorylate Mdm2 at Ser166 *in vitro* and that both MK2 and Akt contribute to the phosphorylation of Mdm2 at Ser166 and the following enhanced p53 degradation (Weber et al 2005).

JNK

The JNK pathway is activated in response to stress such as cytokines, UV radiation and DNA damaging agents. The upstream MAPKKs of JNK are MEK4 and MEK7. JNK phosphorylates transcription factors such as c-Jun and cytoplasmic targets (Roux and Blenis 2004).

1.4 Regulation of cellular division and cellular survival

DNA damage can induce cell cycle arrest, giving the cell time to correct the damage through DNA repair. If the damage is severe it may be irreparable and programmed cell death, apoptosis, is induced. There are a lot of proteins involved in DNA damage response; proteins that signal DNA damage, proteins participating in DNA repair and proteins involved in apoptosis.

1.4.1 DNA damage

DNA damage is frequently occurring in mammalian cells and there are several different causes such as radiation, endogenous metabolites and toxic chemicals. The kind of DNA damage depends on the inducer. Examples of DNA damage are alkylation, single strand breaks, double strand breaks (DSB), DNA-DNA cross-links and DNA-protein cross-links.

DNA damaging chemicals

There are many DNA damaging agents. Many of them are used as cytostatic drugs in cancer treatment because of their common ability to induce cell death. Some examples are mitomycin C which is an antibiotic that alkylates DNA, etoposide which is a topoisomerase II inhibitor that causes DNA strand breaks and 5-fluorouracil (5-FU) which is an analog to uracil and thereby is incorporated in DNA during replication.

Another group of DNA damaging agents, which are found in the environment, is polycyclic aromatic hydrocarbons (PAH) to which benzo[*a*]pyrene (BP) and dibenzo[*a,l*]pyrene (DBP) belongs. PAHs are formed during incomplete combustion of organic materials like coal, oil, wood, waste and tobacco. There are more than hundred different PAHs. Humans are exposed to PAHs mainly via tobacco smoke and food, such as heavily roasted meat, but also through the environment. The environmental exposure of PAHs is most pronounced when contact with polluted soil, water or air occurs. The pollution can come from industries or natural sources such as volcanoes or forest fires. PAHs foremost occur in complex mixtures that are found in combustion products such as soot (ATSDR).

In cells, PAHs can be metabolized by cytochrome P450 enzymes to diol epoxides, products capable of binding DNA. Especially CYP1A1 and CYP1B1 are important for the metabolic activation of PAHs. The DNA adducts formed interfere with replication which may result in mutations and later tumor development. It has been shown that PAHs with fjord regions such as DBP are even more potent carcinogens than PAHs with bay regions such as BP (Baird et al 2005).

Cellular response to DNA damage

Many different proteins and signaling pathways are activated upon DNA damage. In response to ionizing radiation (IR) induced DSB, the MNR complex is recruited to free DNA ends and triggers autophosphorylation of ATM at Ser1981 (Kao et al 2005). ATM has been shown to be a key kinase in DNA damage response and is rapidly activated by this autophosphorylation. ATM has also been shown to be a sensitive marker of DSB since already very low levels of IR induces phosphorylation of more than 50% of all cellular ATM within 5 minutes. Only a few strand breaks are enough to generate an ATM response (Kitagawa and Kastan 2005).

While ATM foremost respond to DSB, causing cell cycle stop and DNA repair initiation, ATR is activated by agents like UV light that inhibits DNA replication. ATM and ATR have been taken for parts of two separate signaling pathways until recently when ATR was found to be activated in response to DSB, in an ATM-dependent manner (Hurley and Bunz 2007).

There are several downstream targets of ATM, proteins that are involved in cell cycle progression, DNA repair and apoptosis. For example, ATM directly phosphorylates p53 at Ser15 and Mdm2 at Ser395, events that contribute to p53 stabilization (Kurz and Lees-Miller 2004). It has also been shown that p53 phosphorylated at Ser15 indicates persistent DNA damage, when bound to chromatin (Al Rashid et al 2005).

The primary focus for the cell in response to DNA damage is to inhibit cell cycle progression and to repair the damaged DNA. If the damage cannot be repaired, the remaining molecular solutions are senescence or apoptosis.

