UNIVERSITEIT GENT

FACULTY OF MEDICINE AND HEALTH SCIENCES

DEPARTMENT OF BIOCHEMISTRY

Faculteit Wetenschappen

FACULTY OF SCIENCES

VIB

DEPARTMENT OF MEDICAL PROTEIN SCIENCES

The role of the glyoxalase system and its substrate methylglyoxal in the induction of growth factor-withdrawal induced apoptosis and the regulation of the cell cycle

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Thesis submitted to fulfill the requirements for achievement of the degree of Doctor (PhD.) in Science (Biotechnology)
Academic year 2007-2008

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The research presented in this thesis was performed at the department of Biochemistry, Faculty of Medicine and Health Sciences, University of Ghent/ Departement of Medical Protein Research, VIB.

Ruben Audiffret was supported by IWT Vlaanderen.

Abbreviations

AG	aminoguanidine
AGE	advanced glycation endproduct
AGE-R	AGE-receptor
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Apaf-1	apoptotic protease activation factor 1
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine 5'-triphosphate
Bcl-2	B cell lymphoma 2
Bcl-x_L	B cell lymphoma-x_L
BH	Bcl-2 homology
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CDK	cyclin dependent kinase
CDPK	calcium dependent protein kinase
C-t-Ab	C-terminus-directed anti-RAGE antibody
C-type	constant type
DHAP	dihydroxyacetonephosphate
Diablo	direct IAP-binding protein with low pI
DNA	deoxyribonucleic acid
$_{DN}$RAGE	dominant negative RAGE
E.C.	enzyme commission
EGF	epidermal growth factor
ERK	extracellular signal-related kinase
G_1	gap 1
G_2	gap 2
G3P	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GF	growth factor
GLO1	glyoxalase I
GLO2	glyoxalase II
GLO3	glyoxalase III
Glut	glucose transporter

GS	glycogen synthase
GSH	glutathione
GSK-3	glycogen synthase kinase-3
GSNO	S-nitrosoglutathione
GTP	guanosine-5'-triphosphate
HK	hexokinase
HLA-DR	human leukocyte antigen-DR
HMGB1	high mobility group box 1
HOG-MAP	high osmolarity glycerol-mitogen-activated protein
Hsp	heat-shock protein
HtrA2	high temperature requirement A2
HUVEC	human umbilical vein endothelial cell
IAP	inhibitory apoptosis protein
IL-3	interleukin-3
IL-7	interleukin-7
INK4	inhibitors of CDK4
IRS-1	insulin receptor substrate-1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
M	mitosis
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	mitogen extracellular kinase
MG	methylglyoxal
MG-AGEs	methylglyoxal-derived advanced glycation endproducts
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
O-GlcNAc	O-linked N-acetylglucosamine

PDGF	platelet derived growth factor
PFK	phosphofructokinase
pI	isoelectric point
PI3K	phosphoinositol-3-kinase
PKA	protein kinase A
PKB	protein kinase B
pRb	retinoblastoma protein
PTK	protein-tyrosine kinase
R	restriction point
RAGE	receptor for advanced glycation endproducts
ROS	reactive oxygen species
rpS6	ribosomal protein S6
S	DNA synthesis
SAPK	stress-activated protein kinase
Smac	second mitochondria-derived activator of caspase
SnRK	SNF1-related protein kinase
sRAGE	soluble RAGE
STAT	signal transducer and activator of transcription
TNF	tumor necrosis factor
TP	triosephosphate
TPI	triosephosphate isomerase
VDAC	voltage-dependent anion channel
V-type	variable type

Summary

Whereas for unicellular organisms growth is controlled mainly by the availability of nutrients, this does not suffice for multicellular organisms. In multicellular organisms nutrients are abundant, so an additional regulatory mechanism is required. This regulatory mechanism is provided by the dependence on growth factors (GFs). These GFs are limited in the environment of the cells, and competition for GFs between cells determines the number, the size and the survival of these cells. Moreover, this competition defines only the fittest cells survive, and they survive only in the right place. GFs control several cellular processes, including the inhibition of the default apoptotic pathways, the rate of metabolism and cell division. Therefore, when GFs are depleted, metabolism collapses, cell division ceases and cell death is initiated.

Results from our group showed that phosphorylation of glyoxalase I (GLO1) is involved in tumor necrosis factor (TNF)-induced cell death. Phosphorylated GLO1 is directly or indirectly involved in modification of specific target proteins with methylglyoxal (MG), an unavoidable by-product of glycolysis. These events are a requisite for TNF-induced cell death and the data suggested that MG may function as a signaling molecule during regulation of cell death.

In this thesis we describe how GLO1 is phosphorylated upon withdrawal of GFs, which leads to a G_1 arrest and induction of apoptosis. GF depletion was also accompanied by MG-modification of specific target proteins. By pharmacological inhibition we could show that both events were GSK-3β-dependent. Inhibition of GSK-3β also significantly inhibited apoptosis induced by GF depletion. Next, we determined for which kinase GLO1 was a direct substrate. It turned out to be not GSK-3β, but by an *in vitro* kinase assay, we found that GLO1 was a direct substrate of Ca^{2+}/calmodulin-dependent kinase II (CaMKII). This was later confirmed in cells by coexpression of CaMKII with GLO1.

In addition we found that phosphorylation of GLO1 also occurs during cell division by a PI3K-dependent mechanism.

Given the possibility that effects of MG-modified proteins can be mediated by the Receptor for Advanced Glycation End products (RAGE), we subsequently set off a study to investigate whether RAGE was regulated during the cell cycle and after GF withdrawal-induced G_1 arrest and apoptosis. We discovered a novel 17 kDa isoform of RAGE. This 17 kDa RAGE isoform was generated by proteolytic cleavage that could be inhibited by the general caspase inhibitor z-VAD-FMK. Processing of RAGE may thus represent an additional regulatory mechanism in RAGE signaling.

Samenvatting

Waar bij eencellige organismen de groei voornamelijk bepaald wordt door de beschikbaarheid van nutriënten, is dit niet afdoende bij meercellige organismen. Bij meercellige organismen zijn nutriënten doorgaans abundant aanwezig in het milieu van cellen. Bijgevolg is er een bijkomend regulerend mechanisme vereist. Dit regulerend mechanisme wordt voorzien in de afhankelijkheid van groeifactoren (GFs). Deze GFs zijn slechts in beperkte mate aanwezig in het milieu van de cel, en de concurrentie voor deze GFs bepaalt het aantal, de grootte en de overleving van cellen. Bovendien zorgt deze concurrentie ervoor dat enkel de gezondste cellen zullen prolifereren en dat cellen van een bepaald type enkel op de juiste plaats zullen overleven. GFs sturen verscheidene cellulaire processen, waaronder het verhinderen van de intrinsieke apoptotische signalisatie, het in stand houden van het cellulaire metabolisme en het reguleren van de celdeling. Bijgevolg, zal, wanneer GFs onttrokken worden, het cellulaire metabolisme vervallen, de celdeling stilvallen en celdood in gang worden gezet.

Resultaten verworven in onze onderzoeksgroep hebben aangetoond dat fosforylering van glyoxalase I (GLO1) betrokken is in tumor necrose factor (TNF)-geïnduceerde celdood. Gefosforyleerd GLO1 is vervolgens rechtstreeks of onrechtstreeks betrokken bij de modificatie van specifieke doelwitproteïnen met methylglyoxal (MG), een onvermijdbaar bijproduct van de glycolyse. Deze gebeurtenissen zijn vereist bij TNF-geïnduceerde celdood en de data suggereren dat MG zou functioneren als een signaalmolecule bij de regulatie van celdood.

In deze scriptie beschrijven we hoe GLO1 ook wordt gefosforyleerd tijdens een groeistop in de G_1 fase van de celcyclus en de hierop volgende apoptose, welke worden veroorzaakt door het onttrekken van GFs. Door farmacologische inhibitie konden we aantonen dat deze gebeurtenissen afhankelijk waren van GSK-3β. Inhibitie van GSK-3β inhibeerde eveneens significant de inductie van apoptose door GF depletie. Vervolgens bepaalden we voor welk kinase GLO1 een rechtstreeks substraat vormt. Dit bleek niet GSK-3β te zijn, maar door een *in vitro* kinase assay vonden we dat GLO1 een rechtstreeks substraat is van Ca^{2+}/calmodulin-afhankelijk kinase II (CaMKII). Dit werd later in cellen bevestigd door co-expressie van CaMKII met GLO1.Daarenboven vonden we dat fosforylering van GLO1 ook voorkomt tijdens de celcyclus, en dit op een PI3K-afhankelijke wijze.

Gezien de mogelijkheid dat de effecten van MG-gemodificeerde proteïnen worden gemediëerd door de receptor voor gevorderde glycatie eindproducten (RAGE), werd vervolgens een aanvang genomen met een studie naar de regulatie van RAGE tijdens de celcyclus en bij GF-onttrekking geïnduceerde groeistop in de G_1 fase en de daaropvolgende apoptose. We ontdekten een nieuwe 17 kDa isovorm van RAGE. Deze 17 kDa RAGE isovorm werd opgewekt door proteolytische verknippen,wat verhinderd kon worden door de caspase inhibitor z-VAD-FMK. Verknippen van RAGE zou bijgevolg een bijkomend regulerend mechanisme kunnen zijn in RAGE signalisatie.

Part I:
General Introduction

Chapter 1: The glyoxalase system

1.1 Introduction

For almost a hundred years the glyoxalase (GLO) system has been known to exist in animals. The discovery dates back to 1913, when two research groups independently found enzymes that converted α-oxoaldehydes into corresponding α-hydroxyacids (1,2). From then on, research focussed on its putative role in glycolysis. However, when the findings of Embden, Meyerhof and colleagues led to the elucidation of the glycolysis (3,4), it turned out that the GLO system did not lie on the main route to glucose degradation. Thus the hypothesis for a role of the GLO system in glycolysis was abandoned. Later it was found that the endproduct of the glyoxalase system was D-lactate and not L-lactate, which is the endproduct of glycolysis (5). This was the definite proof that the GLO system was not involved in glycolysis and interest in the GLO system severely diminished.

The ubiquitous nature of the GLO system, nevertheless, suggests a fundamental and conserved role (6). In the 1960s Szent-Györgyi took an interest in the GLO system. From his research on cancer cells he proposed a role for glyoxalase I (GLO1) in the regulation of cell division (7-9). However, until today his hypothesis could not be proven since a mechanistic link between GLO1 and the regulation of the cell cycle could not be found.

1.2 Detoxification of α-oxoaldehydes

An important function of the GLO system is the detoxification of reactive α-oxoaldehydes, mainly methylglyoxal (MG). This reaction is dependent on reduced glutathione (GSH) (10) and results in the formation of the corresponding α-hydroxyacid (5).

The GLO system comprises of two enzymes: glyoxalase I (GLO1; E.C. 4.4.1.5) and glyoxalase II (GLO2; E.C. 3.1.2.6). α-oxoaldehydes react nonenzymaticaly with reduced GSH, thereby forming a hemithioacetal. GLO1 catalyses the conversion of this hemithioacetal to a S-D-hydroxyacylglutathione (11-14), which, in turn, is hydrolyzed to the corresponding α-hydroxyacid, hereby regenerating reduced GSH (15) (Figure 1.1). The committed step in this pathway is the reaction catalysed by GLO1 since, under physiological conditions, it is considered irreversible (13).

The GLO system has a broad substrate specificity for α-oxoaldehydes (16-18), but the reaction rates are inversely correlated to the hydrophobicity of the side chains of the

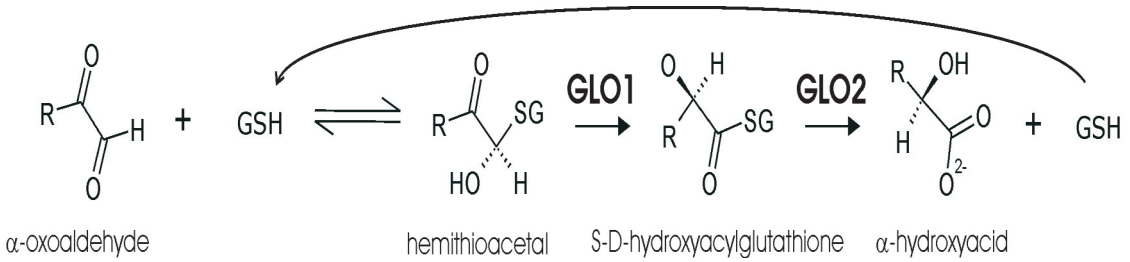

Figure 1.1. The glyoxalase system.
The GLO system comprises of 2 enzymes, GLO1 and GLO2. α-oxoaldehydes spontaneously react with GSH to form a hemithioacetal, which is converted to a S-D-hydroxyacylglutathione by GLO1. This S-D-hydroxyacylglutathione is subsequently hydrolized to the corresponding α-hydroxyacid, hereby regenerating reduced GSH. (R = CH_3 for MG).

substrate (19). MG is generally considered to be the primary physiological substrate for the glyoxalase system. Under physiological conditions the GLO system reaction rates for the catalysis of MG are near diffusion-controlled limits (20-22), making the GLO system very efficient, and keeping the intracellular levels of MG low. The efficiency of the detoxification of MG is primarily determined by the spontaneous formation of the hemithioacetal. In aqueous solutions at pH 7, MG appears in α-oxoaldehyde, but also in a mono- and dehydrated form. When physiological concentrations of GSH are present, the equilibrium is shifted towards the formation of the hemithioacetal, and MG is efficiently removed by the GLO system (23,24) (see also chapter 2: Methylglyoxal).

1.3 Glyoxalase I

As previously mentioned the first step in the detoxification process by the glyoxalase system is the conversion of the hemithioacetal, which is spontaneously formed by MG and GSH, into S-D-lactoylglutathione. This reaction is catalysed by GLO1 (Figure 1.2). The cristal structure of GLO1 shows that the active site is situated in a barrel which is only formed at the dimer interface (25). This active site comprises a Zn^{2+} ion interacting with side chains from both subunits. The side chains involved are Glu_{33} and Glu_{99} from one subunit and His_{126} and Glu_{172} from the other. The structure of the active site suggests the mechanism is a proton transfer between the C-1 and C-2 of the hemithioacetal, whereby the ene-diol intermediate is stabilized in the *cis* configuration with both oxygens orientated towards the Zn^{2+} ion (25-28). The intermediate is rapidly ketonized to the hemithioacetal. It has been proposed that Glu_{172} and Glu_{99} are the catalytic bases for the S-substrate and R-substrate respectively (29-31).

GLO1 has been described to exist in various organisms, going from prokaryotes over yeast to plants, mammals and humans (e.g. (11,20,32-42)). In mammals and higher eukaryotes GLO1 functions as a dimeric metalloenzym, using Zn^{2+} as a cofactor (see

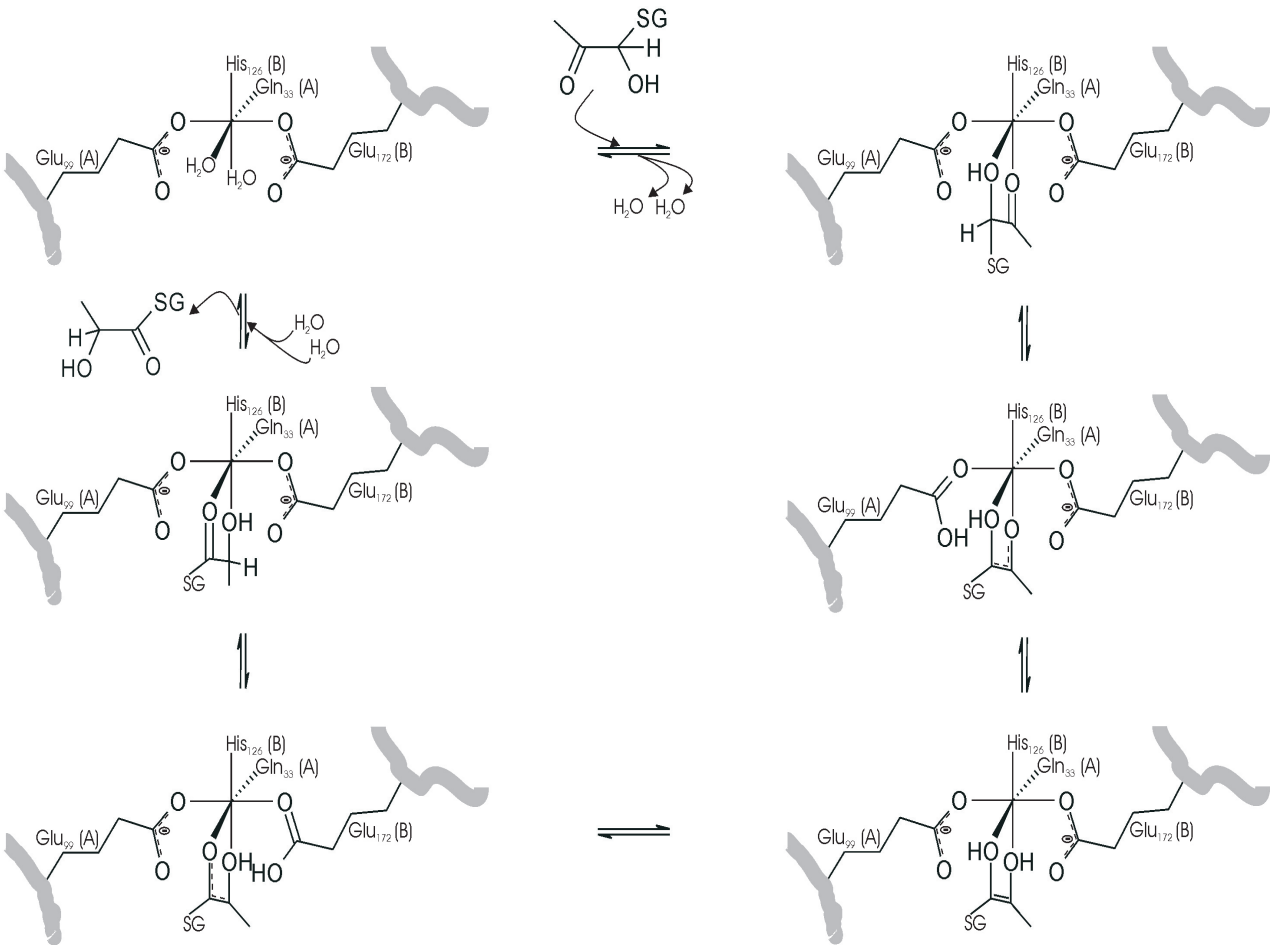

Figure 1.2. Proposed catalytic mechanism for glyoxalase I.
The active site of GLO1 is situated at the dimer interface and contains a Zn^{2+} ion which interacts with side chains from both monomers (designated A and B). The substrate is the R-hemithioacetal and Glu_{99} initiates the reaction as catalytic base. See text for more details (Adaptation from (27,30)).

above). GLO1 from Escherichia coli, however, needs Ni^{2+} for its activation (36). In yeast GLO1 functions as a monomer and contains two active sites (43). Monomeric human GLO1 is a protein of 184 amino acids with a molecular mass of approximately 21 kDa. It has 51% homology at the nucleotide level and 42% homology at the amino acid level with bacterial GLO1 (44). The GLO1 dimer is extremely stable and shows a high resistance to reducing conditions (45). GLO1 is present in a concentration of approximately 0,2 µg/mg protein in most human tissue (46).

The human GLO1 gene is located at chromosome 6 (47) and its locus is linked to the HLA-DR (human leukocyte antigen-DR) (48,49). The gene for GLO1 was first described as one with five exons (45,50), but recent analysis indicates that it contains six exons (51). Human GLO1 is expressed from an autosomal diallelic gene and thus is expressed in three different phenotypes, GLO1 1-1, GLO1 2-2 and GLO1 1-2, representing the two homozygous and heterozygous expression patterns that are characteristic for co-dominant expression (52). GLO1 1-1 is electrophoretically slower than GLO1 2-2 (53). The difference in the two alleles is a single nucleotide polymorphism, resulting in an amino

acid substitution at position 111 from an alanine to a glutamic acid, thereby changing the electrophoretic properties (54). This variation also results in a decrease in glyoxalase activity (55). Several nucleotide polymorphisms have been described in the 5' and 3' untranslated regions and these polymorphisms may affect GLO1 expression, stability or posttranslational regulation. This may also affect the function of GLO1 (51). It has been claimed that some GLO1 phenotypes show correlation with diseases such as autism (55), multiple sclerosis (56), diabetes (57-59) and prostate cancer (60).

1.4 Posttranslational modification of glyoxalase I

There are several reports on posttranslational modification of GLO1. Studies in plants, yeast and mammals show that GLO1 is subject to phosphorylation and responds to nitric oxide (NO), indicating that posttranslational modification is conserved and is important for its biological function.

Phosphorylation: phosphorylation has been described to occur in mammalian cells, in yeast and in plants. Phosphorylation of GLO1 in mammalian L929 cells has been described as a necessity for the induction of necrosis by tumor necrosis factor (TNF) (61). This phosphorylation doesn't alter the detoxification capacity of the glyoxalase system for MG.

In yeast, treatment of the α-type budding strain of Saccharomyces cerevisiae with mating factor also induces phosphorylation of GLO1 (62). In contrast to the phosphorylation in mammalian cells, in yeast the activity of GLO1 was enhanced (63).

In *Arabidopsis*, GLO1 was phosphorylated upon the over expression of the kinase SnRK2.8, a kinase involved in resistance to stress (64). Concomitantly, a rise in GLO1 activity was induced. In rice leafs, gibberellin, a regulator of growth, induces phosphorylation of GLO1 (65), albeit this is a novel type of plant GLO1 (41). Possible changes in GLO1 activity were not reported.

Response to NO: research in yeast and mammalian cells indicates that GLO1 is responsive to NO. In mammalian endothelial cells the addition of NO leads to the inactivation of GLO1 (66). The addition of NO also leads to an increase of the isoelectric point (pI) of GLO1. The effects induced by NO on GLO1 are dependent on the formation of S-nitrosoglutathione (GSNO), suggesting that the substrate recognition site of GLO1 is most likely involved (67). Later it was shown that at least three cystein residues were

sensitive to NO-induced modification (68). Also in yeast and *in vitro* the addition of GSNO inhibits the activity of GLO1 (69).

Interplay between phosphorylation and NO has also been shown. In the TNF-induced necrosis in L929 cells NO-induced modification and phosphorylation occur independently. The NO-mediated modification is not required for the TNF-induced phosphorylation, although TNF-induced phosphorylation does primarily occur on the NO-responsive form of GLO1 (68).

1.5 Glyoxalase II

The second step in the detoxification of α-oxoaldehydes is the hydrolysis of the S-D-hydroxyacylglutathione formed in the first step (see above) to an α-hydroxyacid, hereby regenerating reduced GSH. Although GLO2 is a very efficient enzyme for the hydrolysis of S-D-lactoylglutathione (70), which is considered its physiological and preferred substrate, it is considered to be the rate limiting enzyme of the glyoxalase system (71). GLO2 is a monomer, consisting of two domains. The active site extends over the domain interface and contains a binuclear Zn-binding site. The active site also contains a water molecule, presumably in the form of a hydroxide ion. This hydroxide ion probably acts as the nucleophile catalysing the hydrolysis (72), whereby the Tyr^{175} residue contributes to the binding of the GSH derivative (73).

Like GLO1, GLO2 has been described in various organisms, from prokaryotes over plants to mammals (74-82). GLO2 exists as multiple isozymes and has been located not only in the cytosol, but also in mitochondria. The human gene for GLO2 has been assigned to chromosome 16 (83) and some polymorphism has been described (84).

In mammals, the GLO2 gene gives rise to two distinct mRNA species transcribed from 9 and 10 exons respectively. The former of the two encodes for two protein species, mitochondrial targeted GLO2 and cytosolic GLO2, both initiated from different AUG codons. The latter mRNA species only encodes for cytosolic GLO2 (85). The mitochondrial GLO2 is located in the matrix and intermembrane space (86).

In plants and yeasts however, cytosolic and mitochondrial GLO2 are encoded by different genes (76,87). Multiple forms of mitochondrial GLO2 exist in plants and GLO2 can be located in the matrix and in the intermembrane space (86). Some mitochondrial isozymes have a $Fe^{3+}Zn^{2+}$ metal center instead of the two Zn^{2+} ions described for human

GLO2 (88). In *Arabidopsis thaliana* the different GLO2 isozymes are differently expressed in plants, with cytosolic GLO2 being more abundant in flower buds and mitochondrial GLO2 being more abundant in roots (76).

Quite interesting is the fact that GLO2 appears to be regulated by members of the p53 family of proteins. In the temporal cortex of p53-/- mice GLO2 is decreased (89). Xu and Chen report that GLO2 is up-regulated by p63 and p73, two members of the p53 family that have fundamental roles in development (90). They postulate that GLO2 serves as a pro-survival factor and plays a critical role in the normal development and in the pathogenesis of various human diseases (e.g. cancer and diabetes).

1.6 Glyoxalase III

In *Escherichia coli*, the existence of a novel GLO enzyme, glyoxalase III (GLO3), has been described, which catalyses the conversion of MG to D-lactate (91). GLO3 works independently of the other two glyoxalases and in contrast to GLO1 and GLO2, does not require GSH as a co-factor. Specificity of this enzyme is limited to MG and phenylglyoxal. GLO3 is inactivated by oxidative stress (92). Besides the fact that GLO3 seems to play a role in the survival of non-growing *Escherichia coli* cultures (93), not much is known about this enzyme.

1.7 Possible functions of the glyoxalase system

It is quite obvious that the detoxification of α-oxoaldehydes, MG in particular, is a very important function for the glyoxalase system. However, the complex nature of the glyoxalase system (e.g. the fact that it comprises two enzymes, and not just one), the ubiquitous and conserved nature of the glyoxalase system, the existence of several other detoxification mechanisms for MG (see also the chapter 2: Methylglyoxal) and the existence of MG synthases in eukaryotes and prokaryotes (94-97) suggest that there are other, perhaps more fundamental functions for the glyoxalase system. Moreover, because of the different levels of regulation and modification of the different enzymes it is unlikely that the only role for the glyoxalase system is detoxification of MG. Two possible functions that will be emphasised here are an involvement in cell division and a role during stress.

A possible function in cell division: In plants it was observed that there was a strong correlation between GLO1 activity and cell proliferation (98,99). The observation

was made that in strongly dividing parts of the plant the concentration of MG was low and the GLO1 activity was high. In non-dividing parts, the level of MG was higher and GLO1 activity was undetectable. Also, when the cell cycle was inhibited, a decrease in GLO1 activity was induced and when GLO1 was inhibited, a significant inhibition of the increase in cell number and DNA content were observed. When soybean cell suspensions or carrot cell suspensions were treated with auxin, the activity of GLO1 was modulated together with proliferation (100,101). Gibberellin, another important growth regulator in plants, induces phosphorylation of GLO1 (65).

In yeast GLO1 activity correlates with the overall growth status, although it is not involved in the regulation of cell cycle (102). Furthermore GLO1 activity alters and is phosphorylated after treatment with mating factor (62,63). Mating factors are known to induce G_1 arrest in haploid yeast cells (103,104).

In addition, in animals a correlation between cell proliferation and activity of the glyoxalase system was found. Principato et al. found that GLO1 activity was high in embryonic chicken tissue, and GLO2 activity was low. This relation was inversed in mature chicken tissues, where a low GLO1 activity and a high GLO2 activity were observed (105). Also, in regenerating liver after partial hepatectomy, high GLO1 activity and low GLO2 activity were found. These activities returned to control levels after regeneration (106). During the maturation of human erythrocytes it was found that GLO1 activity increased, but a decreased activity of GLO1 was seen in mature, old erythrocytes (107). A very strong correlation was also found in the development of *Bufo bufo* embryos. Already at the earliest stages of development significant levels of GLO1 activity could be measured, and GLO1 activity rose during development. GLO1 to GLO2 activity ratios are very high at the beginning of development and gradually decrease as the development progresses (108). When differentiation is induced in HL-60 and K526, both leukaemia cell lines, a concomitant decrease in GLO1 to GLO2 activity ratios is seen (71). High expression levels of GLO1 are considered a risk factor for the development of certain types of cancer, and numerous tumours show a significant increase in levels and activity of GLO1 and a high GLO1 to GLO2 activity ratio in comparison to corresponding non-tumour tissues and surrounding tissues (109-117). Albert Szent-Györgyi was a strong believer in a very fundamental role for GLO1 and its substrate MG in the regulation of cell division and built his ‘promine/retine’ theory around it (e.g.(7-9,118,119) reviewed in (120)). However, in general the glyoxalase system is considered to be a ‘marker for cell growth and division’, but a causal relationship between the glyoxalase system and cell growth remains obscure.

A possible function in stress tolerance: A firm link between expression levels and activity of GLO1 and the resistance to abiotic stress has been established in plants. In *Brassica juncea* it was found that GLO1 was upregulated in response to salt, water and

heavy metal stresses (40). Moreover, in tomato plants an induction of GLO1 and GLO2 was observed in response to several forms of stress (121). Transgenic plants over-expressing GLO1 also showed an increase in tolerance towards salt and MG. Research in several plant species showed that in response to salinity, cold and drought, MG concentrations in the plant increase, which results in the inhibition of seed germination. This effect was enhanced by the down-regulation of GLO1 (122). Over-expressing components of the glyoxalase system led to enhanced tolerance towards salinity or heavy metal stress in several plant species (123-126). Furthermore it was observed that overexpression of SnRK2.8, a kinase involved in resistance to several types of stress in plants, induces phosphorylation of GLO1 (64).

In yeast, osmotic stress leads to alteration in GLO1 levels or activity. Inoue and co-workers reported the induction of GLO1 gene expression in *Saccharomyces cerevisiae* in response to osmotic stress (127). GLO1 activity is regulated in *Schizosaccharomyces pombe* in response to osmotic stress, as well. No change in mRNA levels or protein levels was observed in this case, so the effect was induced by posttranslational modification of GLO1 (128). In *Candida albicans* the regulation of GLO1 by the transcription factor Cap1p is part of the stress response induced by oxidative stress (129).

In animals, as well, the GLO1 is involved in resistance to different kinds of stress. Expression of GLO1 is up-regulated in the parasitic nematode *Onchocerca volvulus* under conditions of oxidative stress (37). Miller et al. showed that GLO1 provides protection of human retinal pericytes against hyperglycemia-induced cell death (130). Overexpression of GLO1 in bovine endothelial cells protected against the effects of hyperglycemia (131). It has been reported that tumors with high levels of GLO1 are more resistant to antitumor induced-apoptosis (132). Therefore a lot of effort has been put into finding inhibitors for GLO1 to be used as antitumor agents (e.g. (133-136)).

Regulation of the glyoxalase system may be a requirement for organisms to meet altered metabolic demands imposed by stress. In a sense, cell division can be regarded as a form of stress and the regulation of the glyoxalase system may be a consequence of adaptation to that stress. Indications of this, are, for instance, the involvement of similar signaling pathways that are involved in both stress response and cell division (e.g. Ca^{2+} (136,137) and MAPK pathways (127)).

Chapter 2: Methylglyoxal

2.1 Introduction

Methylglyoxal (2-oxopropanal, MG) has long been thought of simply as a by-product of glycolysis, which is the most fundamental metabolic pathway. Recent research, however indicates that there might be more to this molecule than just a role as cytotoxic by-product.

MG is a reactive dicarbonyl compound known to form covalent adducts with proteins and nucleic acids (138). In aqueous solutions it is present in three forms, the unhydrated, monohydrate and dihydrate forms, which are in equilibrium. The presence of physiological concentrations of glutathione (GSH) shifts this equilibrium towards the formation of the hemithioacetal, and MG is efficiently removed by the glyoxalase system (Figure 2.1) (23,24,139).