1.4.2 DNA repair

Depending on the type of DNA damage an appropriate repair system is activated. If a single DNA strand is damaged, excision repair enzymes are recruited. They cut the damaged DNA sequence away and then use the complementary strand as a template in the repair process, ending up with sealing the nick. There are three different types of excision repair, base excision repair to remove a few bases, nucleotide excision repair to remove up to 30 bases and mismatch repair to excise mismatched bases. DSB are more complicated to repair since there is no intact strand to use as template. There is the simple but less accurate non-homologous end-joining where the two broken ends are rejoined regardless to deletions and there is homologous recombination where the exact sequence is reconstituted by using the sister chromatid (Kao et al 2005).

H2AX

An early cellular marker of DSB is accumulation of H2AX phosphorylated at Ser139 (γH2AX), at the site of breakage. ATM can mediate this phosphorylation. The exact role of γH2AX in DSB repair is not fully understood but it participates in the recruitment of DSB recognition and repair factors to the break. (Fillingham et al 2006).

p53

p53 is involved in DNA repair both indirectly by regulating expression of proteins participating in DNA repair and more directly (Helton and Chen 2007). Concerning the more direct involvement of p53 in DNA repair it has recently been shown that p53 is a chromatin accessibility factor in nucleotide excision repair, relaxing chromatin, which is necessary to enable repair proteins to reach the damaged DNA (Rubbi and Milner 2003).

Mdm2

Mdm2 has been shown to regulate DNA repair by affecting different proteins like DNA polymerase ε and Nbs1. DNA polymerase ε is a protein that is involved in DNA repair, replication, damage sensing and chromatin remodeling among other things. It has been shown that Mdm2 physically interacts with DNA polymerase ε and thereby it has been suggested that Mdm2 may play a role in DNA repair (Ganguli and Wasylyk 2003). Mdm2 is also found to associate with the MNR complex consisting of Mre11, Nbs1 and Rad50. This complex is highly involved in DNA double strand break repair. More specifically it has been shown that Mdm2 binds to Nbs1 and that this complex localizes at DNA damage sites where Nbs1 foci are formed. These foci also include many other proteins like ATM, γH2AX and Mre11. Over-expression of Mdm2 was found to delay DNA repair. It is not fully understood why, but inhibition of Nbs1 localization to or function at foci, are suggested reasons (Alt et al 2005).

2. PRESENT STUDY

2.1 Aim of the study

The aim of this study was to characterize Mdm2 phosphorylations in order to investigate their potential applications in preventive research.

I. Phosphorylation of Mdm2 at Ser166 is important for the ubiquitination capacity of Mdm2 and thereby also for Mdm2 regulated p53 degradation. Akt has been shown to mediate this phosphorylation. Previous studies in the group indicated that TCDD as well as statins can induce phosphorylation of Mdm2 at Ser166. These and other literature data suggested that Ser166 phosphorylation of Mdm2 may be used as a marker for many interacting stresses that may affect the p53 response to DNA damage. In preliminary experiments an Akt independent Ser166 phosphorylation of Mdm2 was indicated. Thereby the regulation of the Ser166 phosphorylation was about to be characterized further in this study.

II. Mdm2 is phosphorylated at Ser395 upon DNA damage, which contributes to p53 stabilization. Ser395 is found within the 2A10 epitope of Mdm2. There were preliminary experiments indicating that low concentrations of genotoxic substances induced 2A10 specific phosphorylation of Mdm2 and the aim of this study was to investigate if Mdm2 could be used as a marker for genotoxicity.

2.2 Materials and methods

Cell lines

The majority of experiments in this study were performed in the human hepatocellular carcinoma cells, HepG2 cells. Another liver cell line was also used, TRL1215 that originates from rat. The human lung carcinoma cell line A549 as well as the human lymphoblast cell line GM00893 was also used.

Western blot

The over all most used method in this study is Western blot, on purpose to analyze protein expression and phosphorylation. Briefly cell samples were subjected to SDS-PAGE and the separated proteins were transferred to a PVDF membrane. The membranes were blocked and incubated with primary antibodies over night. The next day the membranes were incubated with secondary antibodies. A detection kit was used to visualize the results.

Phosphatase alkaline treatment

It has been shown that ATM induced phosphorylation of Mdm2 at Ser395 makes Mdm2 less sensitive to the 2A10 antibody (Khosravi et al 1999) and also that phosphorylation of Ser260 reduced the binding to Mdm2 2A10 antibody (Balass et al 2002). Both these residues are within the 2A10 epitope. By treating the Western blot membranes with phosphatase, the binding of 2A10 antibody to Mdm2 is markedly increased (Maya and Oren 2000). This procedure was used throughout this study. The difference between phosphatase treated membranes and membranes not treated with phosphatase, both subjected to Mdm2 2A10 antibody, was used to measure 2A10 specific phosphorylation.

Immunohistochemical staining

Paper 1 included *in vivo* experiments in rat. Livers from female Sprague-Dawley rats were fixed and analyzed after treatment and sacrifice. The livers were stained for different proteins.

Transfection of cells

This study included transfection of cells with siRNA for different proteins as well as plasmid transfection of cells.

Chromatin isolation

To be able to also investigate proteins bound to chromatin, chromatin was isolated and analyzed with SDS-PAGE and Western blot.

Immunoprecipitation

Proteins that were about to be immunoprecipitated were incubated with the antibody against that protein. Thereafter Western blot analysis was performed which enabled investigation of which proteins the immunoprecipitated protein bound to.

2.3 Results

Paper 1

We demonstrate that the phosphorylation of Mdm2 at Ser166 is mediated via the MEK-ERK pathway in hepatocytes. Different inducers of Mdm2 phosphorylation at Ser166 such as insulin, H_2O_2, bile acids, anisomycin and DEN were used. We also show that Mdm2 is activated by phosphorylation at Ser166 in response to inhibited Akt signaling, and that this activation is ERK mediated. PI3K inhibitors such as LY294002 and wortmannin as well as siRNA for Akt were used, and we show that an up-regulation of Akt inhibits phosphorylation of Mdm2 at Ser166. The ERK mediated phosphorylation of Mdm2 at Ser166 attenuated p53 and p21 responses to DNA damage. Experiments have been performed in different liver cell lines as well as in rat liver *in vivo*.

Paper II

We show that phosphorylation of Mdm2 at the 2A10 epitope is induced by low concentrations of DNA damaging agents such as mitomycin C, etoposide, 5-FU and BP. These Mdm2 alterations were induced at concentrations that did not induce p53 accumulation. Low doses of BP in human lymphoblasts also induce 2A10 specific phosphorylation of Mdm2. Further, two different patterns induced by PAHs in the chromatin enriched fraction, were identified. Low concentrations of BP induced chromatin binding of Mdm2 but neither binding of p53 phosphorylated at Ser15 nor accumulation of γH2AX, while high concentrations of BP and all DBP concentrations used induced the reversed pattern with no Mdm2 binding to chromatin but p53 binding and γH2AX accumulation. We find that acrolein potentiated the effect of BP on p53.

2.4 Discussion

Paper 1

Akt has been shown to activate Mdm2 via phosphorylation at Ser166 (Zhou et al 2001, Mayo and Donner 2001, Gottlieb et al 2002). This phosphorylation increases the ubiquitin ligase activity of Mdm2 and thereby also up-regulates p53 degradation. This signaling mechanism is defined in different cell types. However later publications show a possible involvement of additional proteins in the mediation of Ser166 phosphorylation of Mdm2. By using phage-peptide display it was found that ZIP kinase was able to phosphorylate an Mdm2 peptide at Ser166 but that full length Mdm2 protein seemed to modulate ZIP kinase activity and that the Ser166 phosphorylation of Mdm2 was thereby markedly decreased (Burch et al 2004). It has been shown that MK2 can phosphorylate Mdm2 at Ser166 *in vitro* and that anisomycin which activates p38 and MK2 induces phosphorylation of Mdm2 at Ser166 in cell systems (Weber et al 2005). However, as Weber et al mentions, it is still a possibility that other kinases such as S6K and JNK, which also are activated by anisomycin, contribute to the Ser166 phosphorylation induced. Furthermore their study does not exclude Akt as a contributor of Mdm2 phosphorylation at Ser166. mTOR has been suggested as a mediator of statin induced phosphorylation of Mdm2 at Ser166 (Pääjärvi et al 2005) and the downstream target of mTOR, p70S6K1, affects the basal level of Mdm2 phosphorylation at Ser166 in an Akt-independent fashion in ovarian cancer cells (OVCAR-3), 293 cells and A2780/CP70 cells. It was also clearly demonstrated that p70S6K1 affected Mdm2 expression (Fang et al 2006). These studies seem to have a possible involvement of Akt or S6K in common.