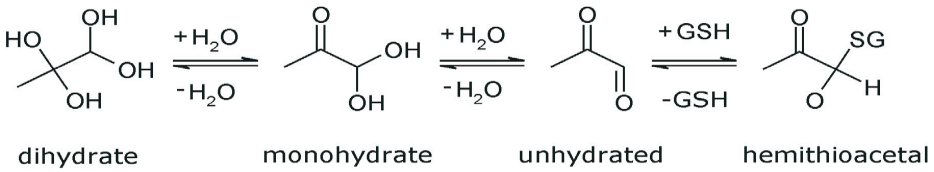

Figure 2.1. Methylglyoxal in aqueous solutions.

The main sources of MG are triose-phosphate (TP) glycolytic intermediates. Non-enzymatic fragmentation and elimination of phosphate from glyceraldehyde-3-phosphate (G3P) and dihydroxyacetonephosphate (DHAP) (140,141) or leakage of phosphor-ene-diolaat from the active site of triosephosphate isomerase (TPI) (142,143) contribute most to the formation of MG (Figure 2.2). Metabolism of acetone and threonine also ads to the formation of MG (144-146), but under normal metabolic conditions both the latter pathways can be considered to be of minor importance. MG can also be formed enzymatically by methylglyoxal synthases (94-97,147-149), although the importance of these enzymes has not been further investigated in higher eukaryotes and therefore remains obscure.

An important regulator of intracellular MG levels is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reduced activity of GAPDH leads to the accumulation of TPs, leading to a subsequent increase in MG formation (150). GAPDH activity is influenced by e.g. oxidative stress (151) and by MG modification (152). This suggests that MG is able to propagate its own formation.

Cells contain considerable amounts of MG. The majority of the MG present *in vivo* however, is bound to proteins (see also below). Chaplen and colleagues detected up to 310 µM of MG in chinese hamster ovary cells and estimate that 5-10% of cellular proteins may be modified to physiologically significant levels (153).

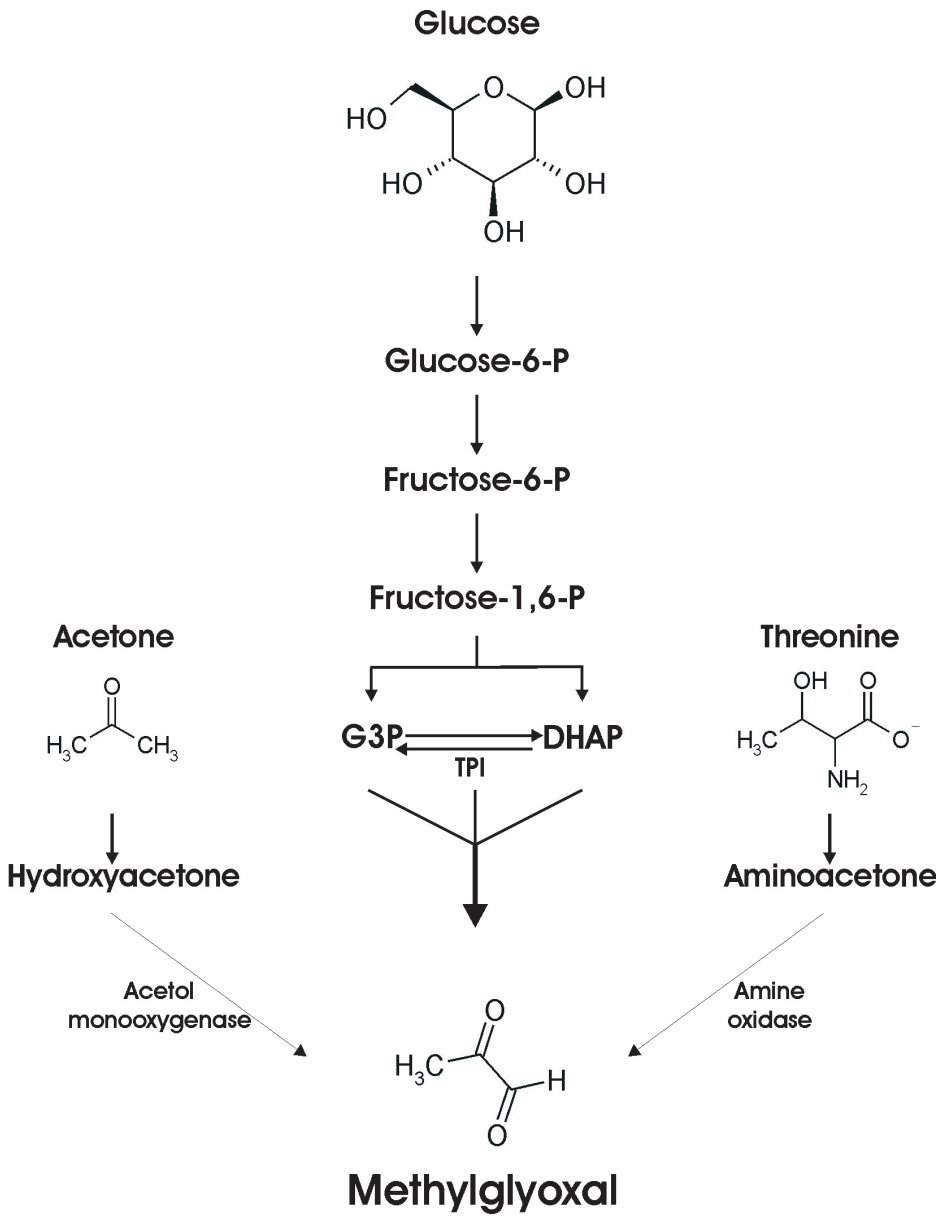

Figure 2.2. Main metabolic origins of methylglyoxal in mammalian cells. MG is mainly formed by non-enzymatic or enzymatic elimination of phosphate from triose-phosphate intermediates from glycolysis. Also acetone and threonine metabolism contributes to MG formation in mammalian cells, albeit considerably less.

2.2 Modification of proteins

MG is able to modify proteins under physiological conditions (154). The reactions proceed even at physiological concentrations of MG and lead to the reversible and irreversible modification of arginine and lysine residues and to the reversible modification of cysteine residues (Figure 2.3). However, when compared to cysteine residues, there is a clear preference for arginine and lysine residues to be modified (155). Although from model studies with Nα-acetylamino acids the reaction of MG with protein is expected to be the most rapid at cysteine residues, *in vivo* MG-modified cysteines have not yet been detected, most likely because of their unstable and reversible nature (154). The modification of cysteine

with MG yields a hemithioacetal (156) (Figure 2.3.A). An example of such a reaction would be the reversible and non-enzymatic reaction between MG and GSH.

Under physiological conditions the primary targets are the arginine residues (157). A major physiological glycation adduct formed from MG with arginine is the imidazolone N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (Figure 2.3.B) (158,159).

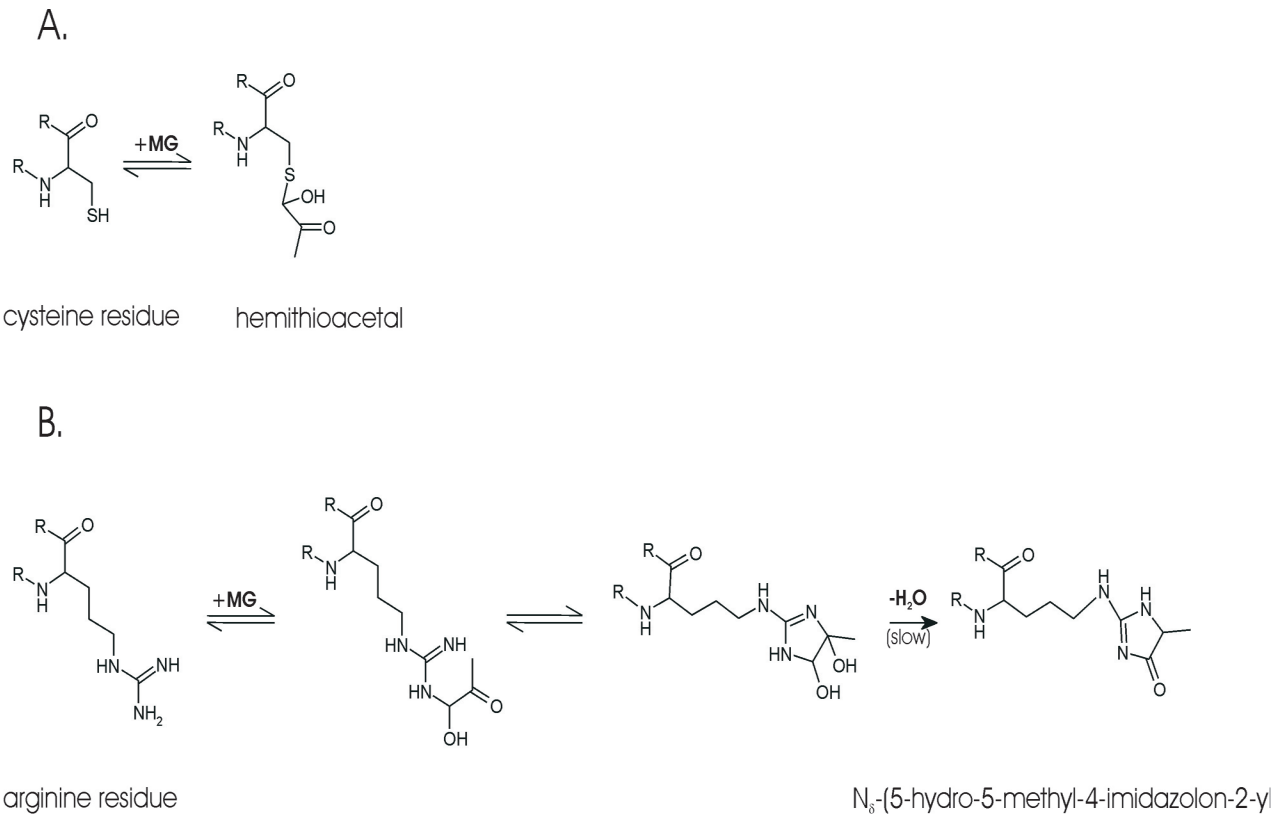

Figure 2.3. The reaction of methylglyoxal with cysteine and arginine residues.
A. Reaction of MG with a cysteine residue (e.g. glutathione). **B.** Reaction of MG with an arginine residue. R = peptide chain.

Further irreversible reactions of the MG-modified arginine and lysine residues lead to the formation of Advanced Glycation Endproducts (AGEs). This reaction is slow and is called the maillard reaction. Formation of these AGEs is often accompanied by oxidative stress and leads to the dysfunctioning of protein and cells. AGEs contribute to the development of numerous pathophysiological conditions, such as diabetes, Alzheimer's disease and atherosclerosis, and aging (for reviews see (158,160-164)).

Glycation of a protein by MG generally induces partial unfolding; this renders proteins instable and induces degradation. MG modification, therefore, might function as a signal for the degradation of protein (154,157).

Interestingly, MG modification displays a high specificity. MG modification is restricted to preferential proteins (165-167), as well as to specific residues of these target proteins (168-170).

2.3 Detoxification of methylglyoxal

Besides the glyoxalase system (see chapter 1: The glyoxalase system) several other pathways contribute to the detoxification of MG.

1. Aldose reductase is dependent on NADPH and catalyses reduction of a wide range of aldehydes; MG is reduced to acetol, or when MG forms the hemithioacetal with GSH, to lactaldehyde. Both can then be reduced to propanediol, also catalyzed by aldose reductase (171-173).

2. Betaine aldehyde dehydrogenase is NAD-dependent and oxidizes numerous aldehydes, among which, MG. The efficiency of MG oxidation is considerably low in comparison with betaine aldehyde, its physiological substrate (174).

3. 2-oxoaldehyde dehydrogenase catalyses the NADP-dependent oxidation of MG to pyruvate. This enzyme seems primarily of importance in the liver, but has been purified from erythrocytes, as well (175-178).

However, under normal physiological conditions the glyoxalase system is the most important pathway for MG detoxification (24,127,171,179). Expression and activity of the glyoxalase system decrease with age, leading to a rise in MG and, consequently, AGE formation (165-167,180,181).

2.4 Methylglyoxal as a cytostatic and cytotoxic molecule

Ever since Szent-Györgyi hypothesized a function of MG as a regulator of cell division ((7-9,118), reviewed in (120)), a lot of attention has been given to this proposed role of MG.

MG exerts its activity on proliferation and survival in different ways:

1. MG signals are mediated by receptors (see chapter on RAGE)

2. MG and S-D-lactoylglutathione, an intermediate in the detoxification of MG, are capable of inhibiting DNA-synthesis and inducing DNA-damage, leading to G_1 arrest and subsequently to apoptosis (182-189).

3. Reactive oxygen species (ROS) are generated during the reaction of MG with amino acid residues (190-193). Cell lines that are more sensitive to oxidative stress are therefore

also more sensitive to the effects of MG (194,195). A first effect of high concentrations of ROS and MG is a depletion of cellular GSH, hereby impairing protection against oxidative stress and detoxification in general (194,196-199). Superoxide formation also leads to the activation of p38 MAPK and JNK, signaling cascades leading to apoptosis (195,200-202) (see also paragraph 'MG as a signaling molecule').

4. In human embryonic kidney cells (HEK293) and a mouse fibroblast cell line (NIH3T3) MG has been shown to render these cells resistant to the mitogenic action of insulin-like growth factor-1 (IGF-1). This effect was associated with a stronger and prolonged activation of ERK (extracellular signal-related kinase) and an up-regulation of p21 (Waf1/Cip1) (203).

5. The impairment of insulin signaling by MG is well documented. MG reacts with insulin, hereby changing the biological functions of insulin (204). In addition, downstream effector proteins of the insulin receptor are modified, impinging on insulin signaling (205). Other receptors, such as the platelet derived growth factor (PDGF) receptor and epidermal growth factor (EGF) receptor, are also inhibited in their signaling by MG (206-208).

6. Glycolysis and mitochondrial respiration are sensitive to MG. Tumor cells seem particularly sensitive to these inhibitory effects (209-215). This inhibition is mainly the consequence of inactivation of GAPDH, but also of hexokinase (HK). At the mitochondrial level, the inhibition occurs at complex I of the respiratory electron transport chain.

7. The covalent modification of proteins by MG plays a role in cell survival, as well. Two examples are the modification of heat-shock protein 27 (Hsp27) (169,216-219) and of the mitochondrial permeability transition pore (220,221). In both cases MG reversibly modifies arginine residues, hereby influencing the structure and function of the involved protein. These MG modifications protect cells against apoptotic stimuli, somewhat in contrast to the other examples, where MG induces cell cycle arrest in the G_1 phase or apoptosis.

2.5 Methylglyoxal in other signaling pathways

Despite its evident cytostatic and cytotoxic properties, MG is inevitably present in every cell under physiological conditions, from prokaryotes to higher eukaryotes. Not only is it a by-product of glycolysis, the most fundamental metabolic pathway, several other metabolic pathways lead to the formation of MG in microorganisms and higher eukaryotes, as well. These facts, together with the well-documented existence of prokaryotic as well as eukaryotic MG synthases, make the explanation of MG as a mere flaw of metabolism unsatisfying. Recent reports suggest a possible role for MG as a signaling molecule.

In plants: Under stress conditions a rise in intracellular MG concentration has been observed (122). This seems to be a general mechanism, as a rise of intracellular MG concentrations has been measured in several different plant systems. This suggests that MG, therefore, could act as a signal for plants to respond to stress.

In yeast: In *Saccharomyces cerevisiae* it has been shown that the activity of Yap1, a transcription factor that mediates the oxidative stress response, is modulated by methylglyoxal (222). The localisation of Yap1 in the cytoplasm or the nucleus is influenced by intracellular concentrations of MG. Yap1 is most likely directly modified by MG, presumably on a cysteine residue. This modification is reversible and independent of ROS formation in the cell. It has also been reported that extracellular MG activates several signal transduction pathways, like the high osmolarity glycerol (HOG)-mitogen-activated protein (MAP) kinase pathway, hereby eliciting the induction of several genes (223,224). In addition, MG transiently activates the uptake of Ca^{2+} from the medium (224). Both responses are independent of ROS formation, neither are they caused by changes in osmolarity due to the higher concentration of extracellular MG. Maeta and colleagues hypothesize there might exist a sensory system for external MG on the cell surface conveying signals to the nucleus. A likely candidate for this is the osmosensor Sln1.

In *Schizosaccharomyces pombe* an alternative mechanism has been shown to exist. Here extracellular MG activated a yeast homologue of the stress-activated protein kinase (SAPK) pathway, leading to activation of the transcription factor Spc1 and independent of this Spc1-SAPK pathway Pap1, a Yap1 homologue in *S. pombe*, is activated, as well (170). The activation of Spc1 through the SAPK pathway takes place irrespective of intracellular MG content. Activation of Pap1, on the other hand, is reversible and dependent on intracellular MG. ROS are not involved in either of the pathways. MG could, therefore, be a metabolic signaling molecule. Intracellular levels of MG vary depending on growth conditions and stress situations. These alterations in MG concentrations could then elicit a response through the modification of different signaling molecules (225,226).

In mammals: In mammalian cells the pathways involved in the mediation of MG-induced signals and the outcome are dependent on the cell type. MG selectively induces apoptosis in Jurkat cells (195,201). The mechanism of MG-induced apoptosis is cell type-dependent since several other cell lines were shown to be insensitive to MG. MG-induced cell death was mediated through JNK (c-Jun N-terminal kinase), showed typical apoptotic morphology (e.g. DNA fragmentation) and was dependent on caspase-3. Administration of MG to rat mesangial cells induces apoptosis. This apoptosis is dependent on the activation of apoptosis signal-regulating kinase 1 (ASK1) through the

formation of ROS and is mediated by the p38α MAPK pathway (202). Up-regulation of p38 MAPK pathway is also involved in the down regulation of glucose transporter-4 (GLUT4) in adipocytes (227). In human umbilical vein endothelial cells (HUVECs) MG triggers several pathways (200,228). A first pathway involves protein-tyrosine kinase (PTK) activation for tyrosine phosphorylation of several cellular proteins, leading to the activation of ERK. Another pathway triggered by MG is the induction of apoptosis. This signal transduction cascade is independent of PTK activity, but involves ROS induced activation of JNK, p38 kinase and c-Jun. Interestingly enough, glyoxal, which differs from MG only by a methyl group, is only capable of inducing the first signal, but unable to induce the second. This strongly accentuates the specificity of MG as a signaling molecule.

In retinal Müller cells and in microvascular endothelial cells from mouse kidney increased glycolysis leads to increased MG modification of the corepressor mSin3A. MG modification of mSin3A results in the recruitment of O-linked N-acetylglucosamine (O-GlcNAc) transferase to an mSin3A-SP3 complex, leading to the O-GlcNAc modification of Sp3. This modification results in a decreased DNA-binding capacity of the repressor complex, thus changing the gene expression pattern of the respective cells (229,230). The extent of this modification is a direct reflection of intracellular MG concentrations which is determined by a variety of cellular processes. This suggests a possible function for MG as an integrator to co-ordinately regulate distinct patterns of gene expression. In the mouse fibrosarcoma cell line L929 tumor necrosis factor (TNF)-induced necrosis is dependent on the accumulation of MG. The formation of specific MG-derived modified protein is observed and these steps are important in these signaling processes (61).

Chapter 3: RAGE, the receptor for advanced glycation endproducts

3.1 Introduction

One mechanism by which methylglyoxal-derived advanced glycation endproducts (MG-AGEs) exert their cellular function is through interaction with specific receptors. Various receptors and proteins that can bind to AGEs have been described, e.g. macrophage scavenger receptors type I and II (231-234), oligosaccharyl transferase-48 (AGE receptor 1; AGE-R1) (235,236), 80K-H phosphoprotein (AGE-R2) (235,236), galectin-3 (AGE-R3) (237), and lactoferrin and lysozyme (238). The one that has been studied the best, however, is the receptor for advanced glycation endproducts (RAGE) (239,240).

3.2 The receptor for AGEs

RAGE was first isolated from bovine lung extract by affinity purification using AGE-modified albumin (240). RAGE is a member of the immunoglobulin superfamily of cell surface receptors. It is composed of one V-type and two C-type immunoglobulin domains which are extracellular, a single-pass transmembrane spanning domain and a short highly charged intracellular domain (239,241). The principal ligand binding domain is the V domain (242), however, one ligand, namely S100A6 has been reported to interact specifically with the C2 domain (243), hereby emphasizing the complex nature of RAGE signaling. Furthermore, recent reports indicate that the V domain and the C1 domain do not function as independent domains, but as a single structural unit (244). The highly charged cytoplasmic tail is important for signal transduction (241).

At least three different major isoforms of RAGE exist, the full length RAGE ($_{FL}$RAGE), an isoform described as a dominant negative RAGE ($_{DN}$RAGE), lacking the intracellular domain, and a soluble RAGE ($_{S}$RAGE), lacking both the intracellular domain and the transmembrane domain (Figure 3.1). These isoforms are the result of alternative splicing from a single RAGE gene (245), although some isoforms may be generated by protein cleavage (246). Other splice variants and polymorphism have also been described (reviewed in (247)).

RAGE expression has been detected in all tissues and at a cellular level RAGE is found at the surface of most cell lines that have been examined (248). The correlation between mRNA levels, detected by Northern Blot, and protein levels seems small, indicating multiple levels of transcriptional, posttranscriptional and translational regulation. Of note is that RAGE expression is very low under physiological conditions, but under pathological

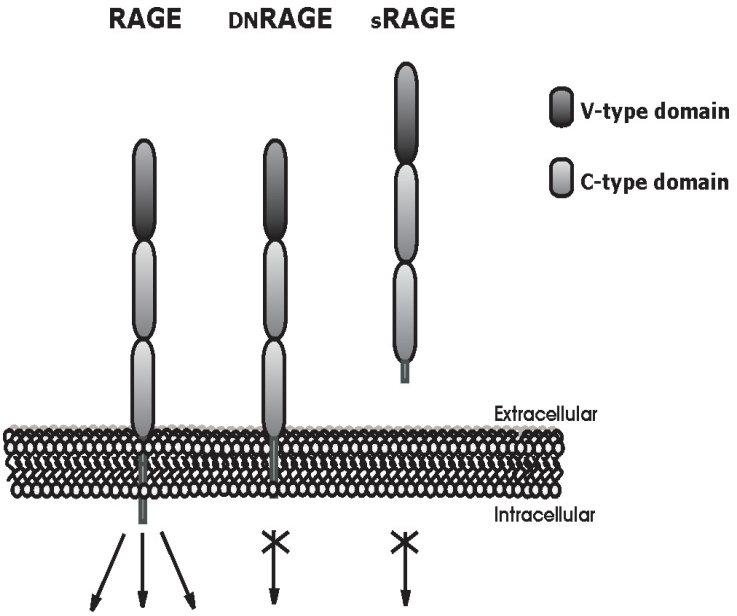

Figure 3.1. Major isoforms of RAGE.
Multiple isoforms of RAGE have been described. The three major isoforms are depicted and all contain an N-terminal V-type immunoglobulin domain and two C-type domains. sRAGE is not attached to the cell and has been hypothesized to attenuate RAGE signaling by capturing ligands prior to membrane interaction. DNRAGE does not posses a signaling function since it lacks the intracellular domain and therefore acts as a decoy receptor. sRAGE and DNRAGE could however exert a signaling function when involved in receptor complex formation. RAGE signaling is in part determined by the orchestrated effects of the different RAGE receptors. Adapted from (247).

conditions RAGE expression is strongly upregulated (249-252).

3.3 Signaling through RAGE

A striking feature of RAGE signaling is its multi-ligand character. These ligands include AGEs (242,253,254), amyloid-β peptide (Aβ) and β-sheet fibrils (255,256), several members of the S100/calgranulin family (257), amphoterin (often referred to as high mobility group box I (HMGB1) DNA-binding protein) (258,259), and Mac-1, a member of the β2-integrins (260). Multiple cellular signaling cascades have been shown to be activated by the binding of ligands to RAGE (Figure 3.2). The first signaling pathway to be described was the activation of $p21^{ras}$ through the induction of reactive oxygen species (ROS), leading to a cascade of mitogen-activated protein kinases (MAPKs) (261,262). This pathway has been reported in several cell types (263-266). The cytoplasmic tail of RAGE could function as a docking site for the extracellular signal-related kinase 1/2 (ERK1/2) MAPK (267). Other signaling pathways induced by the ligand binding of RAGE are other MAPK pathways, such as the p38 pathway and the stress-activated protein kinase/c-Jun N-terminal kinase (SAP/JNK) pathway (266,268-270), several JAK/STAT (Janus kinase/

signal transducer and activator of transcription) pathways (271-273), the cdc42 and rac pathway of rho-GTPases (241), and the phosphoinositol-3-kinase (PI3K) pathway (274). Most of these pathways lead to activation of NF-κB (nuclear factor-κB) (e.g. (241,261,263-266,270,275,276)). Of note is that RAGE signaling hereby provides a positive feedback loop since the RAGE gene contains two NF-κB responsive elements in its promoter (277,278).

The outcome of RAGE signaling is dependent on the cell type, the available ligands and their relative concentrations, the presence of additional splice variants and isoforms, the presence of RAGE polymorphisms and their different affinities for the available substrates, and the interplay between all of these different factors. An additional factor making RAGE signaling even more complex is the observation that some RAGE-mediated effects are independent of the cytoplasmic tail and only require the extracellular domain (284). This suggests engagement of RAGE in receptor complexes.

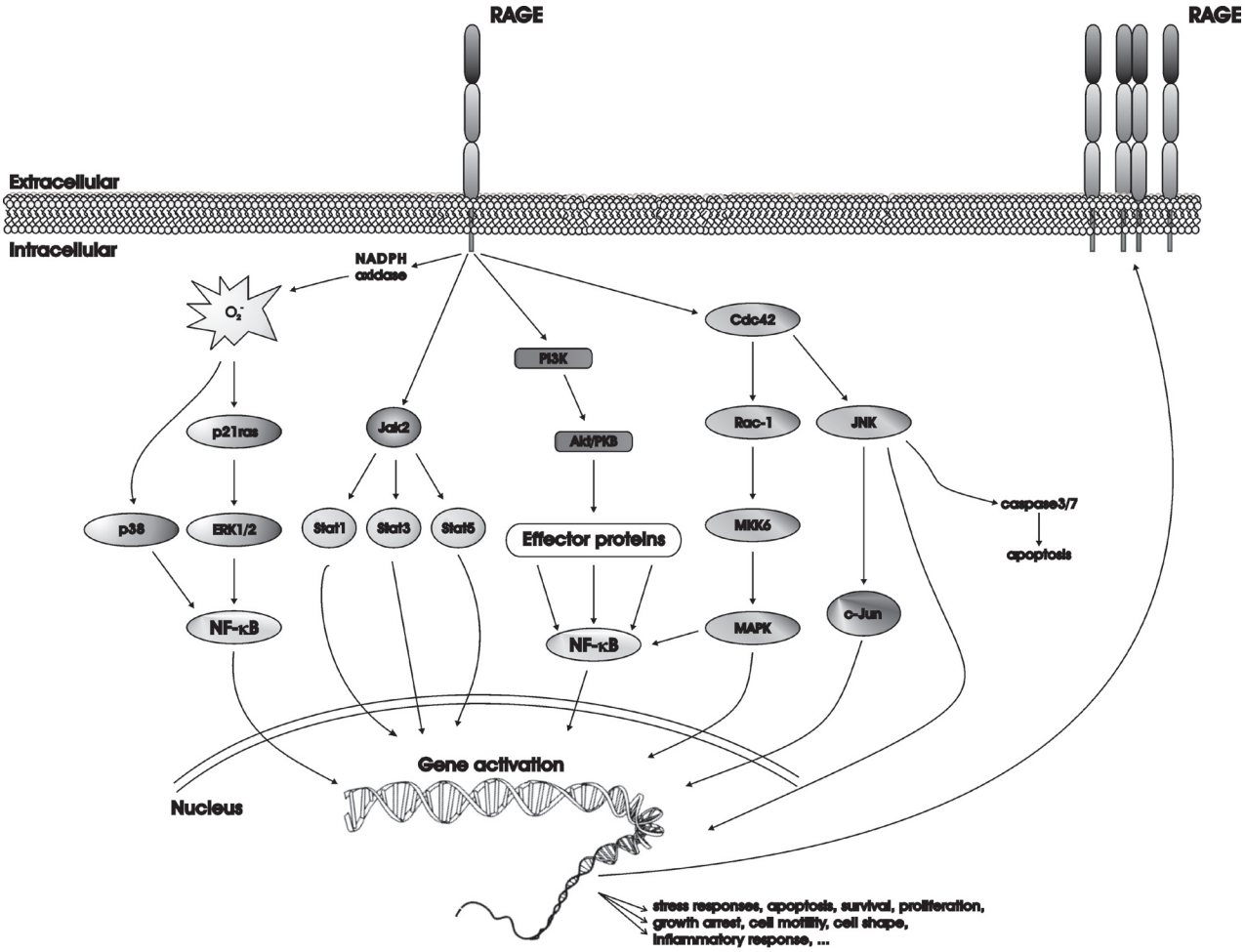

Figure 3.2. Schematic overview of signaling through RAGE.
Numerous signaling pathways can be initiated upon RAGE activation. Of note is the central role of NF-κB. Other transcription factors such as c-Jun are also involved in RAGE-mediated signaling. RAGE activation can also result in apoptosis, independent of gene-induction by activated transcription factors.
See text for more details (recently reviewed in (279-283)).

3.4 Involvement of RAGE in proliferation and survival

Engagement of RAGE by one of its ligands can give rise to the activation of numerous key cell signaling pathways (see also above). The outcome depends on several parameters (e.g. cell type, ligand, ligand concentration, …). Several of these signaling cascades are important in the regulation of proliferation and cell survival, two processes of which the molecular pathways are closely intertwined.

Although several reports implicate the engagement of RAGE in the induction of cell division, RAGE signaling is also involved in differentiation. Proliferation and differentiation are separate and mutually exclusive events that must occur sequentially and in that order. Sajital and co-workers provided evidence for a role of amphoterin/RAGE signaling in growth retardation and survival of neuroblastoma cells when differentiation was induced with retinoic acid. RAGE was shown to play a significant role in cell survival by inducing an increase of the anti-apoptotic protein Bcl-2 (285). In rat L6 myoblasts amphoterin/RAGE interaction also stimulates differentiation and inhibits proliferation through the activation of the p38 pathway (286,287). Amphoterin activates RAGE partly in an autocrine manner in order to exert its activity on myoblasts (288).

When compared to normal tissue, a strong reduction in RAGE mRNA and even more in RAGE protein has been observed in lung cancer cells (289,290). This suggests that RAGE exerts an inhibitory activity on lung cell proliferation and promotes differentiation. Indeed, when full-length RAGE is over-expressed, proliferation is impaired and cells are rendered less responsive to proliferative stimuli. Activity of the p44/p42 MAPK pathway is decreased upon RAGE overexpression and the cells start to differentiate. A more direct link between RAGE and cell cycle control was seen in mesangial cells. Binding of AGE to RAGE leads to the activation of STAT5, which induces the $p21^{waf}$ gene, a potent inhibitor of cyclin dependent kinases (CDKs), key regulators of cell cycle (271).