We report that the phosphorylation of Mdm2 at Ser166 is mediated via ERK in hepatocytes. However, there are data suggesting that ERK cannot directly phosphorylate Mdm2 at Ser166 (Roux and Blenis 2004, Freedman et al 1999). Interestingly there is one study showing a potential link between ERK and mTOR-S6K. ERK can phosphorylate TSC2 and thereby cause dissociation of the TSC1/TSC2 complex. This dissociation activates mTOR and downstream S6K (Ma et al 2005). Another identified link between ERK and mTOR-S6K is that ERK regulates phosphorylation of S6 at Ser235/236, independent of mTOR. S6 is a downstream protein of S6K (Roux et al 2007). Further studies may reveal if mTOR-S6K is involved in ERK mediated phosphorylation of Mdm2 at Ser166 in hepatocytes.

We also demonstrate that a suppression of the Akt pathway in HepG2 cells up-regulates MEK-ERK signaling ending with phosphorylation of Mdm2 at Ser166, possibly depending on an established Akt-Raf cross-talk (Zimmermann and Moelling 1999, Rommel et al 1999). Suppression of the PI3K pathway in A549 cells activated ERK but did not induce phosphorylation of Mdm2 at Ser166. This indicates that the MEK-ERK signaling pathway is not an alternative pathway for Mdm2 Ser166 signaling in A549 cells. A constitutive high expression of Akt in liver cells resulted in decreased pERK and pMdm2 levels, which could be a consequence of an up-regulation of the Akt-Raf cross-talk. Since both PI3K-Akt and Raf-MEK-ERK are survival signaling pathways these data may indicate that survival of hepatocytes is of high importance and that there is a back-up signaling pathway in order to maintain survival mediated via Mdm2.

There are many protective mechanisms in hepatocytes that may prevent cell death such as high levels of the antioxidant glutathione. Back-up systems also exists like an ability of quiescent cells to proliferate or oval cells to take proliferative response. These back-up systems are activated if the functional capacity of the liver becomes too small or if hepatocyte

proliferation is blocked, in order to provide survival of the liver (Schoemaker and Moshage 2004). The maintenance of Mdm2 function in hepatocytes could be a part of this well equipped cell death preventing system in liver cells.

Paper II

New chemical substances are continuously identified for example through drug discovery or via findings of environmental pollutants. These substances need to be characterized in different ways, including toxicity tests. At some stage in the characterization process, and depending on their potential universal interest, animal experiments are used, and the data obtained from these experiments are extrapolated to humans (Buschmann 2006). According to the Committee on carcinogenicity in UK, toxicological methods have to be developed to refine the extrapolation between animals and humans. The Committee also stresses a need for effect biomarkers able to provide information about the potential carcinogenicity risk of the chemical tested (Committee on carcinogenicity 2004). Such biomarkers would be valuable tools in risk assessment.

We report that a cellular system can be used to detect certain genotoxic agents and since genotoxicity may cause cancer our system may also reflect the carcinogenic potential of the substances tested. We find that analysis of Mdm2 phosphorylated at the 2A10 epitope could be used as a marker. This method is very simple and rapid, as well as sensitive. p53 stabilization is a main cellular sign of stress such as DNA damage but we show that the 2A10 specific phosphorylation of Mdm2 is observed at much lower concentrations and at earlier time points than p53 accumulation. As described, our method has many advantages and it has the potential to be frequently used in the future when screening new chemicals for genotoxicity. We also show that low concentrations of BP induced 2A10 phosphorylation of Mdm2 in human lymphoblasts. This indicates that Mdm2 also may be used as a marker for human exposure to genotoxic agents in the future.