RAGE, however, has also been described to promote proliferation. Taguchi and colleagues noted amphoterin/RAGE interaction induces lung tumor proliferation through activation of p44/p42, p38 and SAP/JNK MAP kinase pathways (268). Furthermore, RAGE stimulation with S100B or AGEs could function as a mitogenic stimulus in neuronal cells. Ligand binding of RAGE leads to the activation of the p44/p42 MAPK pathway and results in the upregulation and activation of cyclin D_1/cdk4 and the transition into the S-phase (291).

Of special interest is the complex nature of the involvement of amphoterin/RAGE interaction in T cell activation driven by dendritic cells. In response to inflammatory stimuli, dendritic cells actively secrete amphoterin. This amphoterin is extensively acetylated and serves as an autocrine ligand for RAGE activation, leading to a well-characterized differentiation program, referred to as maturation, through activation of p38, ERK1/2 (p44/p42) and NF-κB. However, the secreted amphoterin also drives proliferation, survival and

polarization of naïve T cells through interaction with RAGE (292,293).

RAGE signaling has been shown to be involved in cell survival. Also here, RAGE exerts a very dualistic function, as it has been implicated in both the survival of cells as in the induction of cell death, sometimes even in the same cell type. Activation of RAGE by amphoterin can promote cell survival by inducing expression of Bcl-2 (B cell lymphoma-2) through activation of NF-κB (294). These effects, however, are seen when nanomolar concentrations of amphoterin are present. When, on the other hand, micromolar concentrations of amphoterin are present, apoptosis was induced. The latter outcome was dependent on the induction of ROS, which led to the activation of mitogen extracellular kinase (MEK) and consequent cytochrome c release from mitochondria and caspase-3 activation. Furthermore, when S100P activated RAGE in an autocrine manner, ERK mediated NF-kB activation leads to proliferation and survival (295,296). This dual role for RAGE is also observed in neuronal cells, where low concentrations of S100B protect cells against hypoxia or ischemia-induced apoptosis, whereas high concentrations of S100B exert a RAGE-mediated cytotoxic effect (249). On the other hand, AGE-modified collagen induces caspase-3, -8, and, to a lesser extent, -9 dependent apoptosis upon binding to RAGE (297,298).

Chapter 4: Growth factors

4.1 Introduction

Growth, proliferation and survival of cells are highly regulated processes and in multicellular organisms depend on the presence of extracellular stimuli (299). The availability of growth factors (GFs) seems decisive herein (300). These GFs control cell survival, either through the inhibition of the intrinsic apoptotic pathways or by actively promoting survival. Both mechanism are strongly intertwined and rely partly on the same signaling pathways. Also, it is clear that neither of the mechanisms is sufficient on its own, both are needed to maintain cell size and viability (301-307). Literature on this subject is vast. A large number of cytokines exist, the web of downstream signaling pathways is complex, and the outcome is largely dependent on the cell type, making knowledge on this matter fragmentary. Valuable tools in the study of GF signaling are hematopoietic cells. For their survival, these cell lines depend on well defined GFs, such as interleukin-3 (IL-3), -2 or -7. A central mediator of interleukin signaling is the phosphoinositol-3-kinase (PI3K) and Akt/protein kinase B (PKB) pathway (308-313). A simplified schematic overview of PI3K/Akt signaling is given in figure 4.1. Akt is a versatile kinase with numerous substrates and Akt signaling contributes to diverse physiological processes, including the regulation of apoptosis, the regulation of the cell cycle and the regulation of glucose metabolism.

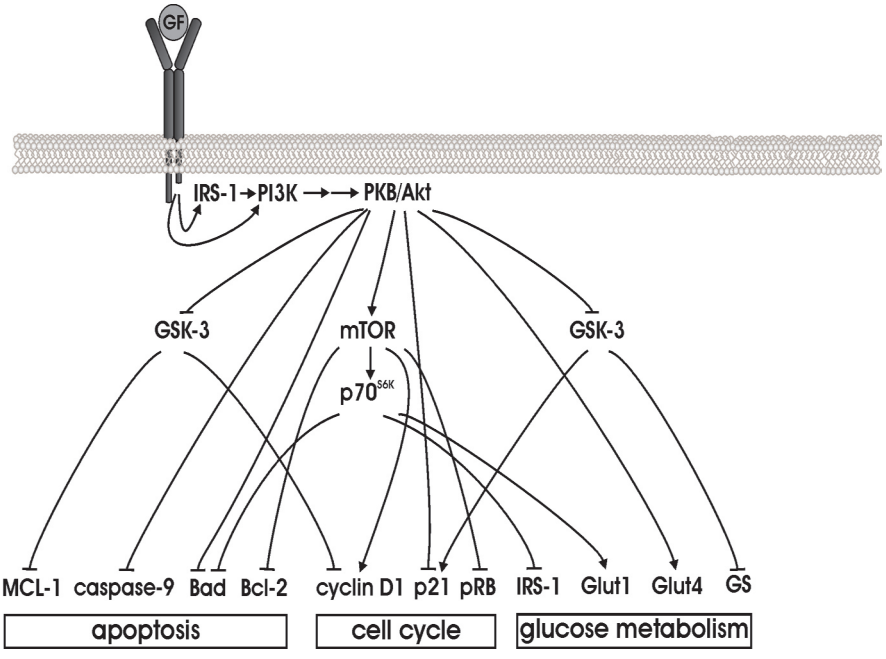

Figure 4.1. The PI3K/Akt pathway has a central role in growth factor signaling. Binding of GFs to their receptors leads to activation of PI3K, either directly or indirectly through scaffolding adaptors such as insulin receptor substrate-1 (IRS-1). These events ultimately lead to the activation of Akt. Subsequently numerous substrates can be phosphorylated by Akt. This phosphorylation can lead to either activation (arrows) or inactivation (blocking arrows). Two major downstream branches in Akt signaling are the mTOR pathway, which gets activated by Akt, and the GSK-3 pathway, which gets inactivated by Akt phosphorylation. Several downstream targets are shown, which are regulated directly by Akt or indirectly through the mTOR or GSK-3 branch. These targets illustrate how Akt signaling is involved in the regulation of different physiological processes, such as apoptosis, the cell cycle and glucose metabolism (see text for further discussion of these processes). Of note, both GSK-3 and mTOR signaling lead to inhibition of IRS-1, hereby negatively regulating Akt signaling. Recent reviews about PI3K/Akt signaling are (314,315), see text for more references.

4.2 Growth factors inhibit intrinsic apoptosis

In the absence of appropriate GFs cells, by default, undergo apoptosis (316) (Figure 4.2). Members of the BH3-only subfamily of the Bcl-2 family[1] are activated upon GF-withdrawal. Once activated, these BH3-only members promote apoptosis, most likely by antagonizing the prosurvival anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-x_L and Mcl-1 or by facilitating the activity of the pro-apoptotic Bcl-2 family members Bax and Bak (321,323-328). Bax and Bak subsequently participate in the permeabilization of the outer mitochondrial membrane, hereby releasing prodeath factors like cytochrome c, Smac, apoptosis-inducing factor (AIF), and endonuclease G. Release of cytochrome c then leads to the activation of caspase-9, which in turn activates caspase-3 (329-331). Numerous studies indicate that Bad is the BH3-only member involved in GF withdrawal-induced apoptosis (332-335). Recent reports, on the other hand, claim that, not Bad, but Puma or Bim are involved (336-339).

GF signaling can intersect with this default apoptosis at several levels. A critical step in the induction of apoptosis is the permeabilization of the outer mitochondrial membrane and the subsequent release of cytochrome c. An important manner in which GFs inhibit apoptosis is, therefore, upstream of this event, by directly hampering the action of the BH3-only proteins. Proteins like Bad, Bim and Puma are under tight transcriptional control and are regulated at the posttranslational level. The best studied example herein is Bad. GFs are able to induce multiple phosphorylations of Bad (309,342-346), hereby marking it for binding to 14-3-3 proteins (342,347-351) or blocking its interaction with Bcl-2 and Bcl-x_L (352-354) and inactivating its pro-apoptotic function. The same mechanism has been shown to be involved in the regulation of the activity of Bim (338). GFs also regulate the BH3-only proteins at the transcriptional level in part by inactivating the forkhead transcription factors (336-339,355-357). In addition, GFs counter apoptosis by inducing the expression of anti-apoptotic members of the Bcl-2 family, partly through the activation of NF-κB (358-366). It is interesting to note that GFs can also regulate this intrinsic apoptotic pathway downstream of cytochrome c release. This inhibition can be obtained in part by the direct phosphorylation of caspase-9 (367-369).

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[1] The control and regulation of mitochondria-related apoptotic events occurs through members of the Bcl-2 (B cell lymphoma 2) family of proteins. Based on the presence of conserved Bcl-2 homology (BH) domains, and their functions, three subfamilies can be described (310,311).

The anti-apoptotic Bcl-2 proteins, such as Bcl-2, Bcl-x_L and Mcl-1, contain all four BH-domains. The pro-apoptotic proteins can be separated into two sub-families. First there is the multidomain Bcl-2 family, including Bax and Bak. This family shares three BH-domains, but lacks the N-terminal BH4 domain (312,313). And finally there is the pro-apoptotic BH3-only subfamily, containing only the BH3 domain. This family includes e.g. Bid, Bad, Bim, Puma, Noxa (314-318).

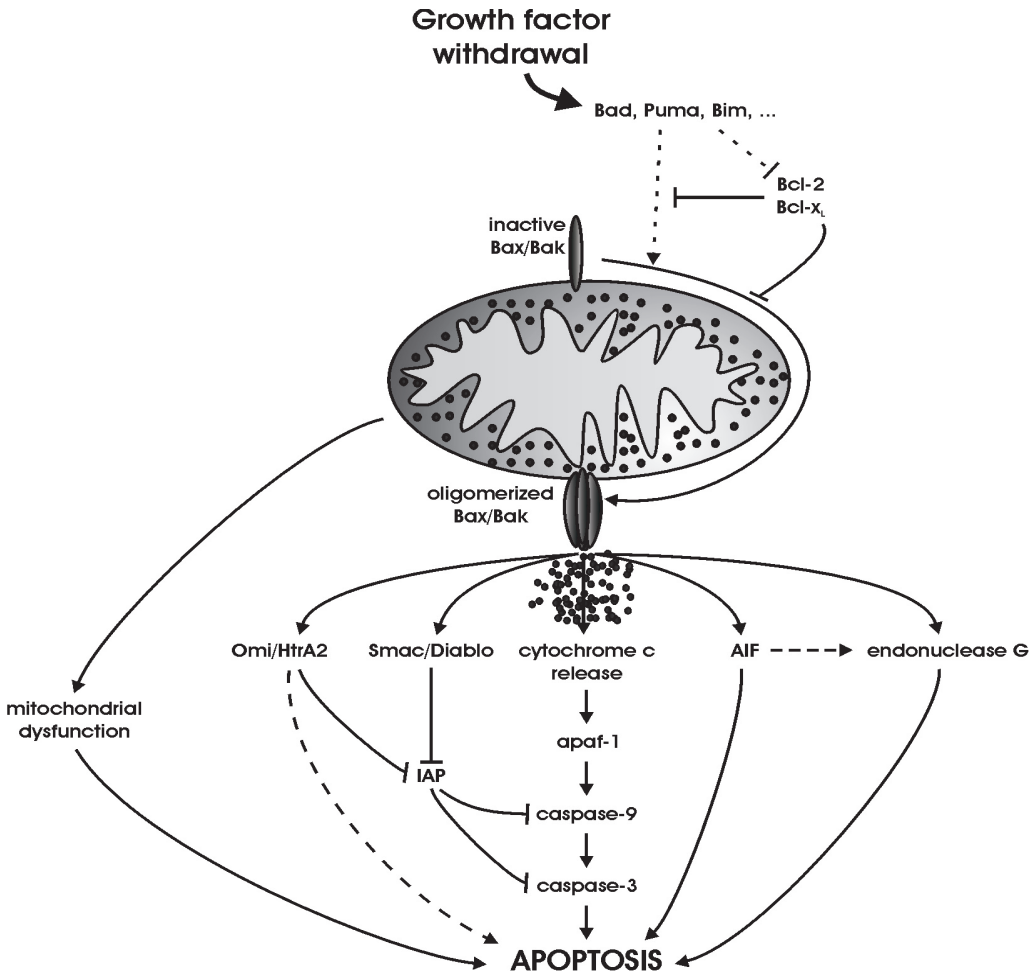

Figure 4.2. Growth Factor withdrawal leads to mitochondria-dependent apoptosis.
Upon the withdrawal of GFs Bax and Bak are activated by the BH3-only proteins (e.g. Bad, Bim, …), leading to the permeabilization of the outer mitochondrial membrane and the subsequent release of cytochrome c and other prodeath factors from the intermembrane space. The precise mechanism of Bax/Bak activation is still unclear. Either BH-3 only proteins bind to and neutralize anti-apoptotic Bcl-2 proteins, leading to the release of Bax and Bak, or BH3-only proteins can interact directly with Bax and Bak. The released cytochrome c, together with Apaf-1 and caspase-9, forms the apoptosome. Smac/Diablo and Omi/HtrA2 relieve the inhibition of caspases induced by IAP. Subsequently the executioner caspase-3 is activated, which results in apoptosis. AIF, endonuclease G, Omi/HtrA2 and the dysfunctioning of the mitochondria further contribute to cell death in a caspase-independent manner (for reviews see (328,340,341)).

4.3 Growth factors regulate metabolism

Although the most obvious way for GFs to regulate survival is by inhibiting apoptosis (see above), GFs are dependent on the presence of nutrients to exert their function (306,370,371). A key indication for this is the observation that even the presence of Bcl-2 or the deficiency of pro-apoptotic Bcl-2 family members fails to rescue cells from cell death upon GF withdrawal (301,372-376). Instead, in the absence of GFs, cells undergo progressive atrophy, ultimately leading to cell death (304,371,377). It is becoming clear

that metabolism is not merely driven by increasing metabolic demands, due to GF-driven proliferation. GFs directly regulate metabolism, in parallel to growth, proliferation and survival (378,379). A decrease or inhibition of glucose metabolism can lead to the initiation of cell death and, inversely, GF withdrawal-induced cell death is associated with a reduced glucose metabolism (380).

A first key step in the regulation of glucose metabolism is the uptake of glucose through facilitative glucose transporters, such as Glut1, the predominant glucose transporter in hematopoietic cells (Figure 4.3). A strong correlation is seen between the ability of GFs to maintain viability and the expression of glucose transporters (380,381). It has also been shown that overexpression of Glut1 can delay the onset of GF starvation-induced apoptosis in IL-3 dependent cells, although this Glut1 overexpression does not maintain long-term survival (306). An important mechanism by which GFs regulate glucose uptake is by promoting localization and retention of Glut1 on the cell surface (379,382). GF withdrawal leads to a rapid decline in membrane expression of Glut1, which can be prevented by activation of PKB/Akt signalling (303,305,306,371,372,383). In addition to the regulation of glucose uptake, GFs have been shown to regulate the membrane expression of several other nutrient transporters, like 4F2hc, an amino acid transporter, transferrin receptor, and low-density lipoprotein (LDL) receptor in a PKB/Akt and mTOR-dependent fashion (384-386).

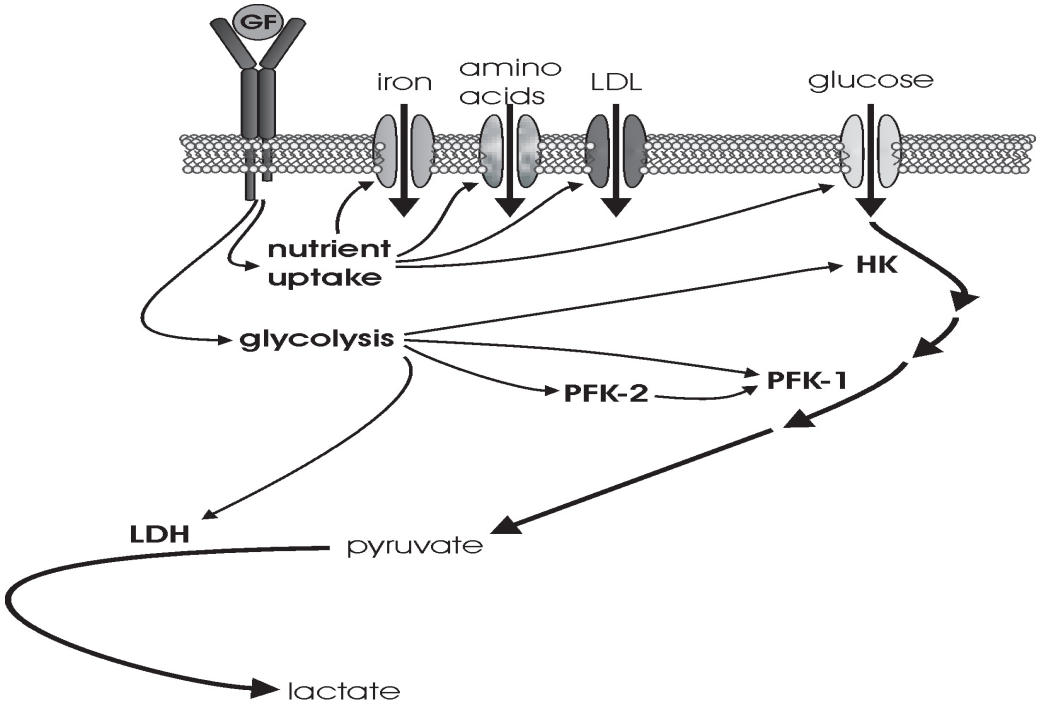

Figure 4.3. Schematic overview of the regulation of the metabolism by Growth Factors.
GLycolytic metabolism is regulated at several steps by GFs. Also the membrane localisation of numerous nutrient transporters is under the control of GFs. See text for more details (adapted from (378,387)).

Besides determining the uptake of glucose and metabolites, GFs strongly regulate glycolysis itself (Figure 4.3). Upon GF withdrawal, the flux of glucose through the glycolitic pathway declines and the production of L-lactate drops (303,306,388). Rate-determining steps in glycolysis are the steps catalyzed by hexokinase (HK) and phosphofructokinase 1 (PFK-1) and a decrease in reaction rate of either one of these enzymes results in a decrease in the overall rate of glycolysis. HK mediates the first committed step of glycolysis, the phosphorylation of glucose to glucose-6-phosphate. GF stimulation leads to an increase of HK expression levels and HK activity (306,389-392). Several reports have confirmed a general association between high levels of HK activity and cell survival (370,393,394). Furthermore, it has been described that overexpression of HK is able to attenuate apoptosis (305,370,395,396). The most important rate-limiting committed step in glycolysis is the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, catalyzed by PFK-1. Besides regulating the expression level (306,397), GFs also control the activity of PFK-2 (phosphofructokinase 2) (397-401). PFK-2 is activated by phosphorylation and this leads to an increase of fructose-2,6-bisphosphate, an important allosteric activator of PFK-1 (402). In addition, other enzymes involved in metabolism, such as lactate dehydrogenase (LDH) and triosephosphate isomerase, are regulated by GFs (389).

4.4 Growth factors maintain mitochondrial physiology

For a long time the maintenance of metabolism and the inhibition of apoptosis were considered to be independent pathways and were studied as such (306,309,331,352,353,370). A central point in the induction of apoptosis is the permeabilization of the outer membrane of the mitochondria, which leads to the release of cytochrome c and other pro-apoptotic proteins. A decrease in metabolism would lead to a perturbation of mitochondrial physiology, resulting in a subsequent rupture of the outer membrane (304,403,404). On the other hand, Bcl-x_L is capable of preventing apoptosis, even in the absence of glucose or GF stimulation (370,388), suggesting that the regulation of metabolism and induction of apoptosis can be dissociated. Several reports, however, point towards a role for HK in the regulation of mitochondria-dependent apoptosis. HKs are known to reside in mitochondria upon GF stimulation and to bind to the voltage-dependent anion channel (VDAC) (370,405). The VDAC is a β-barrel protein, spanning the outer mitochondrial membrane and has been implicated in the induction of apoptosis (406,407). Mitochondrial bound HK is able to attenuate the induction of apoptosis by antagonizing the activity of Bax and Bak (396,408,409). In liver mitochondria HK IV and Bad have been shown to reside in a holoenzyme complex (307). The phosphorylation status of Bad influences HK activity and HK activity is required in order to keep Bad phosphorylated. This mechanism provides strong evidence for the coordination of apoptosis and glycolysis and indicates an interdependence between pathways preventing apoptosis and pathways promoting survival.

4.5 Regulation of the cell cycle is under control of growth factors

Besides preventing apoptosis and stimulating metabolism (see above), GFs are known to exert another function, namely the induction of proliferation. Overexpression of Bcl-2 or Bcl-x_L can protect cells from GF withdrawal-induced apoptosis, but cells undergo growth arrest (404,410,411). Mammalian cells depend on GFs for the regulation of the transgression through the cell cycle (Figure 4.4). It has been known for a long time, however, that this dependency does not last throughout the entire cell cycle. Already during G_1 phase, several hours before the entry into S phase, the restriction point (R) is defined as the moment when a transition occurs from GF dependence to GF independence. After R, cells can complete their division cycle irrespective of the presence of mitogenic signals (412-414). Cell cycle progression relies on a highly regulated chain of signaling and transcriptional events, involving the sequential transcription and activation of cyclin dependent kinases (CDKs, reviewed in (415)). Important herein is the phosphorylation state of the retinoblastoma protein (pRb) (416). pRb binds to the E2F family of transcription factors (417,418) and to chromatin remodeling enzymes, such as histone deacetylase 1 and 2 (419,420), hereby inhibiting their activity and suppressing cell cycle progression. In early G_1 pRb is hypophosphorylated and exerts its inhibitory activity. In order to pass from G_1 to S phase the cyclin D-CDK4/6 complex partially phosphorylates pRb, causing the release of the E2F family of proteins. Many promoters of genes which are important for cell cycle progression, such as cyclins E (421) and A (422) are activated by E2F proteins. Subsequently pRb is further phosphorylated by the cyclin E-CDK2 complex, hereby intensifying the transcription of E2F-regulated genes, leading to G_1-S transition. Phosphorylation of pRb and the subsequent induction of cyclin E are often considered to be the molecular mechanism behind R, although this is contested by several reports (423-426).

GFs regulate cell cycle entry and G_1-S transition mainly by lowering the concentration of CDK inhibitors, consisting of the inhibitors of CDK4 (INK4) and the more broadly acting Cip/Kip families, and by regulating the levels and activity of cyclins and CDKs (415,427,428).

The withdrawal of GFs results in two distinguishable and sequential responses. The first response is a cell cycle arrest, which is followed by apoptosis. Moreover, not only do these processes differ kinetically and mechanistically, it has been shown that lower concentrations of GFs suffice to protect cells from apoptosis, but are not able to induce cell division (429). Of note is the observation that Bcl-2 family proteins have regulatory properties in cell cycle. Bcl-2 and Bcl-x_L are known to enhance G_0 arrest and significantly delay cell cycle progression, in part, by inducing $p27^{Kip1}$ expression (430-434). These effects on cell cycle can not be separated from the prosurvival functions and can be

countered by Bad and Bax (435-437). Recent reports that pro-apoptotic proteins like Bad and caspase-9 are regulated in a cell cycle-dependent manner underline how much cell cycle and apoptotic signaling are interlaced (367,368,438). By combining the regulation of these processes, GFs might provide protection against inappropriate proliferation.

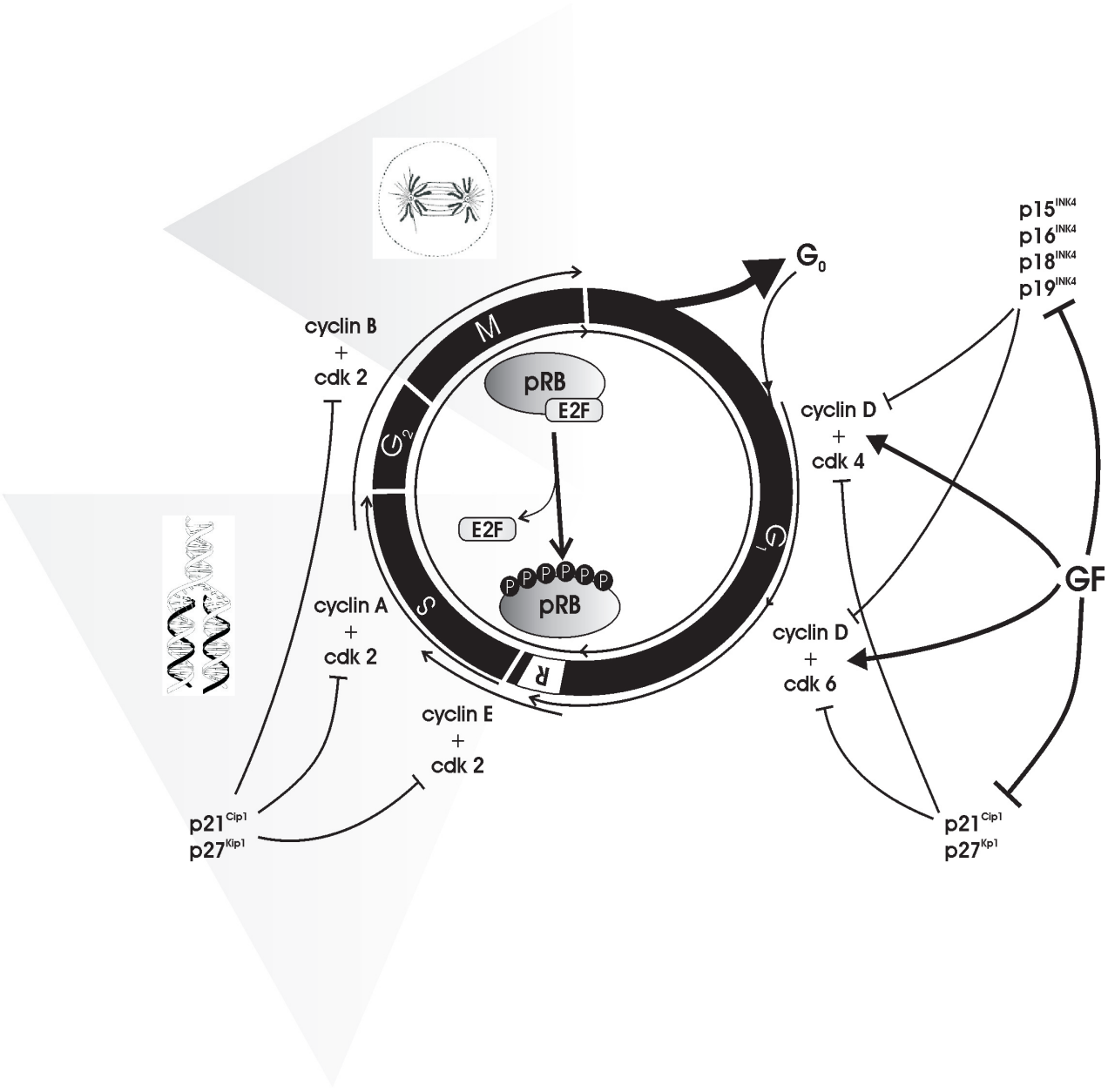

Figure 4.4. Schematic overview of the cell cycle.
Several phases can be distinguished in the cell cycle; G_1 (gap 1), S (DNA synthesis), G_2 (gap 2) and M (mitosis). Late in G_1, cells become independent of GFs to complete their division cycle. This point is referred to as the restriction point (R). Formation and activation of cyclin-cdk complexes determines the progression of the cell cycle and is strictly regulated throughout the different phases. The presence and activity of the respective complexes is indicated with arrows. A central role of the cyclin-cdk complexes is the phosphorylation of retinoblastoma protein (pRB), a general cell cycle inhibitor that prevents G_1-S transition by repressing E2F dependent transcription. The role of GFs is the regulation the levels and activity of G_1 cyclins and CDKs and lowering the levels of inhibitory proteins. Withdrawal of GFs renders cells incapable of passing R and results in G_1 arrest. See also text for more details.

Scope of the thesis

The requirement of growth factors (GFs) is of fundamental importance in physiological homeostasis. For unicellular organism growth regulation is mainly controlled by the availability of nutrients. However, for multicellular organism an additional regulatory mechanism is indispensable. Here nutrient availability is normally not limiting and thus does not suffice to control cellular growth and division in multicellular organisms. This additional regulatory mechanism is provided by the dependence on GFs. GFs exert their activity by regulating several fundamental processes, such as metabolism, proliferation and survival. Although these processes are regulated as independent branches of GF-induced signaling, extensive crosstalk is needed between them.

In this context we set out to study the involvement of the ubiquitous and highly conserved glyoxalase system and its physiological substrate methylglyoxal, an unavoidable by-product of glucose metabolism, in the regulation of cell death and cell cycle. Earlier findings in our group demonstrated that phosphorylated glyoxalase I and its substrate methylglyoxal are important in TNF-induced cell death in the fibrosarcoma cell line L929. However, the ubiquitous nature of the glyoxalase system and the inevitable presence of methylglyoxal under physiological conditions, suggest a more fundamental role. The **scope** of this study was to investigate **the potential involvement of phosphorylated glyoxalase I in the GF-withdrawal induced G_1 arrest and apoptosis and in the regulation of the cell cycle.**

Research in mammalian cells, as well as in prokaryotic and plant cells indicate a strong correlation between the rate of growth on the one hand and the expression level and/or activity of glyoxalase I on the other. However, a mechanistic link has never been found. We therefore investigated whether the phosphorylation of glyoxalase I might be involved in the regulation of the cell cycle. Two cellular model systems were used to study the posttranslational modifications and activity of glyoxalase I in detail. First Ba/F3 cells were depleted of IL-3 to induce G_1 arrest and subsequently apoptosis. GF starvation has been shown to result in a significant decline in glucose metabolism. This would result in a change in intracellular levels of methylglyoxal, making this an attractive model system to study the glyoxalase system and possible modulation of the detoxification of MG. Concurrently attention was given to the **formation of specific methylglyoxal-derived advanced glycation endproducts (MG-AGEs)** and we attempted to situate these events in progression of the IL-3 depletion induced signaling. Time course experiments were also performed on IL-3 dependent Ba/F3 cells going through the cell cycle synchronously. Finally, in vitro studies were commenced to further explore the pathway upstream of the **phosphorylation of glyoxalase I**.

A possible mechanism used by MG-AGEs to exert their influence on the cell cycle and/or apoptosis is through activation of the Receptor for Advanced Glycation Endproducts (RAGE). This multiligand receptor has been reported to be involved in numerous cellular processes, including proliferation and survival. A dual role has been imputed on RAGE in both processes. RAGE has been involved in proliferation as well as in differentiation, and in survival as well as in cell death. The **aim** of the second part of this thesis was thus dedicated to the study of **RAGE during GF-depletion induced G_1 arrest apoptosis, and during the cell cycle**.

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Part II:
Experimental Data and Results

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Chapter 6:

Phosphorylation of Glyoxalase I during growth factor depletion via a GSK-3β-dependent mechanism

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Paper in preparation

Chapter 6: Phosphorylation of glyoxalase I during growth factor depletion via a GSK-3β-dependent mechanism

Introduction

The main function of the glyoxalase system, comprising glyoxalase 1 and 2 (GLO1 and 2), is to detoxify reactive and cytotoxic -oxoaldehydes, mainly methylglyoxal (MG) (1-3). After its discovery in 1913, a function of the glyoxalase system in glycolysis was hypothized. However, this could not be proven (4) and, until today, the full biological function of the glyoxalase system has not been elucidated, but its ubiquitous nature suggests a fundamental and conserved role in life (4,5). In the 1960s and 70s the work of Szent-Gyorgyi indicated a regulatory role for GLO1 and its substrate MG in the control of cell division (for an overview see (1)), but a mechanistic link was never established. However, more and more recent evidence indicates that GLO1 may indeed play a role in the control of cellular growth. For instance, in plants it has been shown that there is a strong correlation between cell proliferation and the expression levels of GLO1, being the highest in strongly proliferating tissues (6). Also in yeast a strong correlation between growth status and GLO1 activity has been described (7). Along with an induction of cell proliferation, the plant growth hormone auxin induces the expression and activity of GLO1 (8). Gibberellin, a growth regulator in plants, induces phosphorylation of GLO1 (9), and calmodulin, a protein involved in cell growth, stimulates GLO1 activity (10).

Methylglyoxal is a cytotoxic by-product of normal metabolism. The main source is the enzymatic and nonenzymatic dephosphorylation from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, both intermediates of glycolysis (for reviews on MG see (1,3)). MG is very reactive and under physiological conditions MG reacts nonenzymaticaly with arginine residues mainly (11), but also with lysine and cysteine residues (12). Under normal conditions MG is detoxified by the GLO system to D-lactate using reduced glutathione (GSH) as a cofactor (13). Pathophysiological conditions can cause a rise of MG concentrations and Advanced Glycation Endproducts (AGEs) can be formed (for an overview see (14)). These AGEs are involved in the development of pathophysiological processes *in vivo*. Reports show that MG is able to inhibit growth. This growth inhibition shows a cell type selectivity and is dependent on divers mechanisms (15-22).

We have previously discovered a phosphorylated form of GLO1 and, in a tumor cell death model, we showed that the TNF-induced phosphorylation of GLO1 is required for cell death. Furthermore, phosphorylated GLO1 is not involved in the detoxification of MG, but instead mediates the cytotoxic effects of MG via a pathway that leads to MG-modification of specific target molecules (MG-derived AGE formation). However, the real biological function of phosphorylated GLO1 remains to be determined. (23,24).

Mammalian cells require stimuli from other cells in order to counter the intrinsic apoptosis machinery (25,26). This serves as a mechanism to ensure cells only survive

in the proper context. Cell death is inhibited partly by interfering directly with apoptosis. Anti-apoptotic proteins are up-regulated or activated (27-30) and pro-apoptotic proteins are down-regulated or inactivated (31-38). Another important mechanism by which growth factors may prevent apoptosis is by regulating basic cellular physiology by promoting nutrient uptake and cellular metabolism (30,39-42). A key regulator in apoptotic signaling is glycogen synthase kinase 3β (GSK-3β (see reviews (43,44)). Also in growth factor deprivation-induced apoptosis, GSK-3β has an important regulatory role (45-48).

In this report we show that depletion of IL-3 induces a phosphorylation of GLO1. This phosphorylation is partly mediated by GSK-3β as inhibition of GSK-3β also inhibits part of the phosphorylation of GLO1. This phosphorylation doesn't show an effect on the detoxification of MG by GLO1 and it occurs before the onset of apoptosis. Also we show that specific MG derived AGEs are formed, which also can be prevented by inhibition of GSK-3β.

Materials and Methods

Cell Lines and Cultures

The IL-3 dependent pro-B cell line Ba/F3 (49) was purchased from DSMZ. Ba/F3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen) supplemented with heat-inactivated FCS (10% v/v) (Cambrex), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and 5% conditioned medium from WEHI-3B cells as a source of mouse IL-3. Ba/F3 cells were cultured at 37°C in a humidified incubator under an 5% CO_2 atmosphere. HekT cells, NIH3T3 cells and L929 cells were cultured in DMEM with glutamax (Invitrogen) supplemented with heat-inactivated FCS (10% v/v), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) at 37°C in a humidified incubator under an 8% CO_2 atmosphere. The acute myeloid leukemia cell line HL-60 was a kind gift from J. Van Der Heyden. The cells were maintained in RPMI 1640 with L-glutamine (Invitrogen) supplemented with 10% heat-inactivated FBS and penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and were cultured at 37°C in a humidified incubator under an 5% CO_2 atmosphere.

Cell stimulations

IL-3 was removed by washing exponentially growing cells 3 times in culture medium without conditioned WEHI-3B medium and resuspended in culture medium without conditioned WEHI-3B medium in the absence or presence of indicated concentrations of methylglyoxal (Sigma), N-acetyl-L-cycteine (NAC; Sigma), LiCl (Fluka), SB216763 (Sigma), SB203580 (Sigma) or Z-VAD-FMK (Promega).

HL-60 cells were depleted of FBS by washing exponentially growing cells 3 times in culture medium without FBS and resuspended in FBS-free culture medium at a concentration of 1 X 10^6 cells/ml. NIH3T3 cells were depleted by washing them 3 times with culture medium without FBS and subsequently cultured in culture medium containing 0.5% (v/v) FBS.

DNA distribution analysis

Cells were stained with propidium iodide (PI, Sigma) containing staining solution (50) and analyzed by a FACSCalibur flow cytometer ($488_{Ex}/590_{Em}$). Cell Quest software was used to analyze cell cycle distribution (FACS Calibur, Becton Dickinson) (50,51). Ten thousand cells were routinely analyzed.

Electrophoresis and immunoblotting

The cells were washed 3 times with ice-cold PBS buffer and lysed with cytosol extraction buffer (10 mM Tris-HCL pH7.4, 50 mM EDTA pH8.0, 25 mM NaCl, 0,7% TritonX-100, 100 mM PMSF, 1 tablet Complete Protease Inhibitor Cocktail (Roche)/50 ml cytosol extraction buffer). Cell lysates were cleared by centrifugation (14,000 x g). Protein concentrations were determined using Bradford method. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the desired antibodies followed by ECL-based detection (Amersham Pharmacia Biotech).

2-Dimensional Gel Electrophoresis

Isoelectric focusing was carried out on 18 cm IPG strips, pH 4-7 (GE Healthcare) according to the manufacturer's instructions. For the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE, 12%).

Assay of glyoxalase1 activity

GLO1 activity was determined by using a spectrophotometric method, which monitors the initial rate of change in absorbance at 240 nm caused by the formation of *S*-D-lactoylglutathione (52). The standard assay mixture contained 2-mM MG and 2-mM GSH in a sodium phosphate buffer (50 mM, pH 6.6, 20°C). The reaction mixture was allowed to stand for 10 min at 37°C before the addition of the cytosolic protein fraction to ensure the equilibration of hemithioacetal formation.

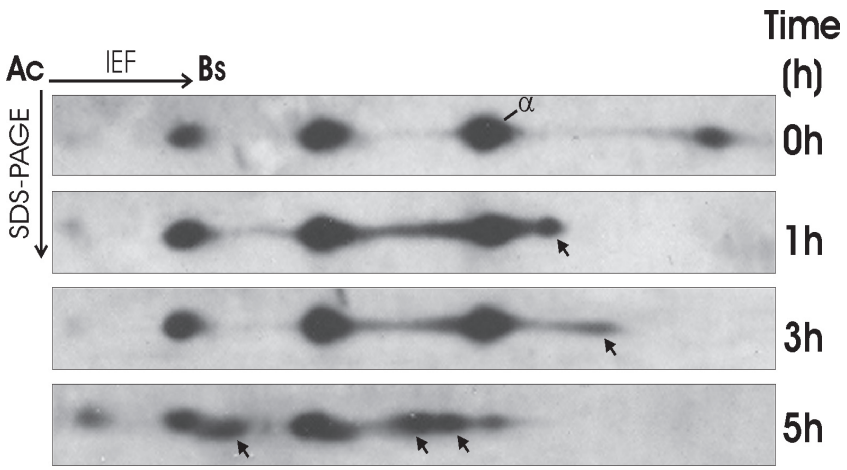

Figure 6.1. Time course of GLO1 phosphorylation in IL-3 depleted Ba/F3 cells.
Ba/F3 cells were depleted of IL-3 and lysed after indicated periods of time. Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with a polyclonal anti-human GLO1 antibody (6) are shown. A reference spot α, which corresponds to the non-NO-responsive form of GLO1 is indicated (6).

Measurement of D-Lactate

A fluorimetric method was used as previously described (53). Briefly, D-Lactate formation is measured by the formation of NADH during oxidation of D-Lactate by a D-lactate dehydrogenase.

Results

Glyoxalase I is phosphorylated upon IL-3 starvation.

We recently described that most cell types, analysed so far express multiple isoforms of glyoxalase1 (GLO1), including a NO-responsive form and a non-NO-responsive form, which we designated as the α-isoform (23). These isoforms can be separated by 2-dimensional gel electrophoresis (2-D) and detected by Western blotting using an anti-GLO1 antibody. We previously discovered phosphorylated GLO1 by means of cell labeling with ^{32}P-orthophosphate (24). Multiple phosphorylation of GLO1 can occur either on the NO-responsive form or the α-isoform and this can be monitored in 2-D gels by a shift of the isoform to the acidic side of the gel (23).

To investigate whether phosphorylation of GLO1 was related to growth factor signaling in mammalian systems, Ba/F3 cells, which are dependent on IL-3 for survival and proliferation, were depleted of IL-3 and cell lysates were prepared at the indicated time points cells. The samples were analysed by high-resolution 2-D in combination with

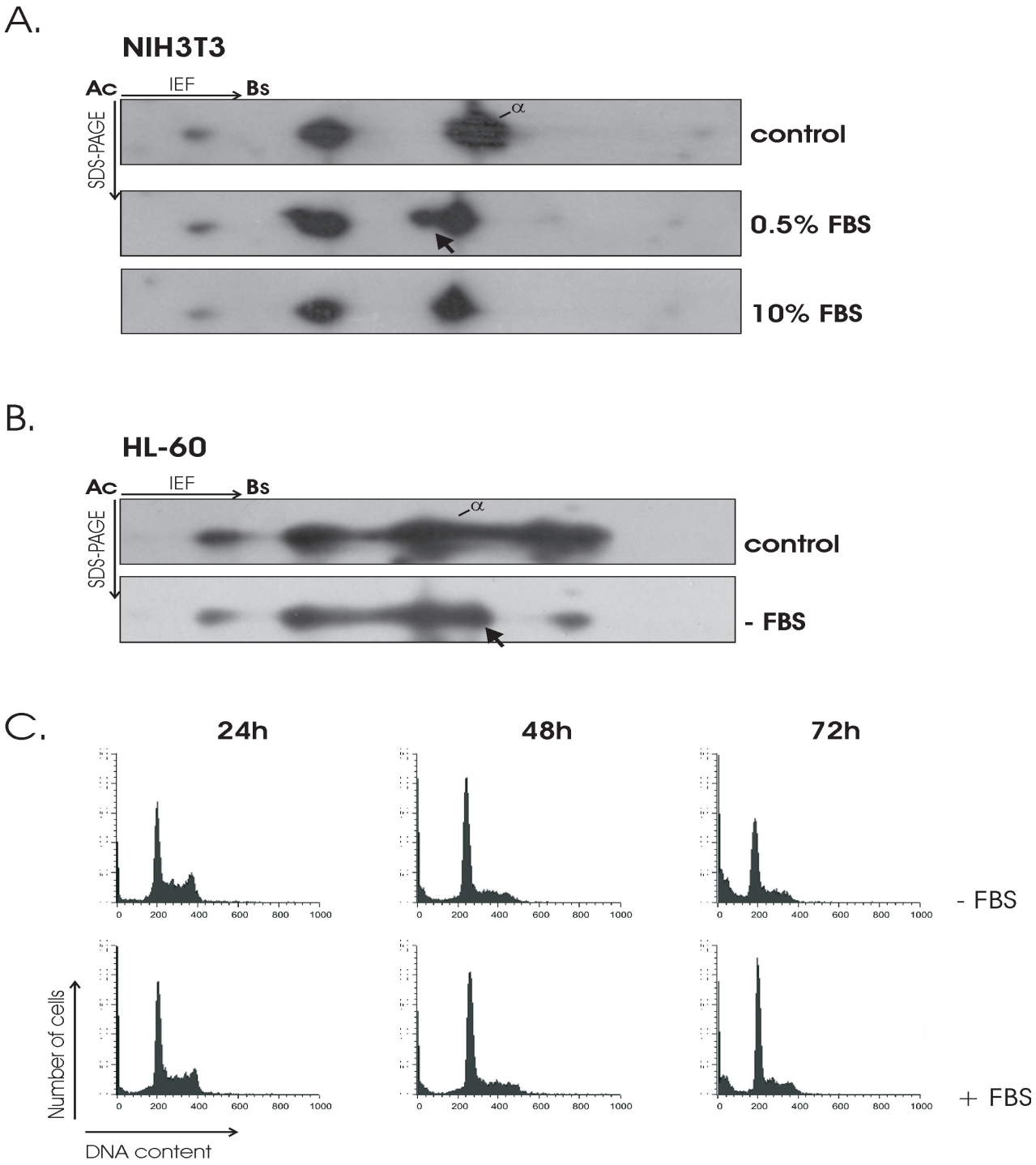

Figure 6.2. Phosphorylation of GLO1 in different cell lines.
A. Phosphorylation of GLO1 in NIH3T3 cells. NIH3T3 cells were washed in culture medium without FBS and cultured in the presence of 0.5 % FBS. Cells were lysed in CEB after 1 hour and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 400 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Representative western blots of 2-DE gels with a polyclonal anti-human GLO1 antibody are shown. **B.** Phosphorylation of GLO1 in HL-60 cells. HL-60 cells were depleted of FBS and lysed after 1 day. Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Representative western blots of 2-DE gels with a polyclonal anti-human GLO1 antibody are shown. **C.** Serum starvation-induced apoptosis in HL-60 cells. HL-60 cells were depleted of FBS. Apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA at the indicated time points.

Western blotting using a polyclonal antibody raised against GLO1 (Figure 6.1). As shown in the upper panel (0h), the isoform pattern of GLO1 in Ba/F3 cells is identical to the one we described previously for several other mouse and human cell types (23). The most basic isoforms corresponds to the NO-responsive form of GLO1 and the non NO-modified form of GLO1 is indicated by α. After one hour of IL-3 starvation, the most basic isoform has shifted to the acidic side, which is indicative for phosphorylation (spot indicated by an arrow). After three hours of IL-3 starvation, the relative amount of the NO-responsive form of GLO1 is reduced and had become partially dephosphorylated (shifted more to the basic side as compared to the 1h time point). After five hours IL-3 starvation, the NO-responsive form of GLO1 is completely disappeared and phosphorylation of GLO1 is induced on the non-No-responsive form (phosphorylated spots are indicated by an arrow). Note that the total amount of phoshorylated GLO1 at 5h of IL-3 starvation is relatively high as compared to the previous time points of IL-3 starvation. In several independent experiments, we also noticed that the time course and degree of phosphorylation of GLO1 induced by depletion of IL-3 could vary according to the experiment and could be induced either earlier or later as shown in Figure 6.1.

Growth Factor withdrawal induced phosphorylation of GLO1 is not exclusive for IL-3

To examine whether phosphorylation of GLO1 was specific for IL-3 depletion or whether it was a more general phenomenon, the phosphorylation of GLO1 upon GF depletion was also examined in two other cell lines.

Mouse fibroblasts are commonly used in the study of serum dependence. After serum withdrawal mouse fibroblasts reversibly exit the cell cycle (54-57) and after a sustained absence of GFs they undergo apoptosis (58,59). The mouse fibroblast cell line NIH3T3 was serum-starved as described in M&M. As shown in Figure 6.2A immunoblots of lysates of cells that were maintained in culture medium with 0.5 % FBS display GLO1 is phosphorylated on the non NO-modified form of GLO1. Immunoblots of lysates from cells that were kept in culture medium with 10 % FBS show that this spot was absent. This phosphorylation occurs after 1 hour of starvation, well before the onset of apoptosis.

Another cell line tested was the human leukemia cell line HL-60. HL-60 is a hematopoietic cell line, like Ba/F3 cells, albeit of the myeloid lineage, whereas Ba/F3 cells are lymphoid cells. It has been reported that HL-60 cells are sensitive to exogenous MG, making HL-60 very interesting to study possible effects on the GLO system. Addition of MG will cause G_1 growth arrest and induce apoptosis in HL-60 cells (19). Upon serum depletion HL-60 also arrest growth and induce apoptosis (60,61). Analysis of immunoblots detected with an anti-GLO1 antibody shows the induction of phosphorylation of GLO1 upon serum starvation (Figure 6.2.B). When compared to the control situation the spot second to the right disappeared and a more acidic new spot appears. Although lysates were made after

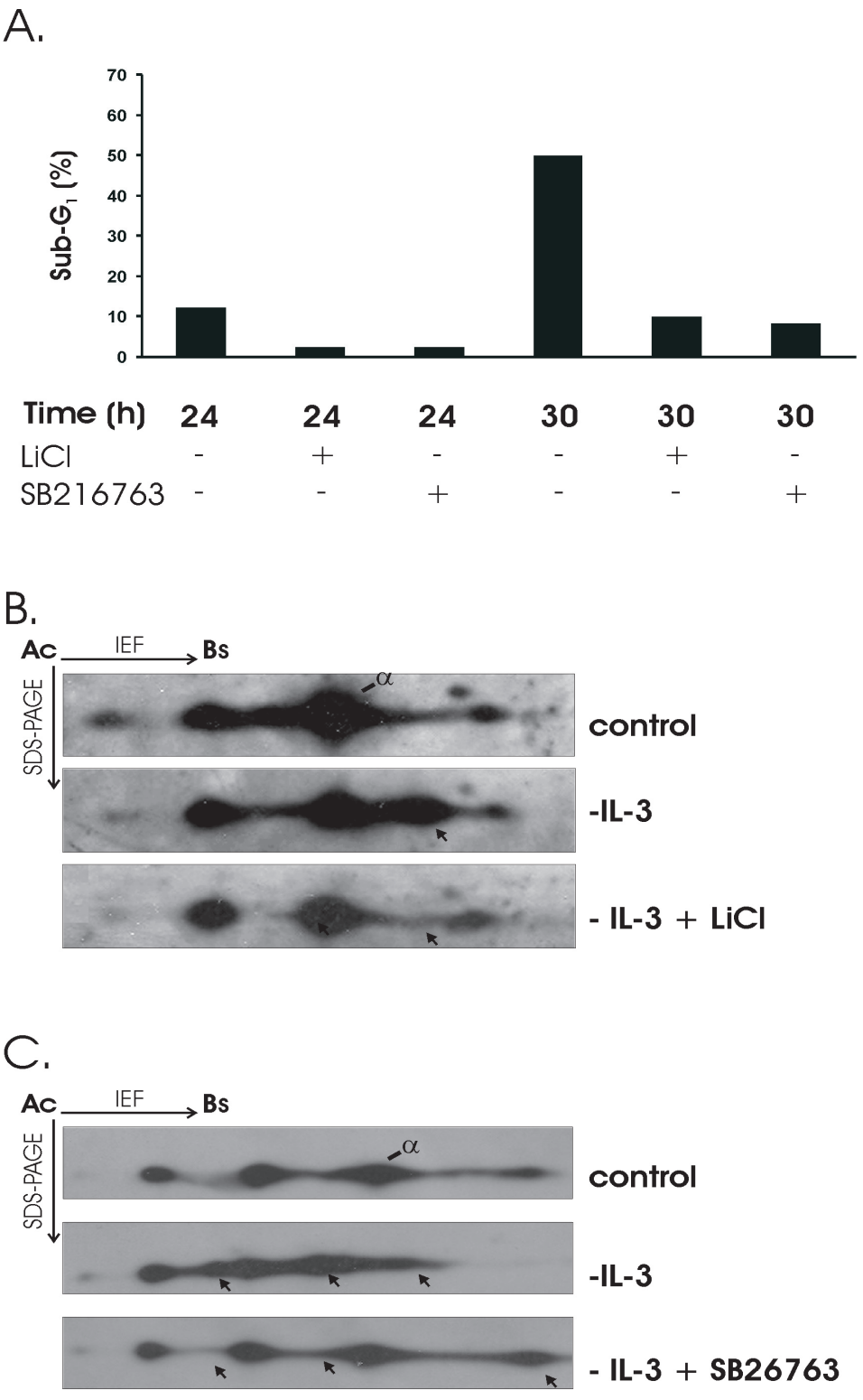

Figure 6.3. Inhibition of GSK-3 interferes with phosphorylation of GLO1.
A. Effect of GSK-3 inhibition on IL-3 depletion-induced apoptosis. Ba/F3 cells were depleted of IL-3 in the presence or absence of the indicated inhibitor. Apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA at the indicated time points. **B and C.** GLO1 phosphorylation in IL-3 depleted Ba/F3 cells in the presence of GSK-3 inhibitors. Ba/F3 cells were depleted of IL-3 and cultured in the presence or absence of LiCl **(A)** or SB216763 **(B)**. Cells were lysed in CEB after 3 hours and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels with a polyclonal anti-human GLO1 antibody are shown. Concentrations used are LiCl: 20 mM and SB216763: 20 µM.

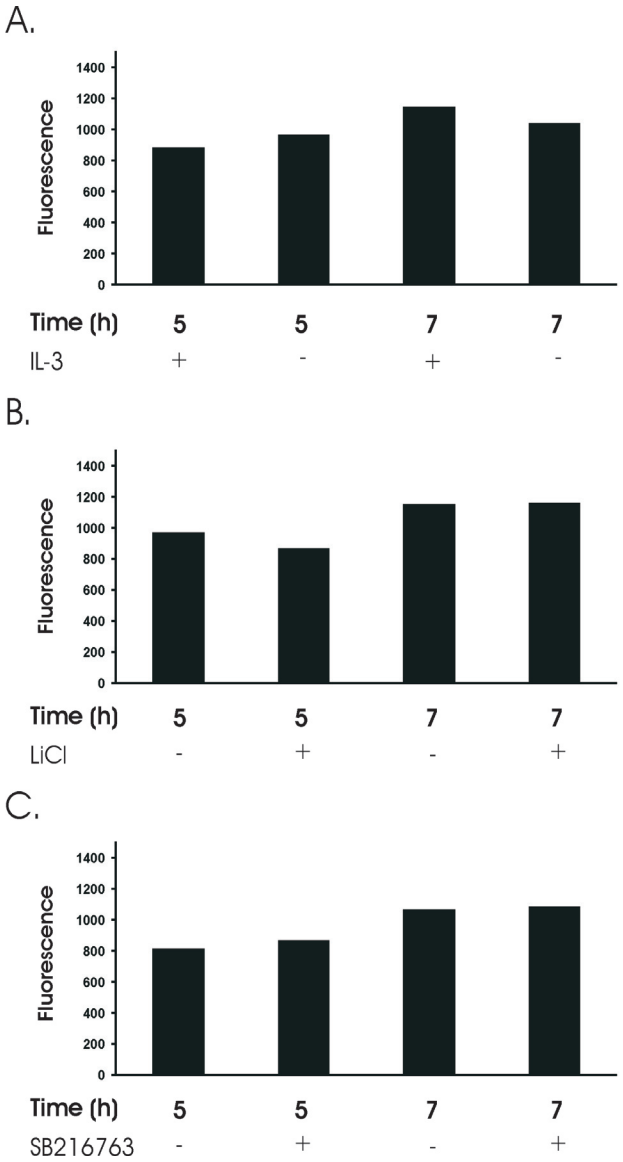

Figure 6.4. Phosphorylation of GLO1 does not affect the formation of D-lactate.
A. A time course of D-lactate formation in Ba/F3 cells. Ba/F3 cells were washed in culture medium without IL-3 and subsequently cultured in the presence or absence of IL-3. At indicated time points the presence of D-lactate in the medium was measured by detection of the formation of NADH upon incubation with D-lactate dehydrogenase. **B and C.** D-lactate production in IL-3 depleted Ba/F3 cells in the presence of GSK-3 inhibitors. Ba/F3 cells were depleted of IL-3 and cultured in the presence or absence of LiCl **(B)** or SB216763 **(C)**. The presence of D-lactate in the medium was measured after indicated time intervals. Concentrations used are LiCl: 20 mM and SB216763: 20 µM.

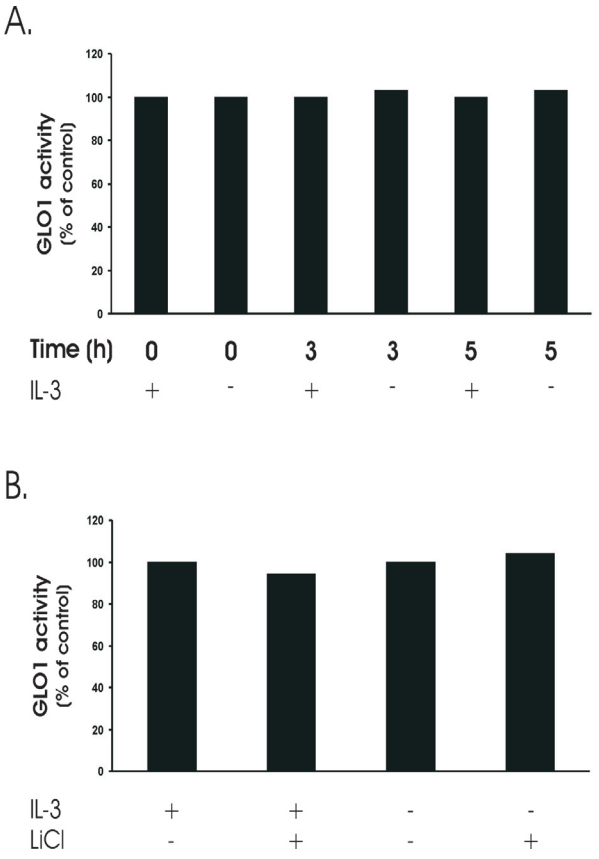

Figure 6.5. *In vitro* GLO1 activity is not altered by phosphorylation.
A. A time course of GLO1 activity in IL-3 starved Ba/F3 cells. Ba/F3 cells were washed in culture medium without IL-3 and subsequently cultured in the presence or absence of IL-3. At indicated time points cells were lysed in CEB. Lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. **B.** GLO1 activity in IL-3 depleted Ba/F3 cells in the presence of GSK-3 inhibitors. Ba/F3 cells were washed in culture medium without IL-3 and subsequently cultured in the presence or absence of IL-3 and LiCl was added. At indicated time points cells were lysed in CEB. Lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. Reaction rates were determined using Ultraspec 1100 software (Amersham, BD Biosciences) and are shown relative to the reaction rate of lysates made of exponentially growing cells, which are set as 100%. LiCl was added in a concentration of 20 mM.

24 hours of serum starvation, phosphorylation is still induced before the onset of apoptosis, which is only observed after 72 hours of serum starvation (Figure 6.2.C).

In conclusion, the data presented here show that the phosphorylation of GLO1 upon GF withdrawal is not restricted to Ba/F3 cells, but can also occur in mouse fibroblasts and

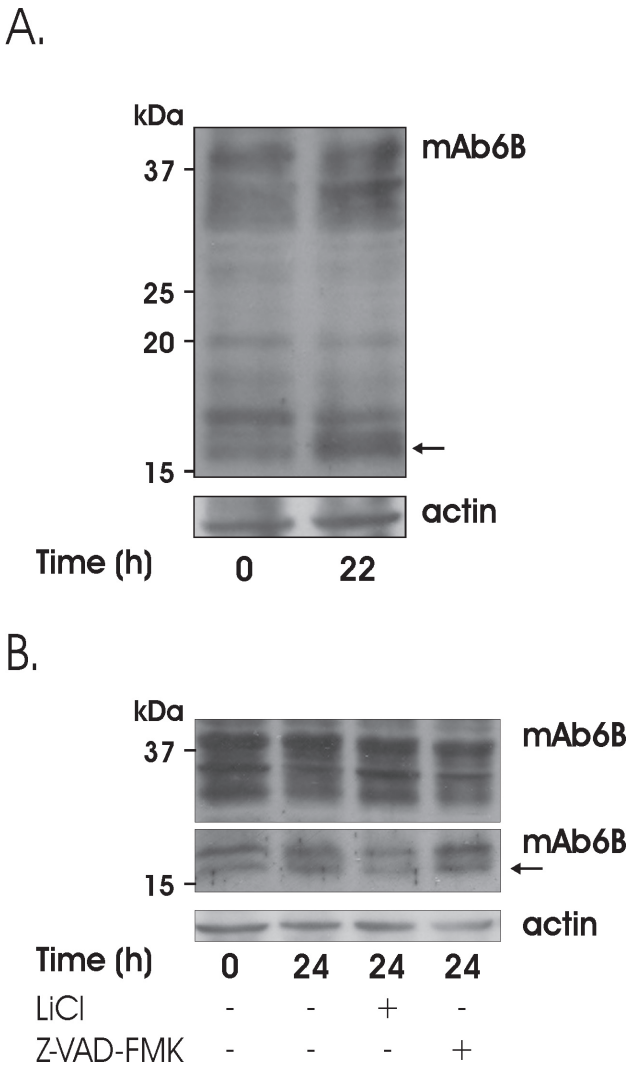

Figure 6.6. IL-3 depletion induces the formation of specific MG-derived AGEs in Ba/F3 cells.
A. Formation of specific MG-AGEs. Ba/F3 cells were depleted of IL-3 and lysed in CEB after indicated periods of time. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Formation of specific MG-AGEs was analysed using the mAB6B antibody (Biologo). As a loading control, blots were subsequently stripped and reprobed with a β-actin antibody (Abcam). **B.** Inhibition of MG-AGE formation. Ba/F3 cells were depleted of IL-3, cultured in the presence of LiCl or Z-VAD-FMK and lysed in CEB after indicated periods of time. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Formation of specific MG-AGEs was analysed using the mAB6B antibody. As a loading control, blots were subsequently stripped and reprobed with a β-actin antibody.

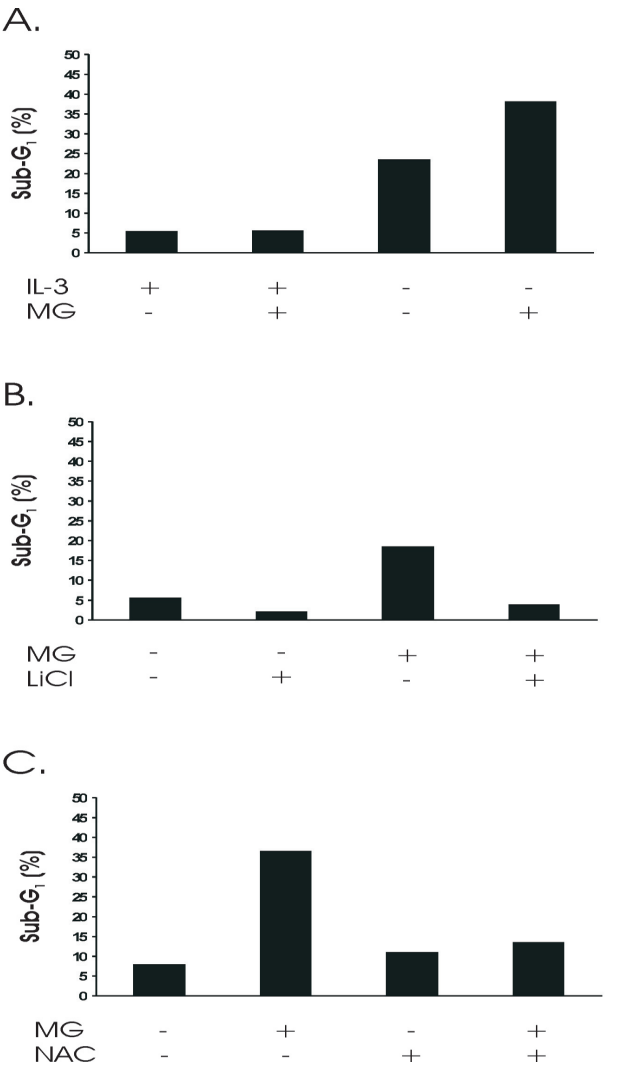

Figure 6.7. MG is synergistic with IL-3 depletion-induced apoptosis.
A. Cell death with exogenous MG. Ba/F3 cells were washed in culture medium without IL-3 and subsequently cultured in the presence or absence of IL-3 and MG was added. After 24 hours apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA. **B. and C.** Inhibition of cell death. Ba/F3 cells were depleted of IL-3 and cultured in the presence of MG and LiCl **(B)** or NAC **(C)**. After 24 hours apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA. Concentrations used are LiCl: 20 mM, NAC: 20 mM, MG: 500 µM.

human leukemic cells upon GF withdrawal. It may therefore represent a more general mechanism. This hypothesis is strengthened by the observation that GF withdrawal-induced phosphorylation takes place in several species, mouse and human, and is not confined to one cell type.