Further, we demonstrate that different patterns of chromatin bound proteins, involving Mdm2, may indicate the severity and repair potential of PAH induced DNA damage. Low concentrations of BP induced chromatin binding of Mdm2 but neither binding of p53 phosphorylated at Ser15 nor accumulation of γH2AX, while high concentrations of BP and all concentrations of the more mutagenic DBP induced the reversed pattern with no Mdm2 binding to chromatin but p53 binding and γH2AX accumulation. Acrolein, that inhibits repair of BPDE induced DNA damage (Feng et al 2006), potentiated the effect of BP on p53. That is in line with our suggestion that a pattern of chromatin bound proteins indicate severe and non-repairable DNA damage. Chromatin analysis thus may be used to further characterize the effects of DNA damage induced by PAHs.

Taken together our findings suggest possible applications of Mdm2 phosphorylation at the 2A10 epitope as a tool in detecting and characterizing certain types of genotoxicity.

2.5 Conclusions

Mdm2 phosphorylation at Ser166, independent of stimuli, is mediated via MEK-ERK in hepatocytes (paper I).

Inhibition of Akt activation in hepatocytes induces phosphorylation of Mdm2 at Ser166 in a MEK-ERK dependent fashion. This Akt inhibition induced phosphorylation of Mdm2 at Ser166 attenuates the p53 response (paper I).

Genotoxic compounds such as mitomycin C, etoposide, 5-FU and BP induce phosphorylation of Mdm2 at the 2A10 epitope, in low concentrations that do not induce p53 accumulation; this is not the case for UV and DBP. Mdm2 could thereby be used as a sensitive marker for certain types of genotoxicity (paper II).

PAHs induce two different patterns in chromatin associated proteins, where lack of Mdm2 binding to chromatin together with binding of p53 phosphorylated at Ser15 and γH2AX accumulation may indicate severe and non-repairable DNA damage (paper II).

2.6 Future perspectives

It would be of interest to further elucidate the pathway responsible for the MEK-ERK mediated phosphorylation of Mdm2 at Ser166 in hepatocytes, documented in paper I. That means to find the protein which is physically responsible for this phosphorylation in hepatocytes. Investigations with focus on mTOR/S6K would be of special interest.

The indications of the ability to use Mdm2 as a sensitive marker for certain types of genotoxicity shown in paper II could be the basis for the development of a test system for genotoxicity. The results in human lymphoblasts further indicate a potential use of Mdm2 as a biomarker. Additional experiments in lymphoblasts are planned in order to develop a biomarker for human exposure to genotoxic agents.

Another future project is to use Mdm2 phosphorylations as a marker in analysis of mixed environmental samples concerning the content of both tumor promoting and genotoxic substances. TCDD is frequently used as a model compound for dioxin-like chemicals. It binds to the aryl hydrocarbon receptor, mediating tumor promoting effects. It has been shown that TCDD induces phosphorylation of Mdm2 at Ser166 and thereby attenuates p53 response to DNA damage (Pääjärvi et al 2005). Phosphorylation of Mdm2 at Ser166 may therefore be used to indicate tumor promoting substances. Statins (Pääjärvi et al 2005) and other factors also induce Ser166 phosphorylation and this endpoint may thus reflect many types of interacting stresses that can affect the p53 response to DNA damage. On the other hand phosphorylation at the 2A10 epitope of Mdm2 contributes to p53 stabilization and indicates genotoxicity. Today BP is often used as an indicator of carcinogenicity in environmental analysis, but our preliminary data presented below, suggest that it is not a good marker for the total content of carcinogenic agents.

In a preliminary study we have investigated soil samples from five Swedish industry settings in collaboration with Staffan Lundstedt from Umeå University. These samples are very well characterized concerning PAH content (Lundstedt et al 2006). They all contain several different PAHs in different amounts. When HepG2 cells were incubated with the samples, diluted to contain the same BP amount (mg/kg), different responses were obtained. Some samples induced 2A10 phosphorylation of Mdm2 (after 24 hours incubation) and the majority of samples induced phosphorylation of Mdm2 at Ser166 (after 3 hours incubation) (figure 5). Phosphorylation of Mdm2 at Ser166 seems to be more rapid (visible at 3h) than the 2A10 phosphorylation (clear response after 24h). Also another marker such as cyclin D1, where a decrease indicates cell cycle stop, was preferably detected after 24h incubation. The preliminary study thereby suggests that incubation time should be chosen according to analysis parameters. Since we got different patterns of Mdm2 phosphorylation and cyclin D1 decrease despite the same BP content, questions about BP as a single marker of carcinogenicity arose.