GSK-3 is involved in IL-3 starvation-induced post-translational modification of GLO1.

GSK-3 has been shown to have a central role in the regulation of cell cycle and apoptosis (43,44). Also, upon growth factor withdrawal, GSK-3 is involved in the induction of G_1 arrest by regulating the levels of cyclin D1 (62,63), and in the induction of apoptosis and inhibition of GSK-3 activity also ameliorates the growth factor-withdrawal-induced cell death (45-48,64). To investigate whether GSK-3 was involved in the phosphorylation of GLO1 upon IL-3 depletion of Ba/F3 cells, we used LiCl, a well known inhibitor of GSK-3 (65). Because it has been previously shown that activation of GSK-3β is required for the induction of apoptosis by GF-depletion, we first tested in our experimental setting whether inhibition of GSK-3β by LiCl also inhibited apoptosis in Ba/F3 induced by depletion of IL-3. Ba/F3 cells were cultured with or without conditioned WEHI-3B medium as a source of IL-3, and in the presence or absence of LiCl. As expected, LiCl inhibited apoptosis by approximately 80% induced by IL-3 depletion (Figure 6.3.A). Next, we examined whether LiCl had an effect on the phosphorylation of GLO1 induced by IL-3 depletion from Ba/F3 cells. As shown in Figure 6.3.B, the phosphorylation of GLO1 (indicated by an arrow) was completely prevented in the presence of LiCl. In addition, the phosphorylation of GLO1 induced by depletion of IL-3 was also completely prevented in the presence of a more specific inhibitor of GSK-3β namely SB216763 (Figure 6.3.C). SB216763 also inhibited apoptosis induced by IL-3 starvation to the same extent as LiCl (Figure 6.3.A). These results indicate that GSK-3β is involved in the pathway that leads to phosphorylation of GLO1 induced by IL-3 starvation. It is however unlikely that GLO1 is a direct substrate for GSK-3β, because coexpression of the catalytic subunit of GSK-3β with GLO1 in serum-starved HEK293 cells did not result in phosphorylation of GLO1 as studied by means of labelling cells with ^{32}P-orthophosphate. Nor could GSK-3β phosphorylate GLO1 in vitro (see chapter 7). By contrast, coexpression of the catalytic subunit of CaMKII with GLO1 in HEK293 did result in phosphorylation of GLO1 (de Hemptinne and Vancompernolle, unpublished results) and GLO1 is also an in vitro substrate for CaMKII (see chapter 7).

Methylglyoxal detoxification is not impaired after IL-3 depletion

Given the cytostatic and cytotoxic properties of MG, a likely hypothesis could be that upon IL-3 depletion, the MG-detoxification capacity of the GLO system could be inhibited by for instance phosphorylation of GLO1. This would cause a rise in the intracellular MG concentration, a condition that could induce growth arrest and if the absence of IL-3 persists, ultimately apoptosis. To investigate whether the MG-detoxification capacity of the glyoxalase system was impaired upon IL-3 depletion, we measured GLO1 activities as well as D-lactate concentrations, the end product of the glyoxalase system. The formation of D-lactate was measured by a fluorimetric enzyme assay as described in (53). The

concentration of D-lactate was determined by the formation of NADH during oxidation of D-Lactate by D-lactate dehydrogenase. As shown in Figure 6.4.A, there is no significant difference in formation of D-lactate between IL-3 starved cells and control cells. GLO1 activity in cell lysates derived from Ba/F3 cells in the presence or absence of IL-3 was determined by a spectrophotometric method, which monitors the initial rate of the GLO1-catalyzed production of S-D-lactoylglutathione from the hemithioacetal that is formed spontaneously by incubating MG in glutathione (see M&M). As shown in Figure 6.5.A, in several separate experiments there was no significant change in GLO1 activity in cell lysates from IL-3-depleted cells compare to control cells. These findings indicate that the MG-detoxification activity of the glyoxalase system is not altered upon GF withdrawal. These data also suggest that phosphorylation of GLO1, which is induced upon GF withdrawal, is not accompanied by changes in the GLO1 activity, which is in agreement with our previous findings in L929 cells (24). This finding is further strengthened by the observation that inhibition of GLO1 phosphorylation with LiCl or SB216763 upon IL-3 depletion, did not result in a change of the D-lactate production or the *in vitro* GLO1 activity (Figure 6.4.B and 6.4.C, and Figure 6.5.B respectively).

Specific MG-derived AGEs are formed during IL-3 withdrawal.

MG has been reported to be involved in several signaling pathways (24,66-69). Recent findings indicate MG has a distinct role as a modulator of cell death (24,70-72). Previous results indicated that phosphorylated GLO1 was involved, directly or indirectly, in a pathway that leads to MG-derived AGE modification of specific proteins. Therefore, we tested whether MG-derived AGE modification was also induced upon GF withdrawal. For this purpose, we used a monoclonal antibody (mAb6B) raised against *in vitro* MG-modified keyhole limpet hemocyanin. This antibody recognizes MG-modified proteins in arterial walls of diabetic kidneys (73) and we have previously shown that this antibody recognizes specific MG-derived AGE epitopes induced during TNF-induced cell death in L929 cells (24). A Western blot of cell lysates derived from IL-3 depleted Ba/F3 cells developed with this antibody is shown in Figure 6.6.A. MG-derived AGE modification is induced on a distinct protein band, with an apparent M.W. 16 kDa (indicated by an arrow), after 22h of IL-3 depletion. The MG-derived AGE formation was already observed after eight hours of IL-3 starvation (data not shown).

Next, we investigated whether phosphorylated GLO1 was involved in the induction of MG-derived AGE-modification of the 16 kDa protein. As already shown in Figure 6.3, inhibition of GSK-3 by LiCl efficiently inhibited the phosphorylation of GLO1, therefore we used LiCl to test whether inhibition of GSK-3 also influenced the MG-derived AGE-formation. As shown in Figure 6.6.B, MG-derived AGE-modification of the 16 kDa protein (indicated by an arrow) was significantly reduced in the presence of LiCl.

Since GF-withdrawal also induces caspase activation (74), we also tested the

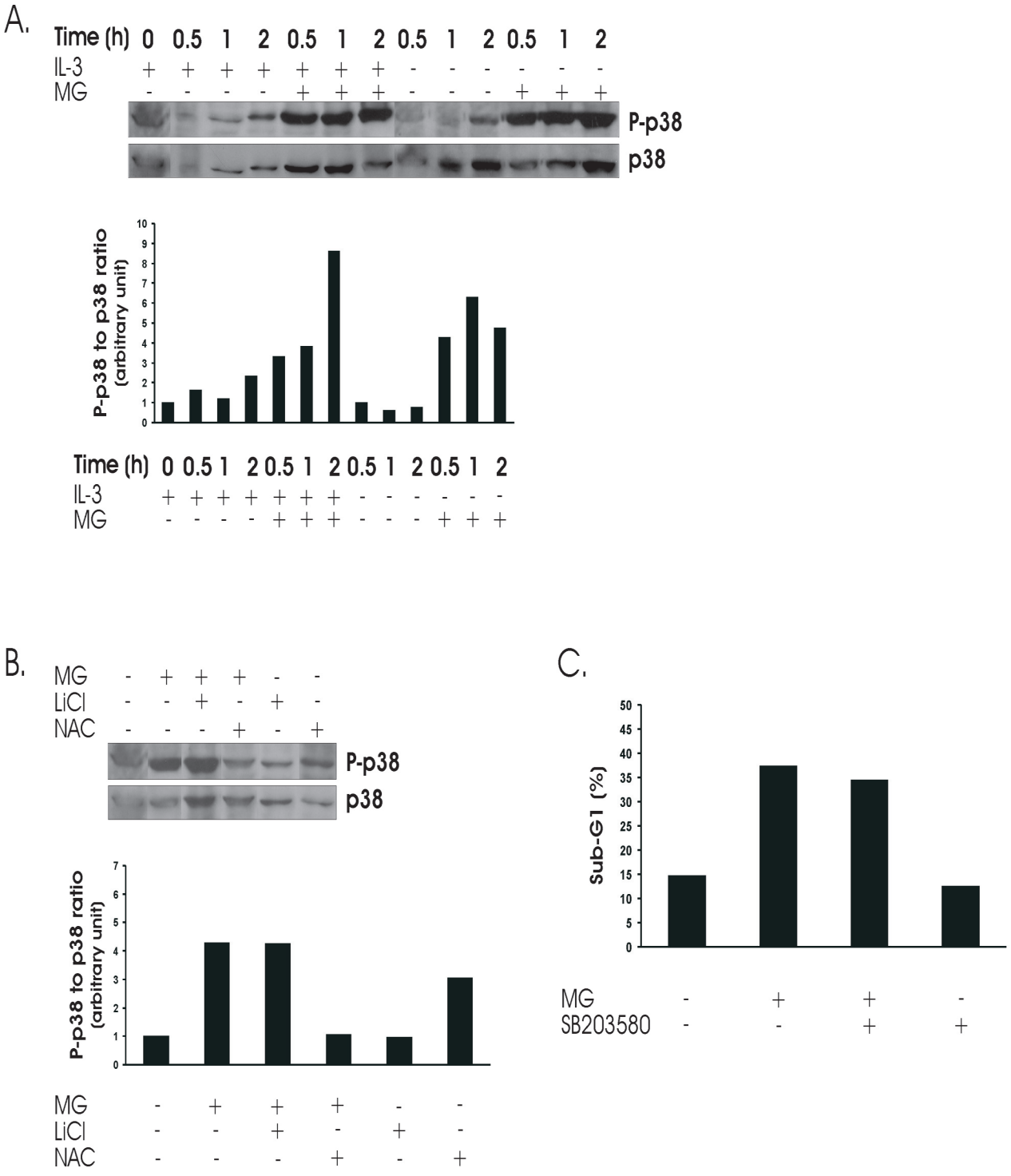

Figure 6.8. Activation of p38 by exogenous MG.
A. Activation of p38. Ba/F3 cells were washed in culture medium without IL-3 and subsequently cultured in the presence or absence of IL-3 and MG was added. Cells were lysed in CEB after indicated periods of time. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Activation of p38 was determined using an antibody directed against phosphor-p38 (Cell Signaling). Subsequently blots were stripped and reprobed with an anti-p38 antibody (Cell Signaling). **B.** Inhibition of p38 activation. Ba/F3 cells were depleted of IL-3 and cultured in the presence of MG and LiCl or NAC. Cells were lysed in CEB after indicated periods of time. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Activation of p38 was determined using an antibody directed against phosphor-p38. Subsequently blots were stripped and reprobed with an anti-p38 antibody. **C.** MG-dependent cell death. Ba/F3 cells were depleted of IL-3 and MG and SB203580 were added. After 24 hours apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA. Concentrations used are LiCl: 20 mM, NAC: 20 mM, MG: 500 µM and SB203580: 10 µM. Protein band intensities were measured using Genetools software (Westburg) and phosphor-p38 to p38 ratios were determined.

possibility whether the 16 kDa MG-AGE-modified protein was formed by caspase-cleavage of a larger pre-formed MG-AGE-modified protein. As shown in Figure 6.6.B, the 16 kDa MG-AGE-modified protein was still formed in the presence of the pan-caspase inhibitor Z-VAD-FMK.

Exogenous MG reinforces the induction of apoptosis after IL-3 withdrawal

To further examine whether MG played a role in the induction of apoptosis in Ba/F3 cells induced by IL-3 depletion, we tested the effect of exogenously added MG. The percentage of apoptotic cells was measured by flow cytometry using the percentage of cells that had a hypoploid DNA profile (expressed as sub-G_1). As shown in Figure 6.7.A, in the presence of 0.5 mM exogenously added MG and in the absence of IL-3, the percentage of apoptotic cells was increased by approximately 55 % compared to the percentage of apoptotic cells induced by IL-3 depletion alone. As a control, addition of 0.5 mM MG did not induce apoptosis in a Ba/F3 cell culture in the presence of IL-3. In the presence of IL-3, exogenous addition of 0.5 mM MG could also not induce formation of the specific 16 kDa MG-AGE-modified protein (data not shown).

We next sought to determine whether GSK-3β was involved in this synergistic effect induced by exogenous MG. As shown in Figure 6.7.B, the synergistic effect by exogenous MG (0.5 mM) on the induction of apoptosis induced by IL-3 depletion was completely abolished in the presence of LiCl. Moreover, addition of LiCl also prevented the synergistic effect on the formation of the specific 16 kDa MG-AGE-modified protein (data not shown). These data suggest that GSK-3β plays a crucial role in this process and that the role of MG and presumably the specific MG-derived AGE-modified proteins is downstream of GSK-3β.

Effects of MG on cells are often dependent on the formation of reactive oxygen species (ROS; (15,16,21,75-78)). Even though the reaction of MG with amino acids by itself leads to superoxide formation (79-81), MG also hampers the activities of the antioxidant mechanisms in cells (21,82-84) and directly modifies components of respiratory complex III in mitochondria, the major site of superoxide production (85,86). In order to investigate the role of ROS in the induction of apoptosis, the effect of N-acetyl-cysteine (NAC) was examined on IL-3 starvation and MG-induced apoptosis. Co-incubation of Ba/F3 cells with NAC strongly impedes the outcome of addition of MG on apoptosis (Figure 6.7.C).

MG-induced effects are not mediated by the p38 MAPK pathway

Several reports implicate the p38 and JNK MAPK pathways in MG-induced signaling to cell death. MG induces a rise in intracellular ROS, hereby leading to the activation of apoptosis signal-regulating kinase 1 (ASK1), and subsequently the p38α MAPK pathway (20). When Ba/F3 cells are deprived of IL-3 in the presence of MG, a strong and rapid

phosphorylation of p38 is induced (Figure 6.8.A). A time-course experiment shows that p38 is phosphorylated within 0.5 hour and peaks after 2 hours. Together with the induction of phosphorylation, an increase in the total amount p38 is also observed. But when the ratios of phosphorylated p38 to total p38 are calculated it is clear that the rise in activated p38 is mainly caused by phosphorylation. However, also in the presence of IL-3 addition of MG leads to a strong activation of p38 (Figure 6.9.A), although MG is not cytotoxic at the concentration used (Figure 6.7.A). This suggest that exogenous MG is able to activate p38 MAPK independent of when IL-3 is present or not.

Next, we tested whether the GSK-3β inhibitor LiCl could prevent the phosphorylation of p38 induced by MG. As shown in Figure 6.8.B, this was not the case although LiCl strongly abrogates the induction of apoptosis by MG. In contrast, NAC could prevent the MG-induced phosphorylation of p38 and also MG-induced cell death (Figure 6.7.C and Figure 6.8.B). Next, we used a specific p38 MAPK inhibitor SB203580, to examine whether p38 played a role in the synergistic effect of MG on the induction of apoptosis. Pre-incubation with SB203508 had no significant effect on MG-induced cell death (Figure 6.8.C). Of note is the observation that NAC by itself is able to induce p38 phosphorylation to a certain degree (Figure 6.8.B). This might be a consequence of the observed pro-oxidant activity of NAC under certain conditions (87). In summary, these data suggest that the synergistic effect of MG on the induction of apoptosis induced by IL-3 depletion occurs independent of p38 MAPK, but is dependent on GSK-3β.

Discussion

MG is a by-product of glycolysis, the most fundamental metabolic pathway. It therefore occurs in every cell and despite the presence of several detoxifying mechanisms Mg can reach high intracellular levels (88). Approximately 10% of total protein content has been estimated to be modified by MG under physiological conditions. Previous reports from our group have implicated MG in TNF-induced necrosis (24). GLO1 is phosphorylated in a PKA-dependent manner and a concomitant rise in MG is observed. As a consequence of these events specific MG-derived AGEs are formed. Inhibition of these events efficiently provides protection against TNF-induced necrosis in L929 cells.

In this study we have demonstrated that depletion of IL-3 induces phosphorylation of GLO1. A possible kinase to be involved in this process would be GSK-3β. GSK-3β is a ubiquitously expressed serine/threonine kinase and is a key regulator in diverse biological processes, including regulation of metabolism and apoptosis (reviewed in (43,44)). A remarkable characteristic of this kinase is the fact that it GFs lead to its inactivation by phosphorylation, and that inactivation of GSK-3β is correlated with survival whereas activation correlates with apoptosis (89,90). Inhibition of GSK-3β with LiCl or the specific inhibitor SB216763 also inhibits phosphorylation of GLO1 (Figure 6.3), showing the

involvement of GSK-3β. The phosphorylation of GLO1 does not seem to influence the activity of GLO1 as measured *in vitro* nor is there a change in detoxification of MG as determined by the production of D-lactate (Figure 6.4 and 5). In spite of the unaltered capacities of GLO1 to detoxify MG, specific MG-AGEs are formed. We hereby provide a possible physiological function for the ubiquitous GLO system and its primary substrate MG. Mammalian cells are dependent on GFs for growth and survival (91). An important mechanism used by GFs is the maintenance of cellular metabolism and glycolysis in particular (41,42,92,93). The intracellular concentration of MG is determined by the rate of glycolysis, and could therefore function as a signal molecule to elicit a response when drastic changes occur in the rate of glycolysis, since this would be reflected in intracellular MG concentration. IL-3 depletion would indeed induce a rapid decline in glycolysis (42,94).

Since *in vivo* induced MG-AGEs are heterogeneous, the choice of the antibody was based on the proven efficacy of the antibody to detect argpyrimidine in immunohistochemical samples of diabetic patients (73). The specific nature of the MG-derived AGEs was substantiated by the observation that addition of exogenous MG was insufficient to induce their formation (Figure 6.6.C). Only after IL-3 depletion MG-AGEs were detected, but this could be inhibited by inhibition of GSK-3β and the consequent phosphorylation of GLO1. This strongly supports the hypothesis that both phosphorylated GLO1 and a rise in MG levels are required for the formation of specific MG-derived protein modification.

MG is known to be a strong inducer of ROS and this ROS formation is often implied in the detrimental activity of MG (15,16,21,77,85). We also found the synergy of MG with IL-3 depletion could be abrogated by addition of NAC, an anti-oxidant which has been shown to be able to quench MG-induced ROS production (76). The concentrations of MG used in this report are within the range commonly used when the biological effects of MG are studied (19,21,75,85,95,96). Moreover, MG levels do not exceed the levels found in living cells (88), and are not cytotoxic by themselves (Figure 6.7.A). Several studies show the p38 and JNK MAPK pathways are activated upon MG-induced ROS formation and mediate the subsequent signaling events (15,16,20,75,95). However, this doesn't seem to be the case in our system. Although a strong activation of p38 was induced by MG, this was not sufficient to induce cell death (Figure 6.8). Nor could it be inhibited by LiCl, even though this strongly inhibited cell death and MG modification of protein. This observation is even strengthened by the inability of the specific p38 inhibitor SB203580 to inhibit the synergistic effect seen between IL-3 starvation and exogenous MG. Phosphorylation of JNK was also observed, but to a much lesser extent than p38 activation (data not shown). Further examination of the specific MG-modified protein or the possible pathways activated by ROS are needed to clarify the action of MG and phosphorylated GLO1 in IL-3 depletion-induced apoptosis.

In conclusion this report shows that upon IL-3 depletion in Ba/F3 cells GLO1 is phosphorylated in a GSK-3β dependent manner. Subsequently specific MG-modification

of proteins occurs. The formation of these MG-modified proteins is not the consequence of a simple rise in intracellular MG concentrations, since neither GLO1 activity or MG detoxification is impaired, nor could they be induced by addition of exogenous MG. Our results could provide a system that allows cells to integrate external signals from GFs with metabolism.

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Part II:
Experimental Data and Results

Chapter 7:

Phosphorylation of Glyoxalase I during the cell cycle

Chapter 7: Glyoxalase I is a direct substrate for Ca^{2+}/Calmodulin Dependent Kinase II (CaMKII)

Introduction

Despite the ample research on the glyoxalase (GLO) system and GLO1 in particular, most of the focus has been on the expression levels and activity, and relatively little is known about posttranslational modification. For human GLO1 it has been described that NO, in cooperation with glutathione, is able to induce modification of GLO1, resulting in a decreased activity and an isoelectric shift towards the basic end on two-dimensional gels (1,2). Similar effects have been described later to occur in yeast and *in vitro* as well (3). Phosphorylation has been described in yeast (4) and mammalian cells ((5,6); chapters 6 and 8 of this thesis), and in plants as well (7,8). Although several different kinases, such as PKA (5,6),GSK-3β (chapter 6) and the PI3K/Akt/mTOR pathway (see chapter 8), have been shown to be involved in the phosphorylation of GLO1 in mammalian cells, to date, it has not been described which kinase can directly phosphorylate GLO1.

In *Arabidopsis*, GLO1 was found to be phosphorylated upon overexpression of SNF1-related protein kinase 2.8 (SnRK2.8 (8)). *In vitro* kinase assays shows that SnRK2.8 directly phosphorylated GLO1 and a concomitant increase of GLO1 activity was found upon phosphorylation. The SnRK family members are the plant homologues of AMPK (AMP-activated protein kinase) and play a broad role in growth and metabolic responses to cellular stress (9). Quite interestingly, SnRK2.8 has been shown to be important in the response to drought and salinity (10), a property shared with GLO1 (11-13). Also, in rice leaf, GLO1 was identified as a phosphoprotein (7). Phosphorylation was induced by gibberellin, which is known to be an important regulator of cell growth, and was mediated by a putative 54-kDa Ca^{2+} dependent protein kinase (CDPK). Furthermore it was shown earlier that Ca^{2+} and calmodulin (CaM) were involved in light-mediated GLO1 activity in plants (14,15) and inhibitors of CaM showed an inhibitory effect on GLO1 activity (16). The data presented in this chapter provide evidence for the phosphorylation of mammalian GLO1 by Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Of note is also the shared consensus sequence for phosphorylation among CaMKII (17) and SnRK2.8 (18,19).

The goal of this study was to find the kinase that directly phosphorylates GLO1. Our data demonstrate that GLO1 is a direct substrate for CaMKII *in vitro*, and CaMKII is able to phosphorylate GLO1 *in vivo*. We could however not yet establish a role for CaMKII in GLO1 phosphorylation induced by IL-3 starvation of Ba/F3 cells, as KN-93, a specific inhibitor for CaMKII, was unable to inhibit IL-3 induced apoptosis.

Results and Discussion

GLO1 is phosphorylated upon coexpression with GSK-3β in Hek293 cells

As described in chapter 6 of this thesis, we showed earlier that inhibition of GSK-3β inhibited the phosphorylation of GLO1 which is induced by IL-3 starvation of Ba/F3 cells. To further study the phosphorylation of GLO1, we cotransfected Hek293 cells with an expression vector encoding wild type GLO1 and a vector containing GSK-3β. Cells were lysed 24 hours after transfection and phosphorylation was examined by 2-dimensional electrophoresis (2-DE) using an antibody raised against rabbit GLO1. Overexpression of GLO1 in itself already induced multiple phosphorylations, as shown by the many isoforms observed in Figure 7.1.A (middle panel; the non-phosphorylated, non-NO-responsive form is designated as the α-isoform). However, when GLO1 was cotransfected with a GSK-3β cDNA, a strong phosphorylation was induced on the NO-responsive isoforms (Figure 7.1.A, indicated with an arrow). Subsequently the same cotransfection was performed as described, but 24 hours after transfection the serum was depleted for 2 hours before cell lysis in order to fully activate GSK-3β and further mimic the conditions from chapter 6. Under these conditions, however, GSK-3β only showed a minor effect on the phosphorylation of the NO-responsive isoform of GLO1 (Figure 7.1.B; indicated with an arrow).

CamKII phosphorylates GLO1 *in vitro*

Next, we studied whether GLO1 is a direct substrate for GSK-3β. Analysis of the amino acid sequence shows the presence of the typical GSK-3β consensus site (consecutive Ser/Thr residues, separated by three amino acids), with Ser94 and Thr98 being the potential sites for phosphorylation by GSK-3β. This site is located at the interface of the GLO1 dimer and is conserved between human and mouse (Figure 7.2.A; the GSK-3β consensus site is underlined; for more information about potential phosphorylation sites see also supplementary figure page 114). We therefore performed *in vitro* kinase assays using purified recombinant GLO1 from *E. coli*. However, as shown in figure 7.2.B, we were unable to detect phosphorylation of GLO1 by GSK-3β *in vitro*.

The consensus sequence for GSK-3β phosphorylation however includes a serine or threonine residue that is already phosphorylated (primed) by another kinase (S/T-X-X-X-phospho-S/T) (20). Analysis of the amino acid sequence from GLO1 reveals the presence of a predicted CaMKII consensus site at Thr98 ((R-X-X-S/T) (17); Figure 7.2.A; indicated by a box) . This CaMKII consensus site overlaps with the predicted consensus site for GSK-3β and could therefore serve as a priming site for GSK-3β. Of note, the CaMKII consensus site is identical to the consensus site for the plant kinase SnRK2.8, which has been shown to be a direct kinase for GLO1 in *Arabidopsis* (8).

To evaluate whether CaMKII could phosphorylate GLO1 we first performed *in vitro*

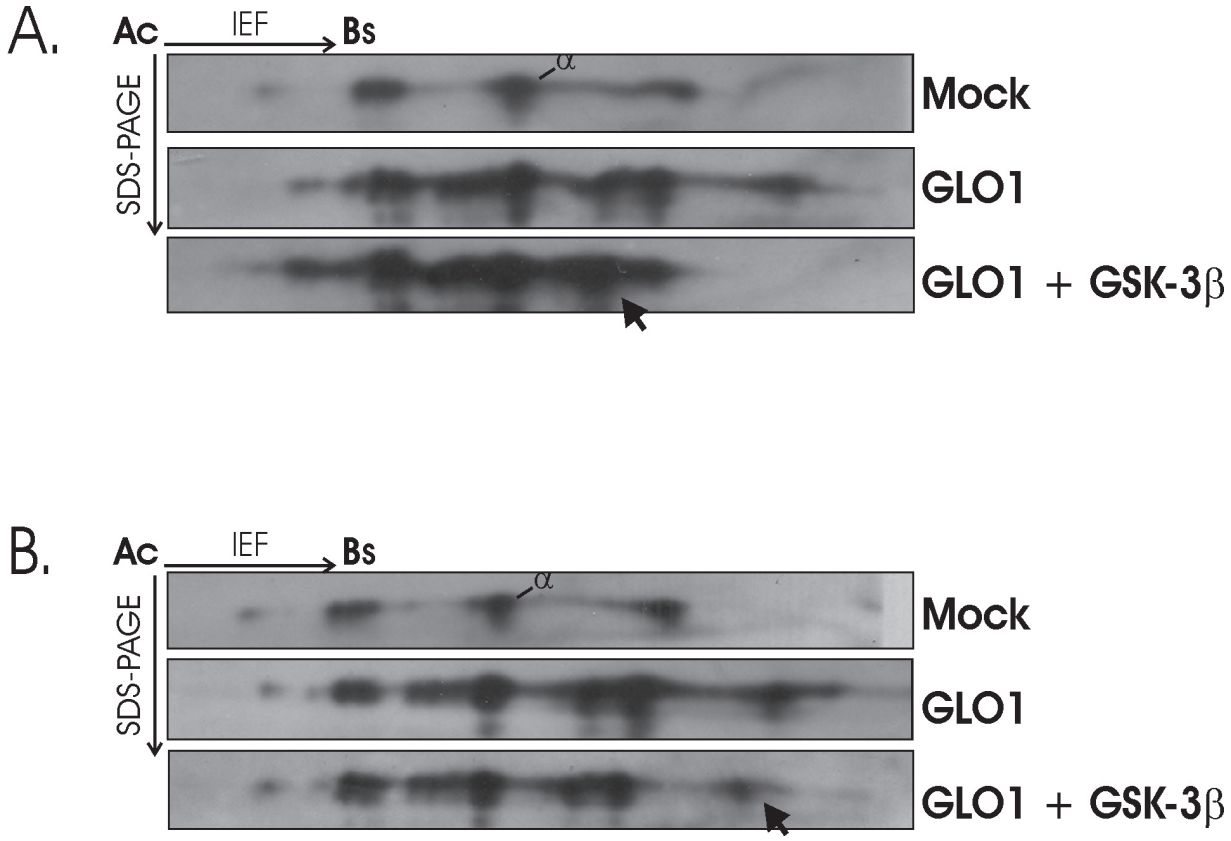

Figure 7.1. Coexpression of GLO1 with GSK-3β.
A. Coexpression of GLO1 with kinases in Hek293 cells. Hek293 cells were cotransfected with expression vectors encoding GLO1 and expression vectors containing GSK-3β. Cells were lysed in CEB after 24 hours and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 10 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with a polyclonal anti-human GLO1 antibody are shown. A reference spot α, which corresponds to the non-NO-responsive form of GLO1 is indicated (5). **B.** Coexpression of GLO1 with kinases in serum-starved Hek293 cells. Hek293 cells were co-transfected with expression vectors encoding GLO1 and expression vectors containing GSK-3β. 24 hours after transfection cells were depleted of serum for 2 hours. Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 10 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with a polyclonal anti-human GLO1 antibody are shown.

kinase assays using purified recombinant GLO1 from *E. coli*. When recombinant GLO1 was incubated in reaction buffer alone in the presence of ^{32}P-ATP, no phosphorylation could be observed (Figure 7.3.A). The presence of contaminating kinases or a possible autophosphorylation activity could thus be excluded. Coincubation of GLO1 with activated CaMKII, on the other hand, clearly induced phosphorylation (Figure 7.3.A).