We continued by diluting the samples in order to contain the same sum of carcinogenic PAHs (mg/kg) instead of equal BP contents. Differences in the response pattern were still observed (not shown), which may suggest that it is not only the PAHs by themselves that constitute a risk but also that the interacting effects between PAHs may be of importance for the over all carcinogenic risk.

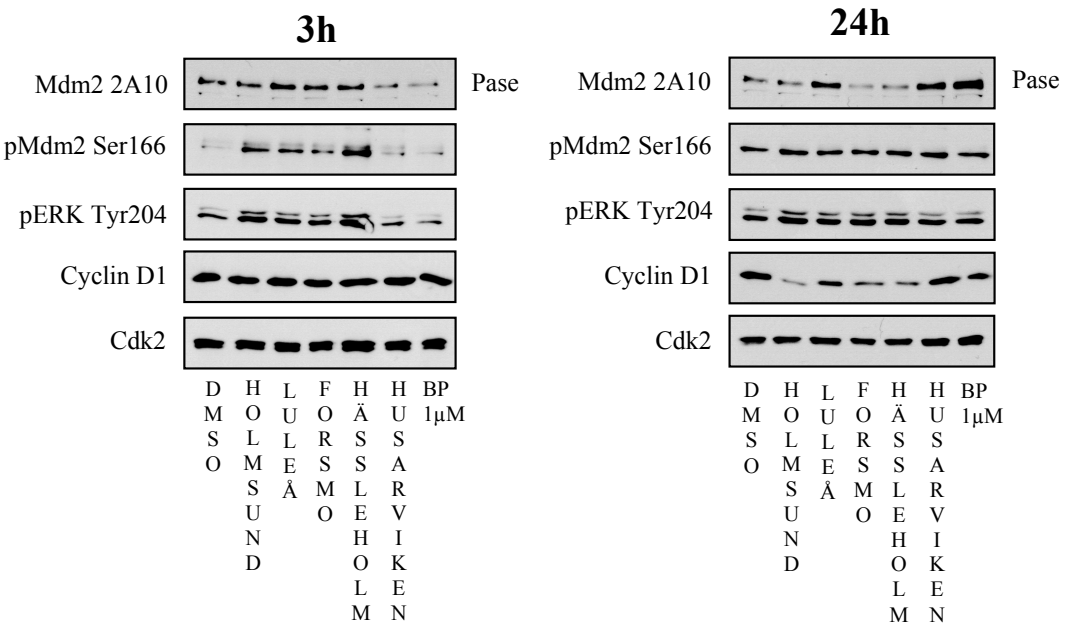

Figure 5. HepG2 cells were incubated for 3h and 24h respectively with extracts from soil samples from Holmsund, Luleå, Forsmo, Hässleholm and Husarviken. BP (1µM) was used as a control. The samples were analysed by Western blotting using antibodies against Mdm2 (2A10), pMdm2 Ser166, pERK Tyr204 and cyclin D1. Cdk2 was used as a loading control. Pase; Western blot with alkaline phosphatase treatment.

A preliminary interpretation of the data obtained, presented in figure 5, suggests that samples from Holmsund, Luleå, Forsmo and Hässleholm contain tumor promoting substances (Ser166 response) and that the sample from Luleå contain genotoxic activity (2A10 response) in addition to the promoting substances. The sample from Husarviken had a strong genotoxic response (2A10) but no tumor promoting response (Ser166). The phosphorylation of Mdm2 at Ser166 induced by these samples is accompanied by a phosphorylation of ERK, which is in line with the Mdm2 Ser166 regulation documented in paper I.

Further experiments are planned in this area to better characterize the observed effects. The possible interactions will also be elucidated further. In this way we hope to find a more reliable marker than BP for carcinogenicity, involving the phosphorylation of Mdm2.

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