We subsequently overexpressed and purified GLO1 from Hek293 cells for *in vitro* kinase assays. As shown in Figure 7.3.B, purified GLO1 from Hek293 cells could also be efficiently phosphorylated by CaMKII *in vitro*. Of note is the appearance of a high molecular weight band on the autoradiograph (indicated by a white arrowhead). Overlay of the autoradiograph with a Western blot performed with an anti-GLO1 antibody however revealed this represents a GLO1-dimer. Mammalian GLO1 requires dimerization for its activity (21) and this dimer is known to be extremely stable and highly resistant against

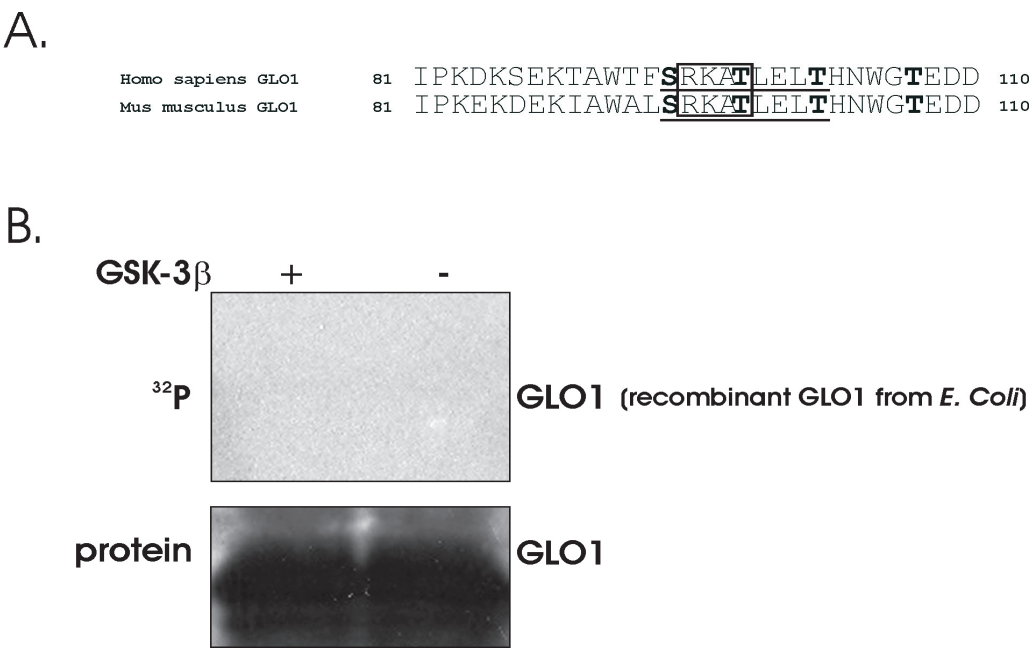

Figure 7.2. GLO1 is no direct target for GSK-3β *in vitro*.
A. Kinase consensus sequences. Alignment of the sequence of human and mouse GLO1 shows the presence of the conserved consensus sequences for GSK-3β (S/T-X-X-X-phospho-S/T; underlined) and CaMKII phosphorylation (R-X-X-S/T; box). Potential targets for phosphorylation are indicated in bold (phosphorylation sites are determined using Netphos software). **B.** Recombinant GLO1 is not phosphorylated by GSK-3β. 2 µg of recombinant GLO1 was incubated with GSK-3β as described in methods. Phosphorylation was detected by autoradiography, followed by western blot with an anti-GLO1 antibody.

reducing conditions (22). These results suggest that phosphorylation does not inhibit dimerization. Inhibition of dimerization would lead to inactivation of GLO1 (21,23,24).

The *in vitro* phosphorylation assays described above were performed on GLO1 which was purified using hexyl-glutatione linked beads and subsequently eluted using 10 mM s-hexylglutathione, dissolved in CaMKII reaction buffer. However, for the required sequential phosphorylation reactions, the priming with CaMKII and the phosphorylation with GSK-3β, the phosphorylation activity of the first kinase needs to be eliminated. This can be achieved by heat inactivation of the enzyme. Alternatively, GLO1 which is still bound to the hexyl-glutathione beads, can be used for the *in vitro* assays, with the advantage that the first kinase can be washed away. An additional benefit would be the possibility to use different reaction buffers for the sequential reactions. We therefore tested an alternative protocol to examine whether GLO1 which was bound to beads could also serve as a substrate for our assays.

When protein-loaded beads were coincubated with activated CaMKII, GLO1 was phosphorylated by CaMKII (Figure 7.3.C left panel). This demonstrates that the binding of GLO1 to beads does not interfere with phosphorylation. Of note, when lysis buffer in which phosphatase inhibitors were omitted was used to lyse cells and wash the beads, phosphorylation of GLO1 by CaMKII could further be enhanced, most likely because any phosphorylation that might have occurred in the cells was removed or reduced (Figure

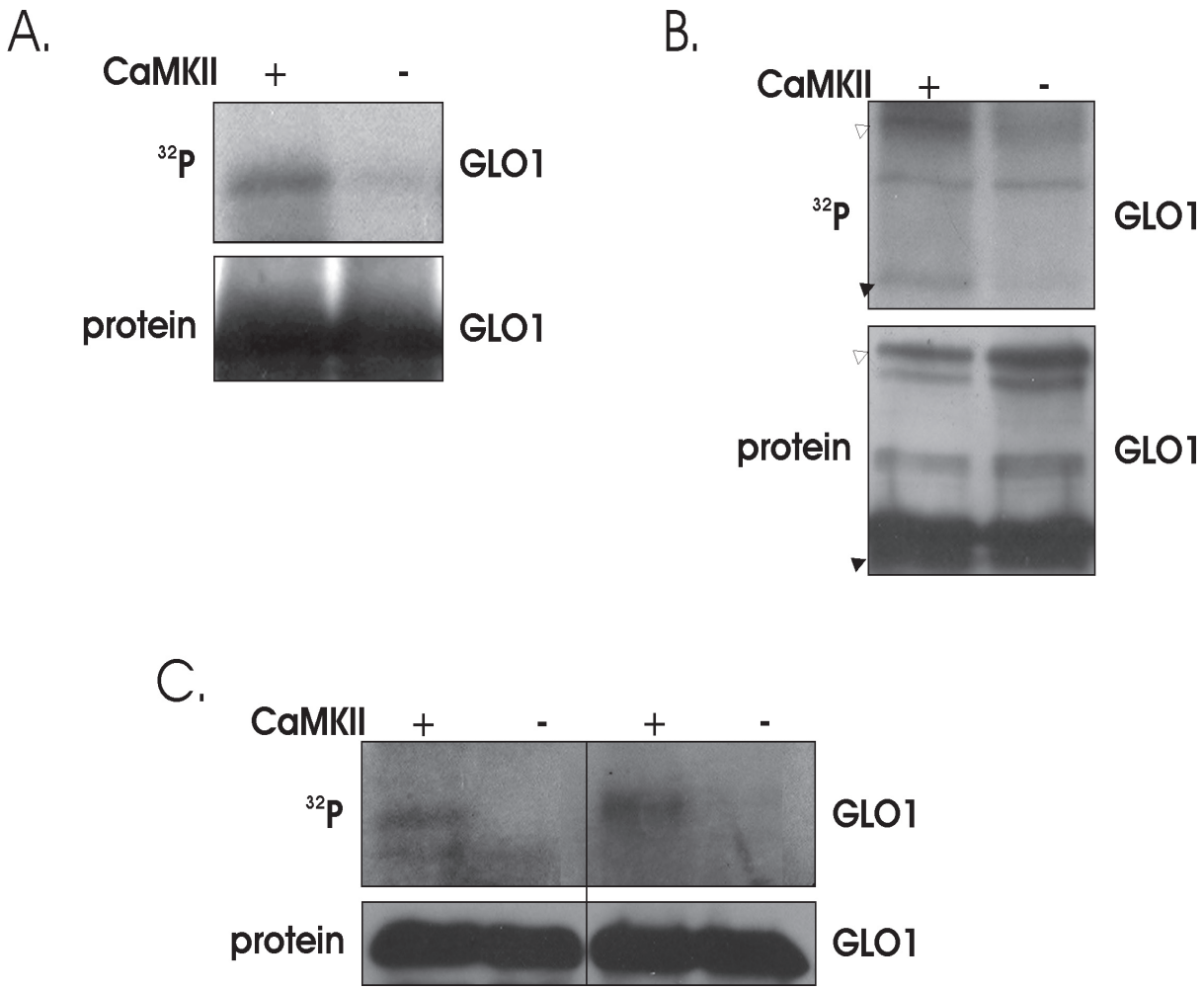

Figure 7.3. GLO1 is a direct substrate for CaMKII *in vitro*.
A. Recombinant GLO1 is phosphorylated by CaMKII. 2 µg of recombinant wild type GLO1 was incubated with activated CaMKII as described in methods. Phosphorylation was detected by autoradiography, followed by western blot with an anti-GLO1 antibody. **B.** Purified GLO1 from Hek293 is phosphorylated with CaMKII. Hek293 cells were transfected with expression vectors containing wild type GLO1. After 24 hours, transfected cells were lysed in CEB and GLO1 was purified as described in methods. Purified GLO1 was subsequently incubated with activated CaMKII. Phosphorylation was detected by autoradiography, followed by western blot with an anti-GLO1 antibody. Momomer GLO1 is indicated with a black arrowhead, dimer GLO1 is indicated with a white arrowhead. **C.** GLO1-loaded beads are phosphorylated by CaMKII. Hek293 cells were transfected with expression vectors containing wild type GLO1. After 24 hours, transfected cells were lysed in CEB (left panel) or in CEB without phosphatase inhibitors (right panel) and GLO1 was purified as described in methods. Protein-loaded beads were subsequently incubated with activated CaMKII. Phosphorylation was detected by radiography, followed by western blot with an anti-GLO1 antibody.

7.3.C right panel).

Subsequently we tested the hypothesis that CaMKII-dependent phosphorylation could serve as a priming phosphorylation for GSK-3β. Recombinant GLO1 from *E. coli* was first coincubated with activated CaMKII in the absence of ^{32}P-ATP for 30 minutes. Then CaMKII was heat inactivated and the reaction mixture was incubated with GSK-3β, in the presence of ^{32}P-ATP. Concomitantly unprimed GLO1 was coincubated with GSK-3β. As shown in Figure 7.4.A, no phosphorylation could be observed with GSK-3β, not even when GLO1 was first primed with CaMKII. However, CaMKII itself significantly phosphorylated GLO1. Also when GLO1 was purified from Hek293 cells and GLO1 bound to beads was used

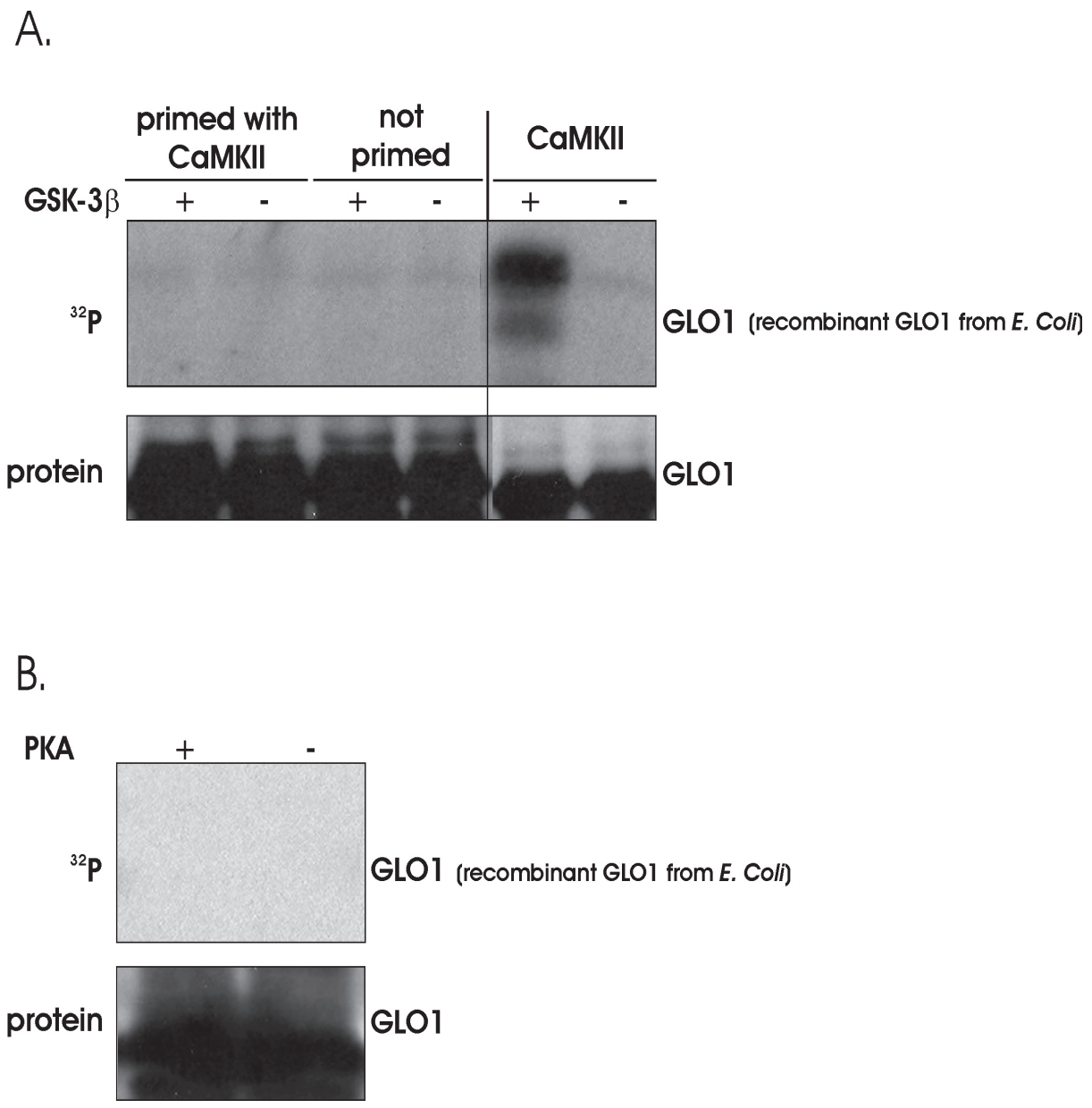

Figure 7.4. Primed GLO1 is no direct target for GSK-3β *in vitro*.
A. Recombinant GLO1 is not phosphorylated by GSK-3β after priming with CaMKII. 2 µg of recombinant wild type GLO1 was incubated with activated CaMKII in the absence of ^{32}P-ATP as described. After heath inactivation, the samples were incubated with GSK-3β in the presence of ^{32}P-ATP. As a control 2 µg of recombinant GLO1 was directly incubated with either activated CaMKII or GSK-3β. Phosphorylation was detected by autoradiography, followed by western blot with an anti-GLO1 antibody. **B.** Recombinant GLO1 is not phosphorylated by PKA. 2 µg of recombinant GLO1 was incubated with PKA as described in methods. Phosphorylation was detected by autoradiography, followed by western blot with an anti-GLO1 antibody.

for *in vitro* phosphorylation, no phosphorylation could be observed with GSK-3β (data not shown).

Another kinase that has been described to be involved in the phosphorylation of GLO1 is PKA (5,6). In spite of the presence of several consensus sites in the amino acid sequence for PKA (not shown), we were unable to detect *in vitro* phosphorylation of GLO1 with PKA (Figure 7.4.B).

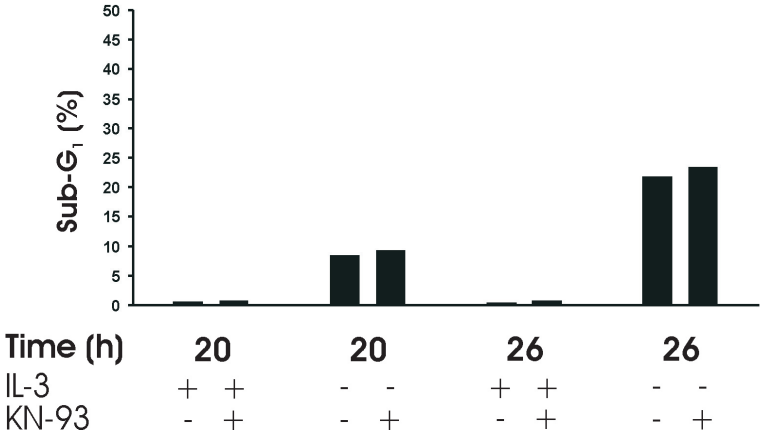

Figure 7.5. Inhibition of CaMKII does not inhibit IL-3 starvation-induced apoptosis.
Effect of CaMKII inhibition on IL-3 depletion-induced apoptosis. Ba/F3 cells were depleted of IL-3 and KN-93 (10 μM) was added. Apoptosis was measured by the analysis of the percentage of cells with sub-diploid DNA at the indicated time points.

Involvement of CaMKII in IL-3 depletion-induced apoptosis

To asses whether CaMKII was involved in IL-3 starvation-induced apoptosis, Ba/F3 cells were depleted of IL-3 in the presence of KN-93, a specific inhibitor of CaMKII. Apoptosis was subsequently determined by measuring DNA breakdown. However, inhibition of CaMKII had no effect on apoptosis induced by IL-3 depletion (Figure 7.5).

Conclusions

Although the GLO system has been discovered almost a hundred years ago, the only known function is the detoxification of reactive α-oxoaldehydes (25). Identification of the kinases involved in the modification of GLO1 might shed a light over the precise role of the GLO system. GLO1 has been shown to be phosphorylated in mammals, plants and in yeast (5-8,26). In spite of the involvement of several signaling pathways, to date, the kinase responsible for the phosphorylation of GLO1 has not been described in mammals. In Chapter 6 it was described how inhibition of GSK-3β also inhibited the IL-3 depletion-induced phosphorylation of GLO1. These observations were corroborated here by the observation that coexpression of GLO1 with GSK-3β also induces phosphorylation of GLO1. On the other hand, *in vitro* phosphorylation assays revealed that GLO1 is no direct substrate for GSK-3β. However, our data shows that GLO1 is a direct substrate for CaMKII. Although the predicted consensus sites for CaMKII and GSK-3β seem to overlap, CaMKII does not seem to be a priming kinase for GSK-3β phosphorylation. It can not be ruled out however that GSK-3β can directly phosphorylate GLO1, but that there is a different priming kinase.

CaMKII is a ubiquitously expressed kinase family, which needs Ca^{2+} and calmodulin for its activation (27). CaMKII is involved in a wide range of cellular processes, among which cell growth and division (28-30), and cell death (28-31), making it a likely candidate to be involved in TNF-induced necrosis and IL-3 starvation-induced apoptosis. However, inhibition of CaMKII did not exert an effect in the induction of apoptosis induced by IL-3 depletion of Ba/F3 cells. Nevertheless, the involvement of CaMKII in the phosphorylation of GLO1 *in vivo* was demonstrated in TNF-induced necrosis in the mouse fibroblast cell line L929 (de Hemptinne et al., in preparation).

CaMKII is probably not the only kinase capable of phosphorylating GLO1. From the 2-DE pattern of GLO1 it could be concluded that, for instance TNF can induce multiple phosphorylations of GLO1 (5). Further research is therefore still needed to fully elucidate all the kinases involved in modification of GLO1.

Materials and Methods

Cell Lines and Cultures

The IL-3 dependent pro-B cell line Ba/F3 (32) was purchased from DSMZ. Ba/F3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen) supplemented with heat-inactivated FBS (10% v/v) (Cambrex), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and 5% conditioned medium from WEHI-3B cells as a source of mouse IL-3. Ba/F3 cells were cultured at 37°C in a humidified incubator under an 5% CO_2 atmosphere. Hek293 cells, were cultured in DMEM with glutamax (Invitrogen) supplemented with heat-inactivated FBS (10% v/v), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) at 37°C in a humidified incubator under an 8% CO_2 atmosphere.

Cell stimulations

IL-3 was removed by washing exponentially growing cells 3 times in culture medium without conditioned WEHI-3B medium and resuspended in culture medium in the absence or presence of conditioned WEHI-3B.

Chemicals and inhibitors used are KN-93 (calbiochem). Duration and concentrations used as described.

Hek293 cells were transiently transfected by Ca precipitation. Vectors used are pCAGGS vectors containing wild type GLO1 and an expression vector for GSK-3β, kindly donated by Dr Jim Woodgett (Department of Medical Biophysics, Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada).

DNA distribution analysis

Cells were stained with propidium iodide (PI, Sigma) containing staining solution (33) and analyzed by a FACSCalibur flow cytometer ($488_{Ex}/590_{Em}$). Cell Quest software was used to analyze cell cycle distribution (FACS Calibur, Becton Dickinson) (33,34). Ten thousand cells were routinely analyzed.

Electrophoresis and immunoblotting

The cells were washed 3 times with ice-cold PBS buffer and lysed with cytosol extraction buffer (10 mM Tris-HCL pH7.4, 50 mM EDTA pH8.0, 25 mM NaCl, 0,7% TritonX-100, 100 mM PMSF, 1 tablet Complete Protease Inhibitor Cocktail (Roche)/50 ml cytosol extraction buffer). Cell lysates were cleared by centrifugation (14,000 x g). Protein concentrations were determined using Bradford method. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the desired antibodies followed by ECL-based detection (Amersham Pharmacia Biotech).

2-Dimensional Gel Electrophoresis

Isoelectric focusing was carried out on 18 cm IPG strips, pH 4-7 (GE Healthcare) according to the manufacturer's instructions. For the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE, 12%).

Recombinant expression and purification of GLO1 in E. coli

Wild type GLO1 was subcloned into pGEX-6P-1 GST fusion vector (Amersham Pharmacia Biotech) and wild type GLO1 was purified according to manufacturers instructions. Protein concentrations were determined using using Bradford method. Aliquots were stored at -80°C until further use.

Purification of GLO1 from Hek293 cells for *in vitro* phosphorylation

Cell cultures were transfected and lysed as described above. The cell lysates were incubated with S-hexylglutathione-agarose beads (Sigma) for 30 minutes. The cell lysates were then removed and the beads were washed 4 times with PBS buffer. GLO1 was eluted from the beads using 10 mM s-hexylglutathione, dissolved in CaMKII reaction buffer.

Phosphorylation reactions

2 µg of recombinant GLO1 or 10 µL of purified GLO1 was incubated with activated CaMKII (New England Biolabs), GSK-3β (New England Biolabs) or PKA (New England Biolabs) in the respective kinase reaction buffer (New England Biolabs) in the presence of 5 µCi ^{32}P-γ-ATP. Reactions were stopped after 1 hour by adding 5x laemmli buffer and boiling at 95°C for 5 minutes.

Alternatively cells were lysed as described above. The cell lysates were incubated with S-hexylglutathione-agarose beads for 30 minutes. The cell lysates were then removed and the beads were washed 4 times with PBS buffer and twice in kinase reaction buffer. Protein-loaded beads were resuspended in kinase reaction buffer and incubated with activated CaMKII or GSK-3β in the presence of 5 µCi ^{32}P-γ-ATP at 30°C for 1 hour. Reactions were stopped by washing the beads in cytosol extraction buffer containing phosphatase inhibitors. Beads were resuspended in 5x laemmli and boiled for 5 minutes.

The phosphorylated GLO1 was analyzed by SDS-PAGE and autoradiography.

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Part II:
Experimental Data and Results

Chapter 8:

Phosphorylation of glyoxalase I during the cell cycle

Chapter 8: Phosphorylation of Glyoxalase I during the cell cycle

Introduction

The glyoxalase (GLO) system is comprised of two enzymes, GLO1 and GLO2. In spite of the fact that it has been discovered almost a hundred years ago (1,2), the only function conferred to the GLO system is the detoxification of reactive α-oxoaldehydes, such as MG (3). The GLO systems ubiquitous nature (4,5), however, suggest a more fundamental and conserved role. Moreover, several other enzyme systems exist that are capable of detoxifying α-oxoaldehydes (6), rendering the GLO system partially redundant for detoxification. Since the work of Albert Szent-Györgyi (5,7-9), the GLO system has often been connected to proliferation. A strong correlation has been observed between high GLO1 activity and the rate of proliferation (10,11). Elevated levels of GLO1 expression or activity have been documented as important for the progression of several tumors and have been proposed to be indicative for the enhanced proliferative status (12-20), although some reports disprove this (21). Also in plants GLO1 activity strongly correlates with cell growth (22-25) and stimulation of cell growth involves an increase in GLO1 activity (26).

The availability of growth factors (GFs) is determining in survival, proliferation and growth. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in many cellular functions in response to GFs (27-30). A requisite for cell growth and division is the presence of glucose and a tight regulation of glucose metabolism (31,32). The involvement of the PI3K/Akt signaling pathway in the regulation of glucose uptake and the regulation of glycolysis has been well-documented (33-36). An important downstream target by which Akt exerts its influence on metabolism is mTOR (37-41). Furthermore, PI3K/Akt/mTOR signaling also directly regulates GF controlled cell cycle progression, primarily by controlling the expression of G_1 cyclins (42-45), but also at the end of G_2/M (46).

In order to study the possible role of GLO1 in the regulation of the cell cycle we analyzed the phosphorylation of GLO1 during the cell cycle. We show here that during progression through the cell cycle different phosphorylations arise. In line with previous reports (47), these phosphorylations could not be associated with a change in GLO1 activity *in vitro*. Inhibition of the PI3K/Akt partially inhibits the phosphorylation of GLO1. Also inhibition of mTOR, one of the downstream targets of the PI3K/Akt pathway involved in control of cellular growth, in part inhibits the phosphorylation of GLO1. These data implicate the PI3K/Akt/mTOR pathway, an important regulator of cell cycle in the modification of GLO1.

Results

Cell cycle dependent phosphorylation of GLO1

Previous studies in our group show that most cell types express multiple isoforms of GLO1, including a NO-modified form and a non-NO-modified form, which was denominated the α-isoform (48). These different forms of GLO1 can be multifariously phosphorylated on the α-isoform as well as on the NO-modified form. These changes in isoform pattern can easily be studied using 2-dimensional gel electrophoresis (2-DE) and immunoblotting with an anti-GLO1 antibody (48). Phosphorylation has been shown to yield a shift of the isoform to the acidic side of the gel, whereas NO-induced modification results in a shift to the basic side.

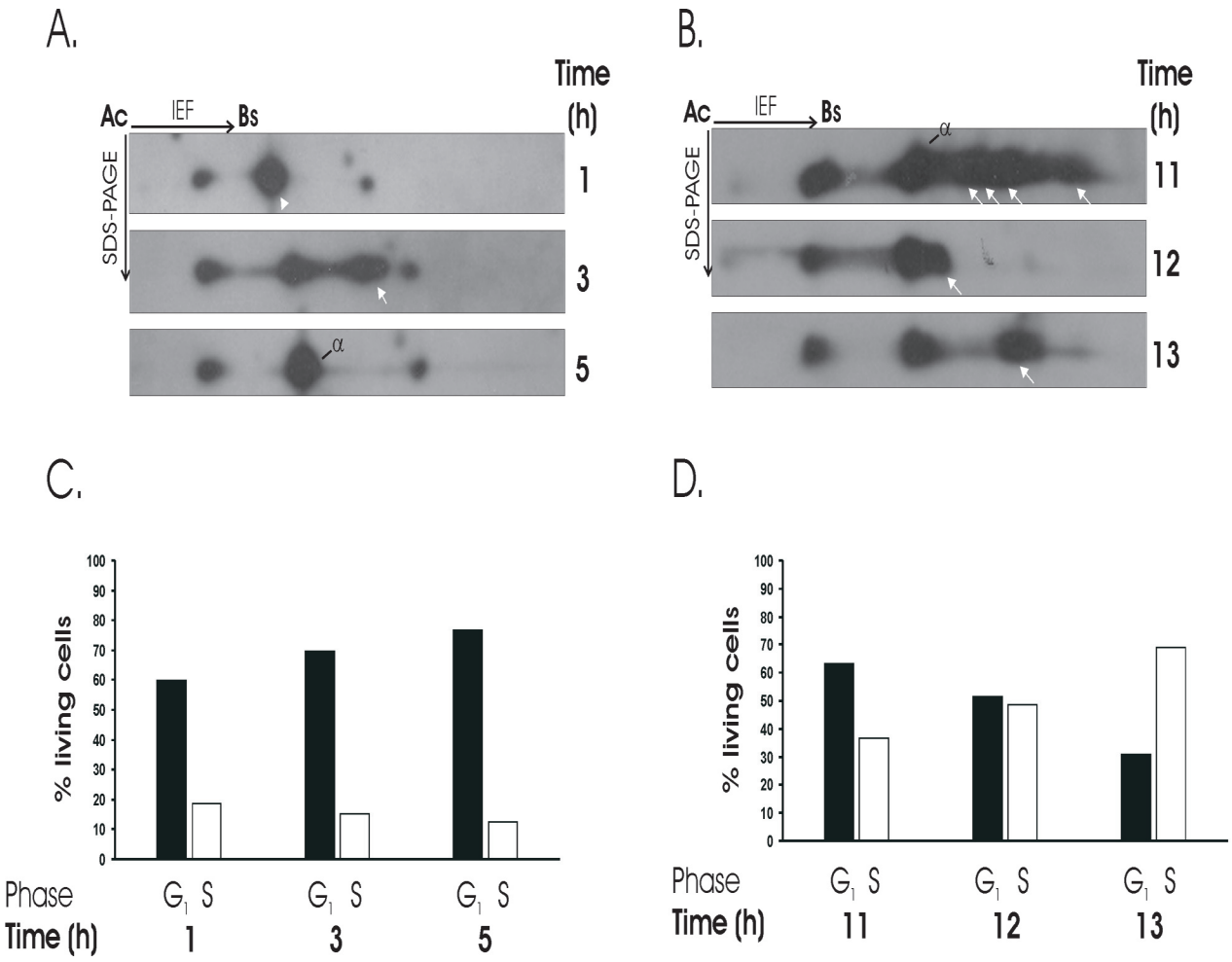

Figure 8.1. Cell cycle dependent phosphorylation GLO1.
GLO1 was phosphorylated during G_1 **(A)** and at G_1/S transition **(B)**. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Cells were lysed in CEB at indicated time points, and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with a polyclonal anti-human GLO1 antibody (48) are shown. A reference spot α, which corresponds to the non-NO-responsive form of GLO1 is indicated (48). **C and D.** Analysis of the cell cycle. The different phases of the cell cycle were monitored by analysis of the DNA content by flow cytometry.

In *Arabidopsis* and rice leaf, regulators of cell cycle have been reported to induce phosphorylation of GLO1 (49-51). To date, in mammalian cells, no posttranslational modifications of GLO1 have been reported in the cell cycle. In chapter 6 we have described how GLO1 is phosphorylated during GF withdrawal induced G_1 arrest. To study phosphorylation during cell cycle, the cell cycle of Ba/F3 cultures was synchronised in the G_1 phase by IL-3 depletion overnight and subsequently released from this block by re-addition of IL-3. Cells were lysed 1 hour, 3 hours and 5 hours after re-addition of IL-3 for the analysis of the posttranslational modifications of GLO1 in G_1 phase using 2-dimensional gel electrophoresis (2-DE) (Figure 8.1). Analysis of the DNA-distribution showed Ba/F3 cells resided in the G_1 phase after all three time intervals (Figure 8.1.C) In lysates made after 1 hour, only a small number of isoforms is displayed. The α-form could not be observed. Instead, the non-NO-modified form is strongly phosphorylated, as can be observed by the localisation of the spot more to the acidic side (Figure 8.1.A, upper panel, arrowhead). At this time, only a very limited presence of the NO-modified form of GLO1 could be detected. In lysates made 3 hours after the release of the cell cycle block, the non-NO-modified form of GLO1 is strongly dephosphorylated, as shown in a shift of the spot to the basic side. Concomitantly, NO-mediated modification of GLO1 occurs, as witnessed by the appearance of a more basic spot (Figure 8.1.A, middle panel, arrow). In the lysates made 5 hours after the re-addition of IL-3, both the NO-modified form of GLO1 and the non-NO-modified form of GLO1 were further dephosphorylated (Figure 8.1.A, bottom panel). The α-form is now visible.

Subsequently, we studied the phosphorylation of GLO1 during G_1-S transition using a same approach (Figure 8.1.B). The percentage of cells in the different phases of the cell cycle was determined by analysis of the DNA-distribution on FACS, indicating at the chosen time points, cells pass from the G_1 phase into the S phase (Figure 8.1.D). Cells were lysed 11 hours, 12 hours and 13 hours after the release from G_1 arrest. 11 hours after the release from G_1 arrest, when 65 % of the cells is still in the G_1 phase (Figure 8.1.D, black bars for the percentage in the G_1 phase and white bars for the percentage in the S phase), we observed a strong presence of several phosphorylated isoforms on the NO-responsive form (Figure 8.1.C, upper panel, arrows). After 12 hours 50 % of the cell population was in the G_1 phase and 50 % of the cells was in the S phase (Figure 8.1.D). At this time point, a hyperphosphorylation of the NO-responsive isoforms could be detected (Fig 1.C, middle panel, arrow). After 13 hours, when 70 % of the cells were in the S phase and only 30 % still resides in the G_1 phase, the NO-responsive form was partially dephosphorylated again (Figure 8.1.C, lower panel, arrow).

All together, our data shows that GLO1 is strongly modified during the early stages of the cell cycle. Both phosphorylation and NO-modification occur. Of note is the observation that the G_1-S transition coincides with a hyperphosphorylated state of the NO-modified

form of GLO1. This hyperphosphorylation is rapidly removed when cells pass on into the S phase.

GLO1 activity does not change throughout the cell cycle

Next, we determined whether the posttranslational modification of GLO1 was accompanied by changes in the activity of GLO1. *In vitro* GLO1 activity in lysates was assayed using a spectrophotometric method, which monitors the GLO1-catalyzed production of S-D-lactoylglutathione from the hemithioacetal that is spontaneously formed when MG is incubated with glutathione (see materials and methods).

Cells were synchronized by overnight IL-3 starvation and subsequent re-addition of IL-3. Cell lysates were made after the indicated time intervals after re-addition of IL-3 and *in vitro* GLO1 activity was assayed (Figure 8.2.A). The chosen time points are situated at the G_1-S transition (Figure 8.1.D). Although hyperphosphorylation and consequent

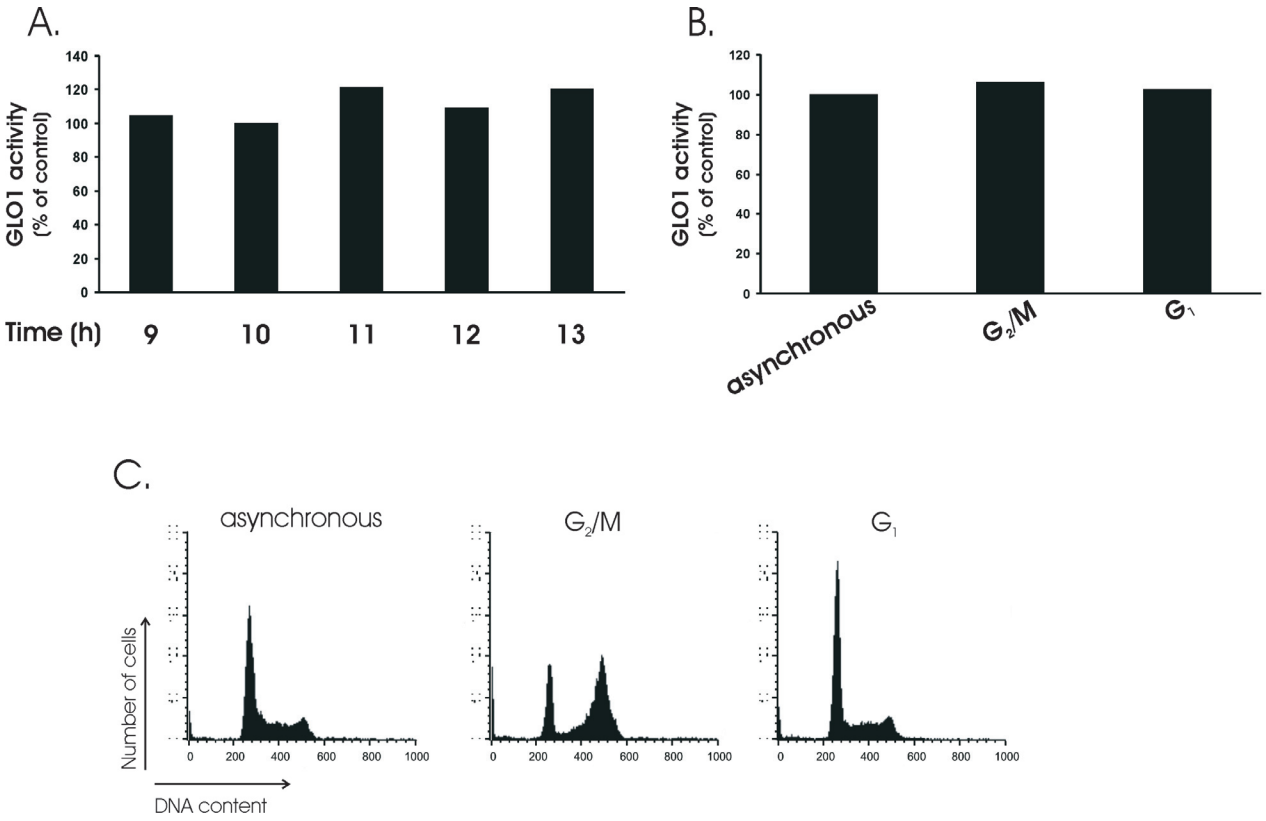

Figure 8.2. *In vitro* GLO1 activity is not altered during the cell cycle.
A. GLO1 activity at G_1/S transition. Ba/F3 cells were synchronized by IL-3 starvation over night. The cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Cells were lysed in CEB at indicated time points after the re-addition of IL-3, lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. *In vitro* GLO1 reaction rates were determined using Ultraspec 1100 software (Amersham, BD Biosciences) and are shown relative to the reaction rate of lysates made of exponentially growing cells, which are set as 100%. **B.** GLO1 activity in different phases of the cell cycle. G_1 arrest was induced by IL-3 starvation of Ba/F3 cells and G_2/M arrest was induced by addition of nocodazole. Cells were lysed in CEB, lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. **C.** Analysis of the cell cycle. The different phases of the cell cycle were monitored by analysis of the DNA content by flow cytometry.

dephosphorylation of GLO1 can be observed at the G_1-S transition (Figure 8.1.C), a concomitant change in GLO1 activity could not be observed (Figure 8.2.A). Some fluctuation can be noticed in GLO1 activity. However, this was not significant and is probably caused by experimental variability.

In order to further compare GLO1 activity in different phases of the cell cycle, we arrested Ba/F3 cells in the G_1 phase and in G_2/M, respectively by IL-3 depletion or addition of nocodazole for 8 hours. Nocodazole inhibits microtubule polymerization and blocks mitotic spindle formation, resulting in the accumulation of cells in M. The arrest in respective cell cycle phases was verified by analysis of the DNA distribution (Figure 8.2.C). Cell lysates were prepared and *in vitro* GLO1 activity subsequently assayed (Figure 8.2.B). Neither G_1 arrest or G_2/M arrest was accompanied by a change in *in vitro* GLO1 activity when compared to a culture of asynchronously growing cells.

We therefore conclude that GLO1 activity as assayed by its ability to catalyze the formation of S-D-lactoylglutathione, is not modulated by post-translational modification. These results are in line with our previous observations in Ba/F3 cells (chapter 6), but also in L929 cells (47) and bovine aortic endothelial cells (BAEC; Laga et al., submitted). Our findings however contradict the reported rise in GLO1 activity observed during G_2/M in carrot cell suspension (52).

Phosphorylation of GLO1 is mediated by the PI3K/Akt pathway

IL-3 has been shown to induce activation of several signaling pathways, including Jak-2/STAT-5, ras/raf/MEK/ERK, and PI3K/Akt kinase cascades (53-55). The PI3K/Akt signaling pathway has been implicated in regulating metabolism and proliferation (56-59). We therefore first investigated whether the phosphorylation of GLO1 was dependent on PI3K/Akt signaling.

Ba/F3 cells where synchronized as described previously. 8 hours after the release from cell cycle arrest, three dominant spots can be observed. The α-form of GLO1, and two more acidic spots. Only little NO-responsive GLO1 is present. 11 hours after re-addition of IL-3, the α-form of GLO1 and the middle isoform both are phosphorylated (Figure 8.3.A, second panel, black arrowhead and white arrowhead respectively). Also NO-mediated modification of GLO1 is induced at that time (Figure 8.3.A, second panel, arrow). One hour later, the most acidic spot shifts to the left, indicating this form of GLO1 is subject to additional phosphorylation (Figure 8.3.A, third panel, arrowhead), and the phosphorylation on the α-form has disappeared again. The NO-responsive form is hyperphosphorylated at this time (Figure 8.3.A, third panel, arrow).

However, upon addition of wortmannin, a specific inhibitor for the PI3K/Akt pathway,

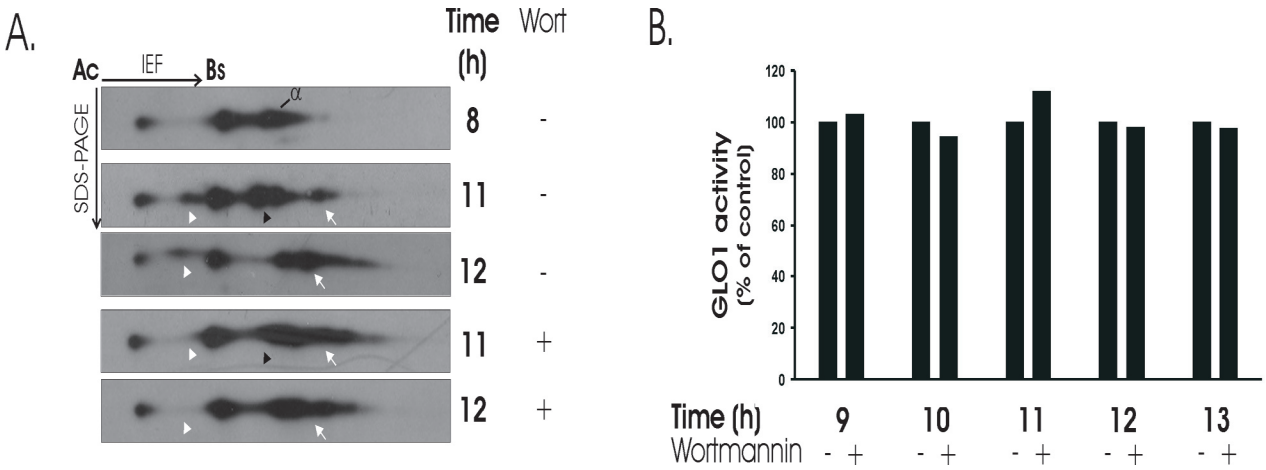

Figure 8.3. Inhibition of PI3K/Akt interferes with phosphorylation of GLO1 during the cell cycle. **A.** Phosphorylation of GLO1 with PI3K/Akt inhibitors. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Wortmannin was added after 8 hours. Cells were lysed in CEB after indicated time intervals, and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with an anti-GLO1 antibody are shown. Differential spots are indicated with an arrow or an arrowhead. **B.** Effect of PI3K/AKT inhibitors on *in vitro* GLO1 activity. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Wortmannin was added after 8 hours. Cells were lysed in CEB after indicated time points, lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. *In vitro* GLO1 reaction rates were determined using Ultraspec 1100 software (Amersham, BDbiosciences) and are shown relative to the reaction rate of lysates made of exponentially growing cells, which are set as 100%.

strong inhibition of phosphorylation could be observed. 11 hours after re-addition of IL-3 (and three hours after addition of wortmannin), both the phosphorylation on the acidic isoform of GLO1 and the phosphorylation on the α-form were severely impaired (Figure 8.3.A, fourth panel, black arrowhead and white arrowhead respectively). NO-modification still occurred, but the NO-responsive form was phosphorylated to a lesser extent than in the absence of wortmannin (Figure 8.3.A, fourth panel, arrow). When the 2-DE pattern of lysates made 12 hours after initiation of the cell cycle was studied, the hyperphosphorylation of the NO-modified form of GLO1 was reduced in the presence of wortmannin, when compared to the absence of the inhibitor (Figure 8.3.A, fifth panel, arrow). Also the phosphorylation on the more acidic isoform was absent (Figure 8.3.A, fifth panel, arrowhead).

Also the *in vitro* activity of GLO1 was determined. In line with the observations discussed above, no differences were observed in the level of GLO1 activity at the G_1-S phase transition and the addition of wortmannin, which did show a drastic effect on phosphorylation, did not alter the activity of GLO1 *in vitro* (Figure 8.3.B).

In order to corroborate our findings in the cell cycle, wortmannin was also added to an asynchronous exponentially growing Ba/F3 culture for 3 hours (Figure 8.5.A). Whereas a phosphorylation could be observed under control conditions, possibly caused by the manipulation of the cells, this was not the case when PI3K/Akt was inhibited with wortmannin (Figure 8.5.A, arrowhead). Next, we assayed lysates from asynchronous

Ba/F3 cells that were cultured in the presence or absence of wortmannin. In spite of the differences in phosphorylation between the different conditions, no change in GLO1 activity was induced (Figure 8.5.C).

In summary, our data show that cell cycle dependent phosphorylation of GLO1 is at least in part dependent on the PI3K/Akt pathway. Our data also strengthens the observation that phosphorylation of GLO1 can not be correlated with a difference in *in vitro* GLO1 activity.

Phosphorylation of GLO1 is partially inhibited by rapamycin

Although inhibition of PI3K strongly influences the phosphorylation status of GLO1, phosphorylation of GLO1 still occurs (cf. Figure 8.3.A, fifth panel, where the NO-responsive form is still phosphorylated, albeit less). An important downstream target of PI3K/Akt is mTOR. mTOR and its target p70S6K1 have been shown to be important mediators of the effects of PI3K on cell cycle regulation and proliferation (42,45,60-62). Furthermore, mTOR is considered to be a central regulator in cellular growth and is involved in the coordination of cell growth with cell cycle (63-65).

Ba/F3 cells were synchronized as described above and cells were lysed at the indicated time points after re-addition of IL-3. Analysis of cells lysed after 8 hours show the presence of multiple phosphorylations on both the non-NO-modified form and the

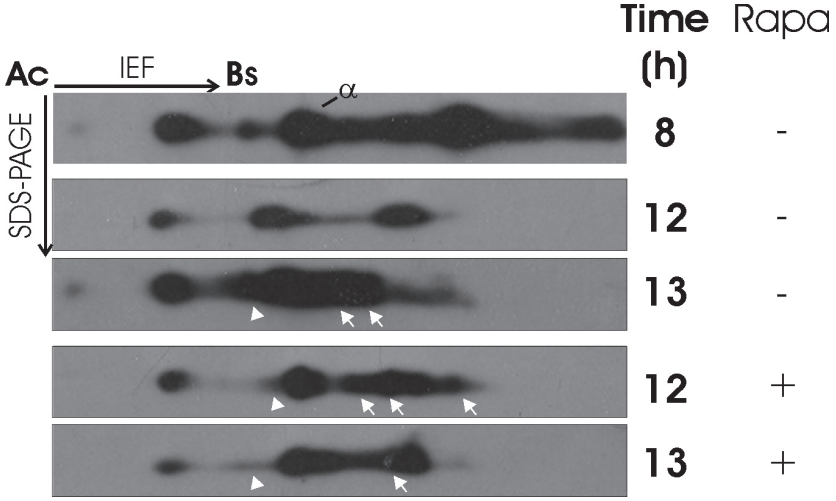

Figure 8.4. Inhibition of mTOR interferes with phosphorylation of GLO1 during the cell cycle.
Phosphorylation of GLO1 with mTOR inhibitors. Ba/F3 cells were synchronized by IL-3 starvation over night. The cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Rapamycin was added after 8 hours. Cells were lysed in CEB at indicated time points, and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with an anti-GLO1 antibody are shown. Differential spots are indicated with arrows on the NO-modified form and with an arrowhead on the non-NO-modified form.

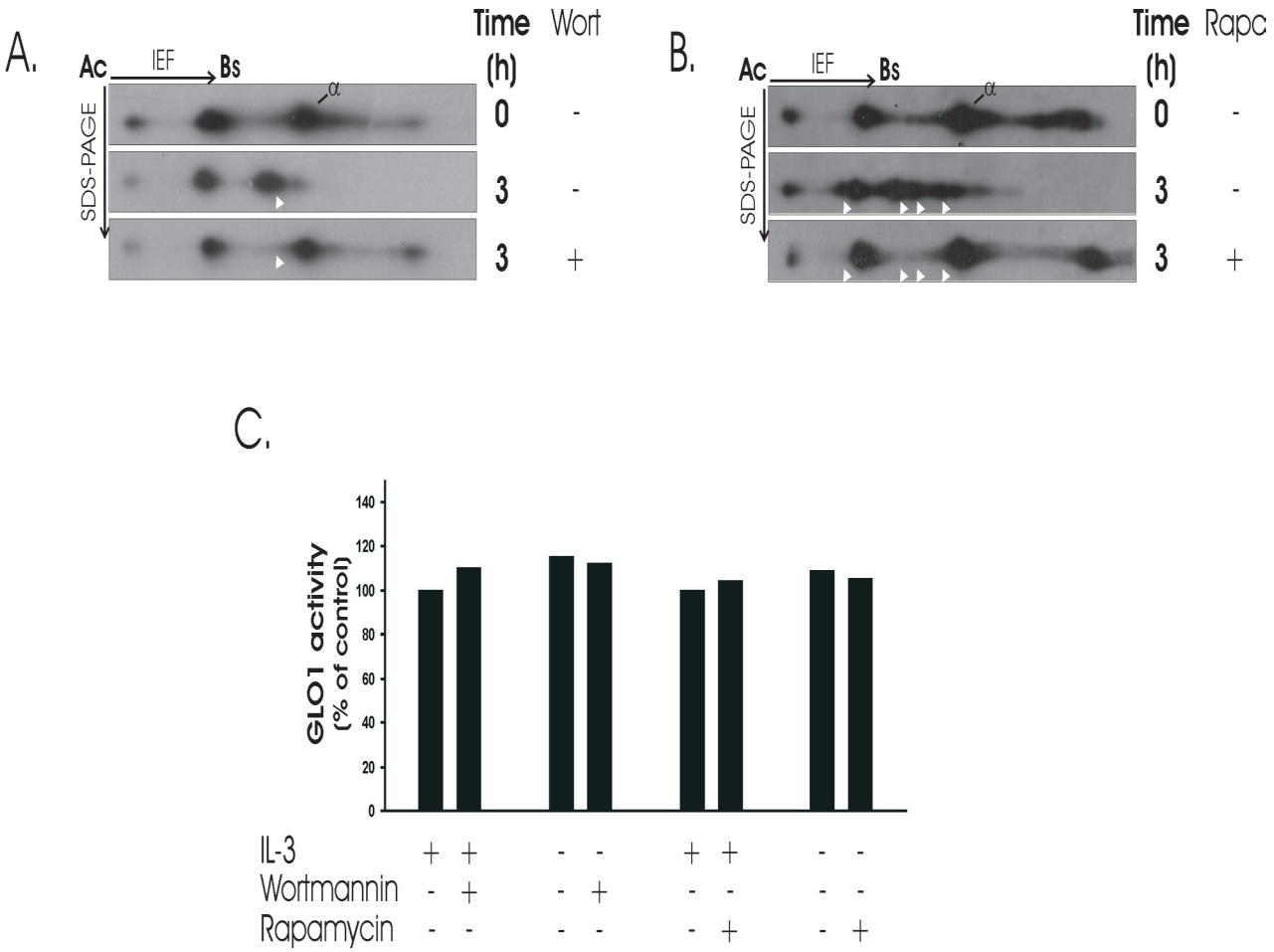

Figure 8.5. Inhibition of PI3K/Akt or mTOR inhibits phosphorylation of GLO1 in asynchronously growing cells.
A. Phosphorylation of GLO1 with PI3K/Akt inhibitors. Wortmannin was added to exponentially growing Ba/F3 cells. Cells were lysed in CEB after 3 hours and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with an anti-GLO1 antibody are shown. **B.** Phosphorylation of GLO1 with rapamycin. Rapamycin was added to exponentially growing Ba/F3 cells. Cells were lysed in CEB after 3 hours and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with an anti-GLO1 antibody are shown. **C.** *In vitro* GLO1 activity of exponentially growing Ba/F3 cells. Wortmannin or rapamycin was added to exponentially growing Ba/F3 cells. Cells were lysed in CEB after 5 hours, lysates were brought to equal concentrations and cell lysates were used to assay GLO1 activity. Reaction rate of lysates made of exponentially growing cells are set as 100%.
Differential spots are indicated with an arrowhead.

NO-modified form (Figure 8.4, upper panel). After 12 hours, the number of different phosphorylations was reduced, with only a phosphorylated NO-modified form, and a phosphorylated non-NO-modified form remaining (Figure 8.4, second panel). After 13 hours, additional phosphorylation can be observed on the non-NO-modified form (Figure 8.4, third panel, arrowhead), as well as on the NO-modified form (Figure 8.4, third panel, arrows). Although the phosphorylations induced at the 13 hour time point, both on the NO-modified form and the non-NO-modified form, can be inhibited with rapamycin (Figure 8.4, fifth panel, arrow and arrowhead respectively), this was not the case after 12 hours. In contrast, at 12 hours, addition of rapamycin seemingly enhanced phosphorylation

on the NO-modified forms (Figure 8.4, fourth panel, arrows). On the other hand, the phosphorylation on the non-NO-modified form was inhibited (Figure 8.4, fourth panel, arrowhead)

Likewise, incubation of asynchronously growing Ba/F3 cells with rapamycin for 3 hours, strongly changed the phosphorylation of GLO1. Whereas multiple phosphorylations could be observed in the absence of rapamycin, these were abolished upon the addition of rapamycin (Figure 8.5.B, second and third panel, arrowheads). As well as in the case of inhibition with wortmannin, no effects on the activity of GLO1 could be found after incubation of Ba/F3 cells in the presence of rapamycin (Figure 8.5.C).

In conclusion, these observations show that phosphorylation of GLO1 in the cell cycle occurs in part in a mTOR dependent manner.

Discussion

GLO1 expression levels and activity have been shown to associate strongly with proliferation. This has been reported to be the case in animal cells (10,11,66) as well as in plants (22,23,25) and yeast (67). In general it is observed that strongly dividing cells and tissues display high GLO1 activity, whereas GLO1 levels and activity drop in more quiescent or matured cells and tissues (11,22,68,69). Furthermore, under various pathophysiological conditions GLO1 is regulated and higher levels of GLO1 are associated with increased proliferation of tumors (14,16-19). The data presented above show that during the cell cycle, GLO1 is strongly subjected to phosphorylation. This is in agreement with the findings described in chapter 6, where was shown how phosphorylation of GLO1 occurred when G_1 arrest was induced by GF withdrawal in a GSK-3β dependent manner. This took place in several cell lines, suggesting a general mechanism. Of note is the apparent absence of the NO-responsive isoform in early G_1. It has been described that phosphorylation primarily occurs on the NO-responsive isoforms of GLO1, but both types of posttranslational modification are independent and neither type of modification is required for the other type to occur (48). Phosphorylation could be severely altered by use of wortmannin, a PI3K inhibitor, and rapamycin, an inhibitor for mTOR. This suggests the involvement of the PI3K/Akt/mTOR signaling cascade.

Interestingly, in spite of the strong effects observed with both wortmannin and rapamycin, neither one could however completely inhibit GLO1 phosphorylation. Possibly this is the consequence of feedback mechanisms or compensatory mechanisms that exist in this pathway. Elaborate evidence exists for such mechanisms. Although mTOR activation is downstream of PI3K/Akt, mTOR reciprocally phosphorylates Akt, hereby

either further activating Akt, when mTOR is part of the mTOR complex 2 with rictor (70), or serving as a negative feedback mechanism when mTOR is bound in the raptor containing mTOR complex 1 (71,72). Alternatively, there might be several kinases involved in phosphorylation of GLO1, depending on the stimulus, or acting in a redundant manner. It would also be plausible to hypothesize that inhibition of either Akt or mTOR would result in (partial) activation of GSK-3β since both kinases are able to phosphorylate and inhibit GSK-3β *in vivo* (73-77). Moreover, we have shown that GLO1 is a direct substrate for CaMKII (chapter 7).

Cycling cells require a dynamic regulation of glucose metabolism. The presence of glucose is a requisite for cells to proliferate and it has been observed that cells upregulate glucose uptake and glucose metabolism during G_1 and S phase and downregulate glucose uptake during G_2/M phase (31,32,78). GFs play an essential role by promoting cell metabolism and determining the rate of cell division. The PI3K/Akt pathway is critically involved in GF signaling in hematopoietic cells and directly affects the glucose metabolism (34,36,39,79) as well as the cell cycle (42,80-82). It would therefore be reasonable to hypothesize that GFs would attach a dynamic regulation of the GLO system to the cell cycle determined regulation of glycolysis, since a rise in the rate of glycolysis would be expected to automatically implicate higher intracellular levels of the cytotoxic MG. Our data show, however, that different phases in the cell cycle did not display different levels of *in vitro* GLO1 activity (Figure 8.2.A), in contrast with the described rise in G_2/M, which has been described in carrot cell suspension cultures (52). Also at the G_1-S transition, no higher levels of GLO1 were observed *in vitro* (Figure 8.2.B), in spite of the fact that from literature we would expect a rise in glucose uptake and glycolysis (32,83-86). On the other hand, it has been shown that higher GLO1 activity, even when adequate concentrations of reduced glutathione are present, not necessarily suffices to keep intracellular concentrations of MG low (87), which could make a rise in GLO1 activity redundant. Furthermore, addition of rapamycin or wortmannin did not have an effect on GLO1 activity *in vitro*, despite the drastic changes that were induced on phosphorylation. These data should however be interpreted cautiously since they do not exclude the possibility that in the cells GLO1 activity might differ locally, hereby creating microenvironments with different levels of MG. Alternatively GLO1 isoforms have been described that are not involved in detoxification of MG (88), and possibly these isoforms are phosphorylated during the cell cycle.

In summary, our data shows that GLO1 is subject to several phosphorylations during the cell cycle, but this does not seem to influence the activity of GLO1. These phosphorylations are in part mediated by the PI3K/Akt/mTOR signaling cascade.

Materials and Methods

Cell Lines and Cultures

The IL-3 dependent pro-B cell line Ba/F3 (89) was purchased from DSMZ. Ba/F3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen) supplemented with heat-inactivated FBS (10% v/v) (Cambrex), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and 5% conditioned medium from WEHI-3B cells as a source of mouse IL-3. Ba/F3 cells were cultured at 37°C in a humidified incubator under an 5% CO_2 atmosphere.

Cell stimulations

Ba/F3 cells were synchronized by washing exponentially growing cells 3 times in culture medium without conditioned WEHI-3B medium and resuspension in culture medium without conditioned WEHI-3B medium for 8 hours or overnight. At the end of the synchronisation conditioned WEHI-3B medium (5% v/v) was re-added. This time point was designated 0 hours. Alternatively was added for 8 hours or overnight. At the end of the synchronisation nocodazole was removed by washing cells 2 times in fresh culture medium. This time point was designated 0 hours.

Chemicals and inhibitors used are nocodazole (Sigma aaldrich), wortmannin and rapamycin (Calbiochem). Duration and concentrations used as described.

DNA distribution analysis

Cells were stained with propidium iodide (PI, Sigma) containing staining solution (90) and analyzed by a FACSCalibur flow cytometer ($488_{Ex}/590_{Em}$). Cell Quest software was used to analyze cell cycle distribution (FACS Calibur, Becton Dickinson) (90,91). Ten thousand cells were routinely analyzed.

Electrophoresis and immunoblotting

The cells were washed 3 times with ice-cold PBS buffer and lysed with cytosol extraction buffer (10 mM Tris-HCL pH7.4, 50 mM EDTA pH8.0, 25 mM NaCl, 0,7% TritonX-100, 100 mM PMSF, 1 tablet Complete Protease Inhibitor Cocktail (Roche)/50 ml cytosol extraction buffer). Cell lysates were cleared by centrifugation (14,000 x g). Protein concentrations were determined using Bradford method. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the desired antibodies followed by ECL-based

detection (Amersham Pharmacia Biotech).

2-Dimensional Gel Electrophoresis

Isoelectric focusing was carried out on 18 cm IPG strips, pH 4-7 (GE Healthcare) according to the manufacturer's instructions. For the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE, 12%).

Determination of GLO1 activity

GLO1 activity was determined as described earlier (47), using the spectrophotometric method developed by Oray and Norton (92). Equal concentrations of cell lysates were used.

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A.

```
Homo sapiens GLO1  1   MAEPQPPSGGLTDEAALSCCSDADPSTKDFLLQQTMLRVKDPKKSLDFYTRVL  53
                               +                 *
Mus musculus GLO1  1   MAEPQPASSGLTDETAFSCCSDPDPSTKDFLLQQTMLRIKDPKKSLDFYTRVL  53
                             +                 *

Homo sapiens GLO1  54  GMTLIQKCDFPIMKFSLYFLAYEDKNDIPKEKDEKIAWALSRKATLELTHNWG 106
                                                              *
                                                              ✹
                                                              ✖
Mus musculus GLO1  54  GLTLLQKLDFPAMKFSLYFLAYEDKNDIPKDKSEKTAWTFSRKATLELTHNWG 106
                                                         *    *
                                                              ✹
                                                              ✖

Homo sapiens GLO1  107 TEDDETQSYHNGNSDPRGFGHIGIAVPDVYSACKRFEELGVKFVKKPDDGKMK 159
Mus musculus GLO1  107 TEDDETQSYHNGNSDPRGFGHIGIAVPDVYSACKRFEELGVKFVKKPDDGKMK 159

Homo sapiens GLO1  160 GLAFIQDPDGYWIEILNPNKMATLM 185
Mus musculus GLO1  160 GLAFIQDPDGYWIEILNPNKIATII 185
```

B.

Phosphorylation sites predicted: Ser: 4 Thr: 2

Serine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	8	PQPPSGGLT	0.664	*S*
Sequence	18	EAALSCCSD	0.441	.
Sequence	21	LSCCSDADP	0.329	.
Sequence	26	DADPSTKDF	0.997	*S*
Sequence	45	DPKKSLDFY	0.645	*S*
Sequence	69	IMKFSLYFL	0.032	.
Sequence	94	AWALSRKAT	0.980	*S*
Sequence	114	DETQSYHNG	0.431	.
Sequence	120	HNGNSDPRG	0.193	.
Sequence	137	PDVYSACKR	0.009	.

Threonine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	12	SGGLTDEAA	0.152	.
Sequence	27	ADPSTKDFL	0.033	.
Sequence	35	LLQQTMLRV	0.053	.
Sequence	50	LDFYTRVLG	0.013	.
Sequence	56	VLGMTLIQK	0.041	.
Sequence	98	SRKATLELT	0.726	*T*
Sequence	102	TLELTHNWG	0.041	.
Sequence	107	HNWGTEDDE	0.768	*T*
Sequence	112	EDDETQSYH	0.013	.
Sequence	182	NKMATLM--	0.041	.

C.

Phosphorylation sites predicted: Ser: 6 Thr: 2

Serine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	8	PQPASSGLT	0.482	.
Sequence	9	QPASSGLTD	0.640	*S*
Sequence	18	ETAFSCCSD	0.695	*S*
Sequence	21	FSCCSDPDP	0.267	.
Sequence	26	DPDPSTKDF	0.997	*S*
Sequence	45	DPKKSLDFY	0.645	*S*
Sequence	69	AMKFSLYFL	0.023	.
Sequence	86	PKDKSEKTA	0.986	*S*
Sequence	94	AWTFSRKAT	0.963	*S*
Sequence	114	DETQSYHNG	0.431	.
Sequence	120	HNGNSDPRG	0.193	.
Sequence	137	PDVYSACKR	0.009	.
Sequence	201	PDVYSACKR	0.009	.

Threonine predictions

Name	Pos	Context (v)	Score	Pred
Sequence	12	SSGLTDETA	0.054	.
Sequence	15	LTDETAFSC	0.046	.
Sequence	27	PDPSTKDFL	0.092	.
Sequence	35	LLQQTMLRI	0.013	.
Sequence	50	LDFYTRVLG	0.013	.
Sequence	56	VLGLTLLQK	0.025	.
Sequence	89	KSEKTAWTF	0.132	.
Sequence	92	KTAWTFSRK	0.089	.
Sequence	98	SRKATLELT	0.726	*T*
Sequence	102	TLELTHNWG	0.041	.
Sequence	107	HNWGTEDDE	0.768	*T*
Sequence	112	EDDETQSYH	0.013	.
Sequence	182	NKIATIIDP	0.286	.
Sequence	246	NKMATLM--	0.041	.

Supplementary figure 1. Prediction of phosphorylation sites in glyoxalase I.
A. Alignment of the amino acid sequence of human and mouse GLO1 as found in UniProtKB/Swiss-Prot. Potential phosphorylation sites as predicted by Netphos software are indicated in bold. The potential phosphorylation sites that were described for human GLO1 in (1) are indicated within boxes. Predicted phosphorylation sites for CaMKII (*), GSK-3 (+), PKA (✹) and Akt (✖) were determined using Motifscan software (low stringency).
B and C. Scores of predicted phosphorylation sites as obtained using Netphos software for human **(A)** and mouse **(B)** GLO1.

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Overview of known phosphorylations of GLO1.

	Stimulus	Kinase	Cell line	*In vitro* phosphorylation	Change in GLO1 activity	Reference	Of note
mammalian cell lines	TNF	PKA	L929	no	no	(1)	established using specific inhibitors
		CaMKII	L929, Hek293	yes	/	de Hemptinne, unpublished results; chapter 7 of this thesis	established using specific inhibitors confirmed with phospho-specific antibody
	cell cycle	PI3K/Akt	Ba/F3	/	no	chapter 8 of this thesis	established using specific inhibitors
		mTOR	Ba/F3	/	no	chapter 8 of this thesis	established using specific inhibitors
	IL-3 starvation	GSK-3β	Ba/F3	no	no	chapter 6 and chapter 7 of this thesis	established using specific inhibitors confirmed using coexpression in HEK293
	Serum starvation	/	HL-60	/	/	chapter 6 of this thesis	
		/	NIH3T3	/	/	chapter 6 of this thesis	
yeast	addition of mating factor		*S. cerevisiae*	/	+	(2,3)	
plants	addition of gibberelin	CDPK	rice leaf	/	/	(4)	
	overexpression of SnRK2.8	SnRK2.8	*Arabidopsis*	yes	+	(5)	shares its consensus sequence with CaMKII SnRK family are plant homologues of AMPK

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Part II:
Experimental Data and Results

Chapter 9:

The Receptor for Advanced Glycation Endproducts (RAGE) is regulated at several levels during the cell cycle

A novel proteolytically generated form of RAGE

Chapter 9: The Receptor for Advanced Glycation Endproducts (RAGE) is regulated at several levels during the cell cycle

A novel proteolytically generated form of RAGE

Introduction

The receptor for advanced glycation endproducts (RAGE) was first isolated as a receptor for advanced glycation endproducts (AGEs), which are adducts formed by glycation and subsequent oxidation of proteins (1,2). RAGE is a member of the immunoglobulin superfamily of receptors and has been suggested to be involved in embryonic development, since it has been reported to be expressed during development, repressed in adulthood and re-expressed in several pathologies (3). Although the original ligand to be identified for RAGE were AGEs, a remarkable feature of RAGE is its ability to be activated by different other specific ligands such as amphoterin (often referred to as high mobility group box I (HMGB1) DNA-binding protein) (4), several members of the S100 Ca^{2+}-binding protein family (5,6), and β-sheet fibrils and amyloid-β-peptide (7,8). Multifarious signaling pathways are induced upon RAGE activation, including the reactive oxygen species (ROS)-dependent activation of mitogen-activated protein kinases (MAPKs) (9-12), other MAPK pathways, such as the p38 pathway and the stress-activated protein/c-Jun N-terminal kinase (SAPK/JNK) (11,13,14), Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (15,16), the small GTPases cdc42 and rac (17) and the phosphoinositol-3-kinase (PI3K) pathway (18,19). To date, the full range of different signaling pathways initiated by RAGE activation has not been elucidated. Also the intracellular factors that bind to the cytoplasmic tail of RAGE remain to be determined. The outcome of RAGE signaling is highly dependent on the cell type, the available ligands and their concentrations, and the different levels and isoforms of RAGE, all of this adding further to the complexity of RAGE signaling.

RAGE activation has been implicated in cell cycle related signaling. Although proliferation and differentiation are sequential and mutually exclusive events, RAGE has been involved in both, depending on the ligand and cell type. RAGE activation is often considered to promote tumor growth and metastasis, and the level of RAGE expression is regarded as a measure for metastatic potential of tumor cells (13,20-29). In neuronal cells it was reported that both AGEs as well as S100B could elicit a mitogenic response through RAGE, hereby upregulating cyclin D_1 expression and urging cells into S phase in a p42/44 MAPK dependent manner (30,31). Also in other cell types, such as fibroblasts (29), osteoblasts (9), smooth muscle cells (26) and mesangial cells (23), activation of RAGE induced MAPK activation and subsequent cell growth.

However, in spite of the elaborate evidence for a role of RAGE in proliferation, this has been contradicted by several reports. In melanoma and neuroblastoma cells RAGE activation by amphoterin led to inhibition of metastasis *in vivo* (32). Further it has been reported that overexpression of full-length RAGE (flRAGE) did not promote metastasis of lung cancer cells *in vivo*, but instead showed a diminished proliferation *in vitro* (33,34). It was also shown that higher tumor stages and several other metastatic tissues displayed a downregulation of RAGE (35). The same was observed after overexpression of RAGE in rhabdomyosarcoma cells, where binding of amphoterin to RAGE stimulated differentiation in a cdc42 and p38 dependent manner (36). In the development of diabetic nephropathy it was observed that AGE interaction with RAGE activated STAT5–mediated upregulation of $p21^{waf}$ and consequent growth arrest (37). Furthermore, both amphoterin and AGE-BSA are able to stimulate maturation of dendritic cells (38,39).

The ambiguous function of RAGE in the regulation of cell cycle may find its basis partly in the presence of different isoforms of RAGE. RAGE signaling is not mediated by the action of a single type of RAGE receptor, but relies on the interplay of different factors, such as the level of RAGE expression, the presence and relative concentrations of different ligands, the large number of different isoforms and their possible splice variants, and possible posttranslational modifications of RAGE (reviewed in (40,41)). Furthermore, the outcome of RAGE signaling is, above all, highly dependent on the cell type. We commenced the study for the involvement of RAGE in the regulation of the cell cycle by studying the differential expression of RAGE isoforms. A twofold approach was used. Cells were synchronized and the different phases and phase transitions were studied. Alternatively, G_1 arrest was induced in Ba/F3 cells or Hek293 cells by IL-3 depletion or serum starvation respectively.

Results

Ba/F3 cells exhibit different isoforms of RAGE

First, we studied the expression of the different isoforms of RAGE. Several different isoforms have already been described, the major isoforms being flRAGE, the soluble RAGE (sRAGE) which lacks the transmembrane domain and the cytoplasmic domain and the dominant negative RAGE (dnRAGE) which lacks the cytoplasmic domain (reviewed in (40)). Several other isoforms have also been described, but their expression is cell type dependent (41,42). The antibody used was an antibody raised against the C-terminus of RAGE (C-t-Ab; Abcam). Soluble isoforms of RAGE would therefore be missed in our study since they have alternative C-termini (43,44). However, the specificity of this antibody was confirmed by the specific recognition of RAGE on Western blots from wild type mouse lung

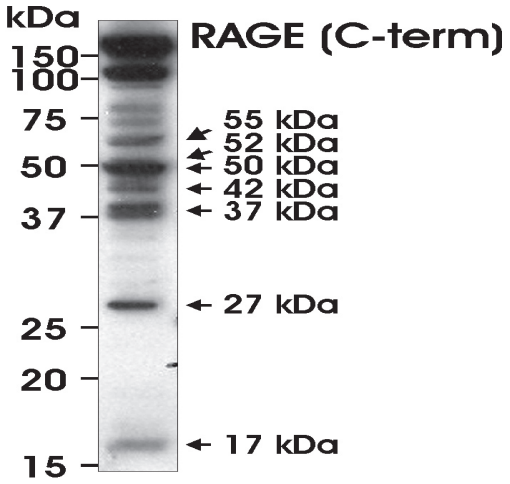

Figure 9.1. RAGE isoforms in Ba/F3 cells.
Isoforms of RAGE in Ba/F3 cells. Exponentially growing Ba/F3 cells were lysed in CEB. 40 µg of protein was separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-terminus-directed anti-RAGE antibody (C-t-Ab; Abcam).

extracts, whereas RAGE was not detected in lung extracts from RAGE$^{-/-}$ mice (Salliau et al., submitted).

In total lysates from asynchronously growing Ba/F3 cells several isoforms are present (Figure 9.1). The band migrating at 50 kDa is $_{FL}$RAGE (44,45). $_{FL}$RAGE can be severely glycosylated, which is known to result in bands of higher molecular weight, such as the 52 kDa, the 55 kDa and the two bands near the 75 kDa marker (44,46). Further we observed the presence of several high molecular weight bands. These high molecular weight bands are hypothesized to result from oligomerization or complexes with other proteins (43,47). Also a band of 42 kDa is present in Ba/F3 cells. This band has been described as a N-terminal truncated form of RAGE (44). Further bands of 37 kDa and 27 kDa can be observed, which are hypothesized to be processed and truncated forms of RAGE ((43); Salliau et al., submitted). Furthermore, a band of approximately 17 kDa can be seen. This is a novel proteolytic processed isoform and will be discussed below.

It should be noted that RAGE not only appears on the plasmamembrane. Some isoforms, among which $_{FL}$RAGE, also appear in the cytosol and the nucleus (Salliau et al., submitted).

RAGE expression is altered during the cell cycle

We then analysed whether different phases are associated with differences in the expressed RAGE isoforms. G_1 arrest was induced by IL-3 depletion and G_2/M arrest by addition of nocodazole. The analysis of DNA distribution on FACS confirmed the respective phases arrests (Figure 9.2.B). Lysates of cells arrested in either G_1 or G_2/M were compared with lysates of asynchronously growing cells. Nocodazole-treated cells showed an upregulation of the expression of a band with an apparent molecular weight of 42 kDa (Figure 9.2.A lane 2, indicated with an arrowhead). This band corresponds to the band that has been described to be a N-terminal truncated isoform, which lacks the N-terminal V-

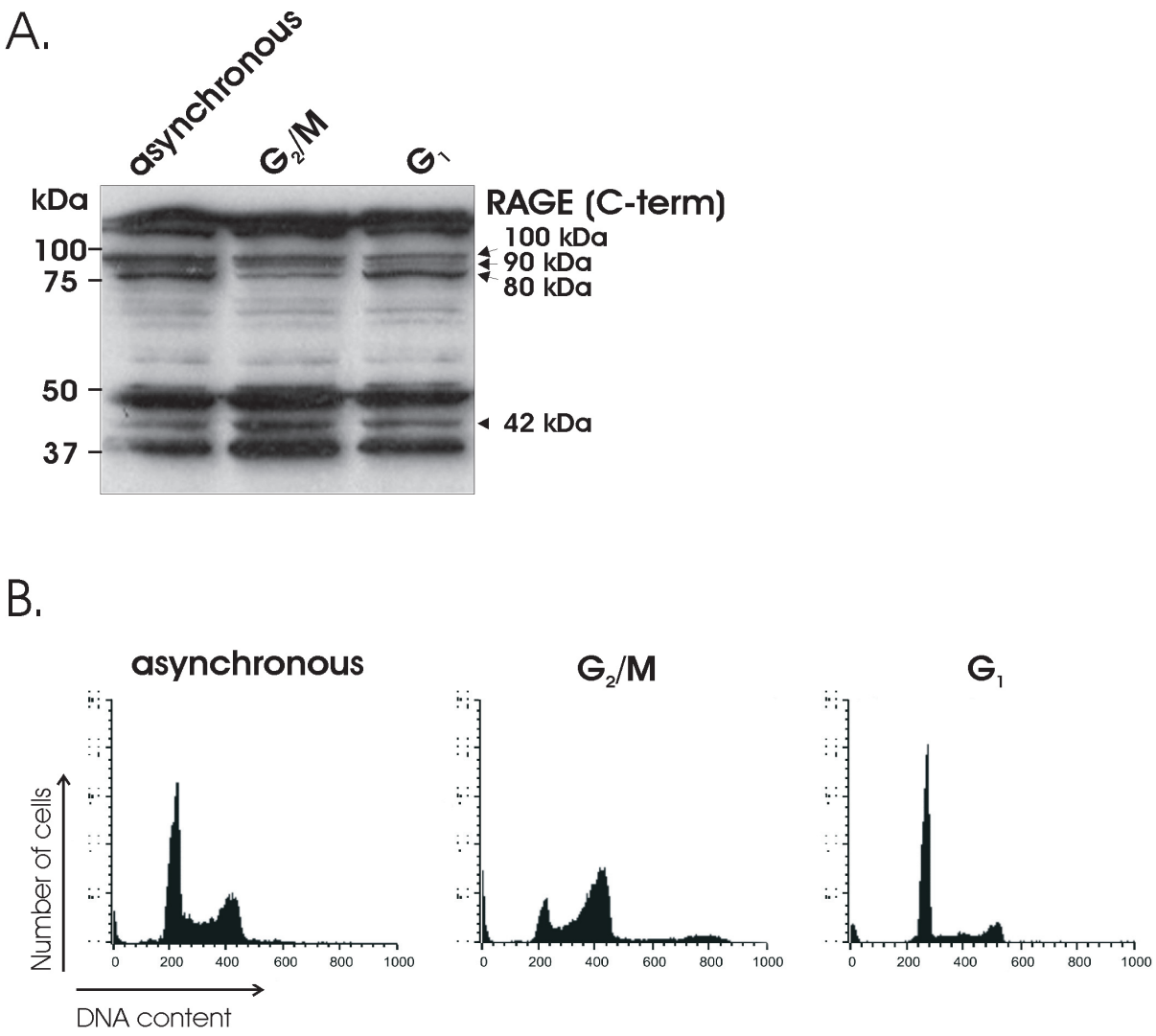

Figure 9.2. Cell cycle phase specific RAGE expression.
A. Analysis of RAGE in different phases. G_1 arrest was induced by IL-3 starvation of Ba/F3 cells and G_2/M arrest was induced by addition of nocodazole. Cells were lysed in CEB, equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab. **B.** Analysis of the cell cycle. Induction of the different phase arrests were monitored by analysis of the DNA content by flow cytometry.

type immunoglobulin-like domain (43,44). This upregulation was not observed in the cells that were arrested in the G_1 phase (Figure 9.2.A, lane 3). Also the high molecular weight isoforms displayed different levels of expression when the G_1 and G_2/M arrested cells were compared with asynchronously growing cells. These high molecular weight bands were specific for the cytosolic fraction and could not be observed in nuclear fractions (data not shown). In the lysate of the asynchronously growing culture, two intense protein bands are observed between with molecular weights of approximately 80 kDa and 100 kDa, with in between them a less intense band of approximately 90 kDa (Figure 9.2.A, indicated with arrows). The 80 kDa band was downregulated in the G_2/M-arrested cells, whereas in the G_1–arrested cells the inverse was observed, and the 90 kDa band and the 100 kDa band were expressed substantially less.

In this context, we additionally studied whether the change of the expressed

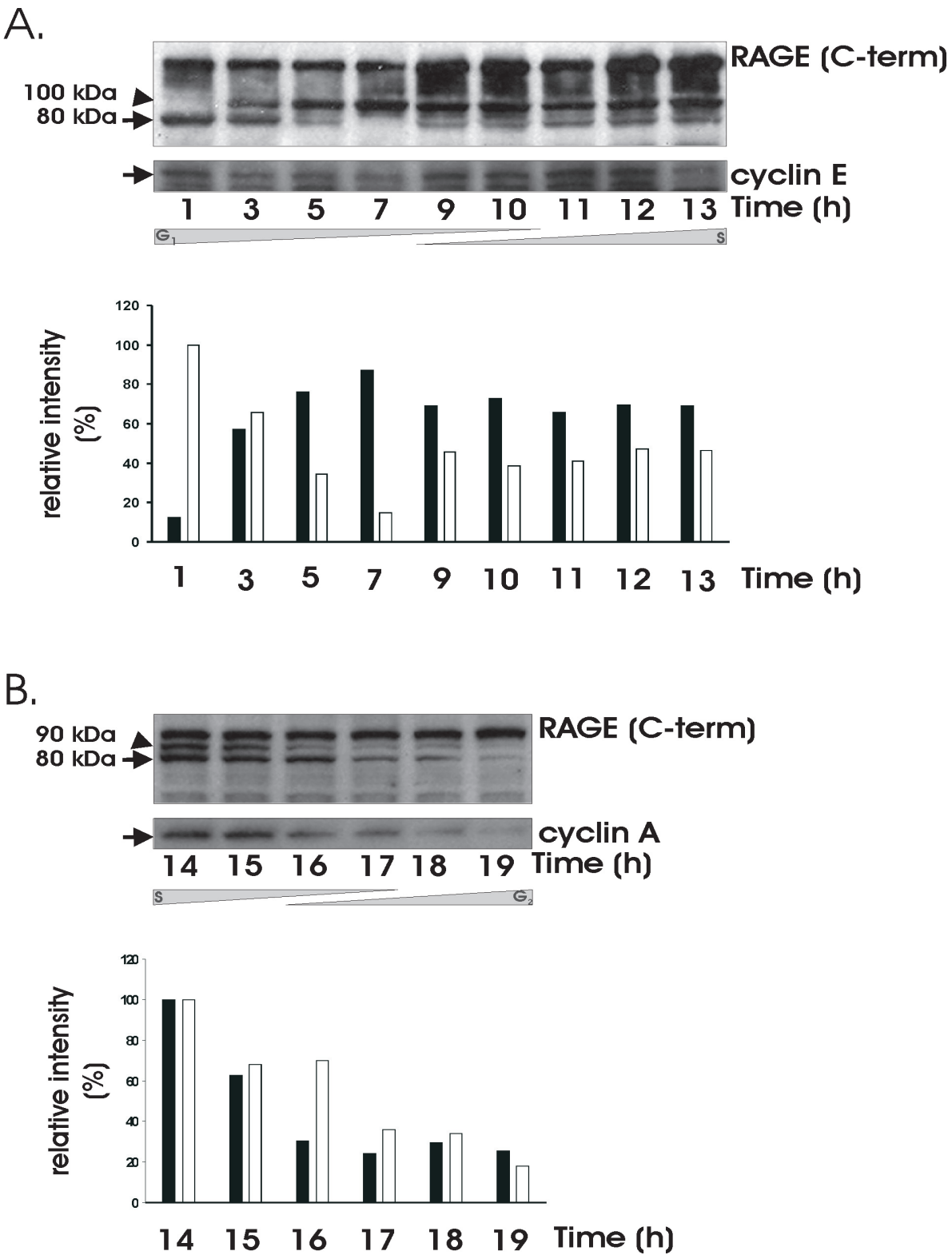

Figure 9.3. Differential expression of RAGE at phase transitions.
A. Isoforms of RAGE at G_1-S transition. **B.** Isoforms of RAGE at S-G_2/M transition. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by readdition of IL-3. This time point was designated 0 hours. Cells were lysed in CEB at indicated time points and equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab. Blots were subsequently stripped and reprobed with an anti-cyclin E antibody (Abcam) **(A)** or an anti-cyclin A antibody (Santa Cruz) **(B)**. Protein band intensities were measured using Genetools software (Westburg) and corrected for equal concentrations.The relative intensities of the 100 kDa isoform and the 90 kDa isoform are represented by black and white bars respectively in **(A)** and the 90 kDa isoform and the 80 kDa isoform are represented by black and white bars respectively in **(B)**.

isoforms coincided with the transition of different phases. Hence, cells were synchronized by IL-3 withdrawal and lysates were made after the appropriate time intervals after re-addition of IL-3 to release cell cycle arrest (indicated by hours after re-addition of IL-3). The transgression of the cells through the different phases of the cell cycle was observed by analysis of the DNA distribution on FACS and the transition of G_1 to S was confirmed by the rise of the level of cyclin E (Figure 9.3.A, lower panel indicated with an arrow), a marker for late G_1 and S phase. Immunoblotting of the obtained lysates endorsed the observation that differential expression of RAGE isoforms coincides with specified phases (Figure 9.3). When the progression through G_1 phase towards S phase was studied (Figure 9.3.A), we observed a strong decline of the 80 kDa isoform of RAGE throughout G_1, reaching its lowest level at 7 hours, where it only retains 15 % of its initial intensity (Figure 9.3.A, upper panel indicated with an arrow and chart, white bars). 9 hours after the re-addition of IL-3 to remove the cell cycle block, this 80 kDa isoform is then upregulated to approximately 40 % of its initial level. This time corresponds to the beginning of the S phase. Concomitantly with the downregulation of 80 kDa band, the 100 kDa isoform of RAGE (Figure 9.3.A) is upregulated throughout G_1, reaching its maximum level at the end of G_1, when expression levels of the 80 kDa isoform is lowest, and its expression level slightly decreases after the onset of S, 9 hours after the release of the cell cycle block (Figure 9.3.A, upper panel indicated with an arrowhead and chart, black bars).

Also at the transition of the S to the G_2 phase was studied. While 100 kDa RAGE isoform remained constant throughout this study, a rapid decline was observed in the

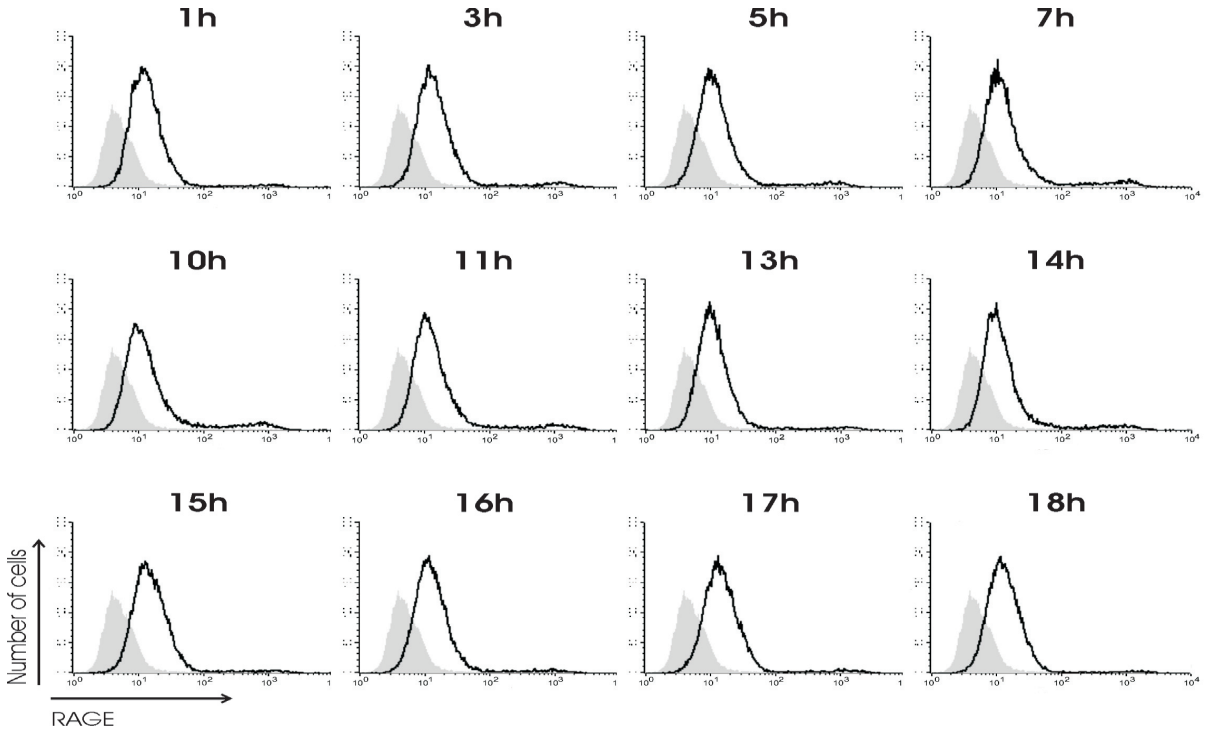

Figure 9.4. Cell surface staining for RAGE expression.
Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by readdition of IL-3. This time point was designated 0 hours. Staining occurred at indicated timepoints using a N-terminus-directed anti-RAGE antibody (Santa Cruz). Gray filled histograms represent isotype control and black histograms represent RAGE expression.

90 kDa band, reaching its lowest levels of approximately 20 % of the initial intensity 16 or 17 hours after the release of the cell cycle arrest. This time point coincides with the transgression from the S phase to the G_2 phase (Figure 9.3.B, upper panel indicated with an arrowhead and chart, black bars). The 80 kDa RAGE isoform also decreased during S phase, and reached minimum levels after 19 hours in late G_2 phase (Figure 9.3.B, upper panel indicated with an arrow and chart, white bars). S to G_2 transition was marked by the decrease of the level of cyclin A, a marker for S phase (Figure 9.3.B, lower panel indicated with an arrow).

Subsequently, we measured the membrane expression levels of RAGE on FACS. In spite of the strong alterations in high molecular weight isoforms of RAGE during the cell cycle, we could not correlate these observations to differences in the membrane expression levels (Figure 9.4).

In summary, we show that expression of RAGE is strongly regulated throughout the cell cycle. This regulation occurs mainly intracellularly and is not related to different levels of localization of RAGE on the plasmamembrane.

RAGE is processed into a 17 kDa isoform during G_1 arrest

As shown in Figure 9.1 we detected a novel isoform of RAGE of approximately 17 kDa in asynchronously growing Ba/F3 cells. This isoform, which will be designated 17 kDa RAGE, has not been reported to date. Cellular fractionation revealed this isoform was present predominantly in the nuclear and membrane fractions, and could be distinguished much less in cytosolic fractions (data not shown).

To examine whether this novel isoform could be connected to a particular phase, we compared lysates of asynchronously growing Ba/F3 cells with lysates from Ba/F3 cells that were arrested in G_1 by IL-3 depletion and nocodazole-induced G_2/M arrested Ba/F3 cells. As shown in Figure 9.5.A, a strong induction of 17 kDa RAGE was observed specifically in the G_1 arrested cells (Figure 9.5.A, arrow). This induction was specific, since no upregulation of FLRAGE was observed (Figure 9.5.A, arrowhead). The induction of G_2/M arrest and G_1 arrest by nocodazole and IL-3 depletion respectively were confirmed by analysis of the DNA-distribution on FACS (Figure 9.5.B). Subsequently Ba/F3 cells were synchronized by IL-3 depletion and re-addition of IL-3 and RAGE expression was studied throughout the cell cycle. The 17 kDa RAGE isoform could also be detected in the S phase (Figure 9.5.C), where it seemed to be up- and downregulated in an alternating fashion (Figure 9.5.C, arrow), in contrast to FLRAGE, which appears to remain constant (Figure 9.5.C, arrowhead).

Together we show the existence of a novel isoform of RAGE, which is strongly induced during G_1 arrest, although it also occurs during the cell cycle.

Kinetics of 17 kDa RAGE generation

Since prolonged absence of IL-3 also leads to the induction of apoptosis in Ba/F3

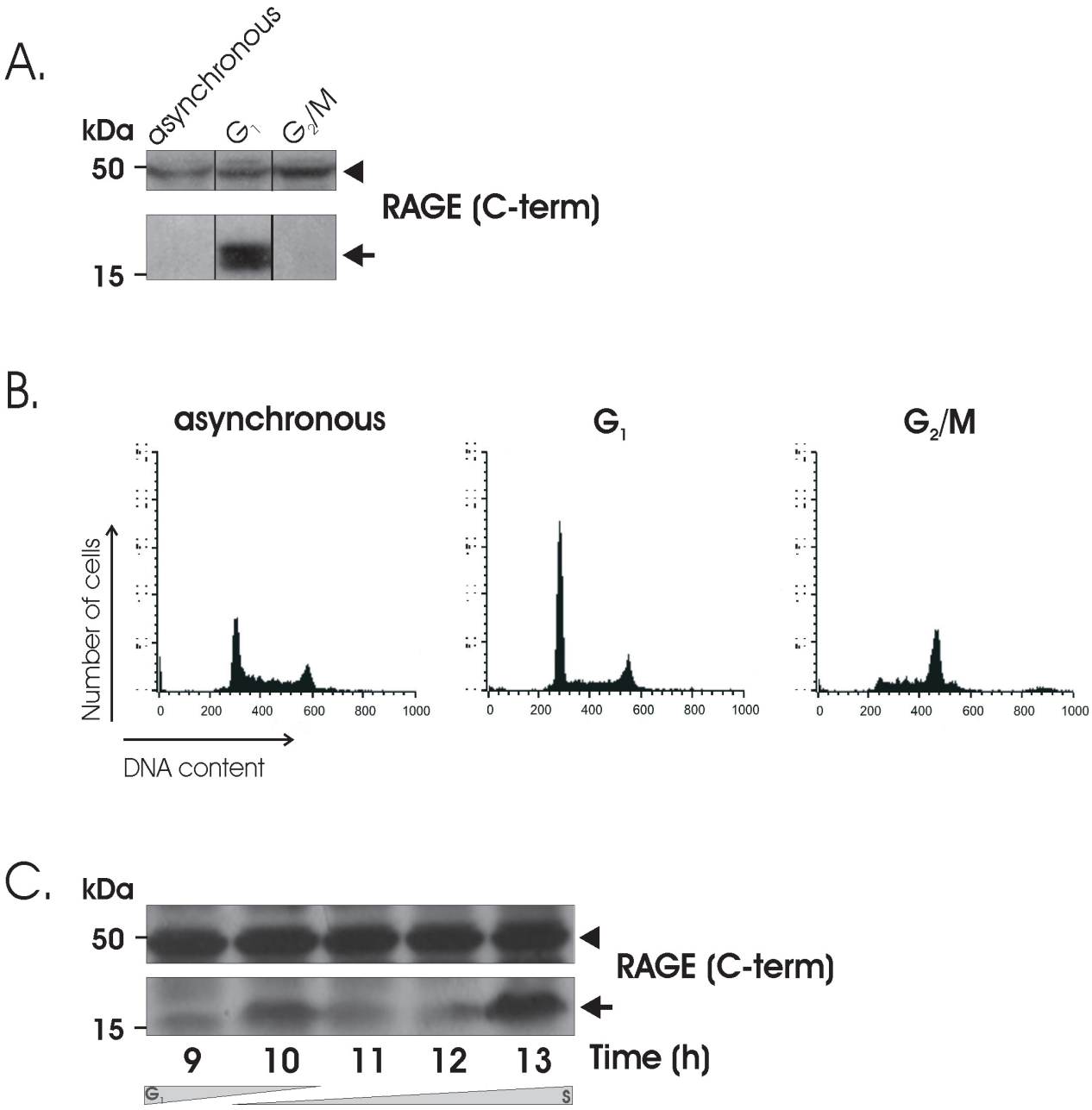

Figure 9.5. Novel 17 kDa isoform of RAGE.
A. Analysis of 17 kDa RAGE in different phases. G_1 arrest was induced by IL-3 starvation of Ba/F3 cells and G_2/M arrest was induced by addition of nocodazole. Cells were lysed in CEB, equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab. **B** Analysis of the cell cycle. Induction of the different phase arrests were monitored by analysis of the DNA content by flow cytometry. **C.** 17 kDa RAGE at G_1-S transition. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by re-addition of IL-3. This time point was designated 0 hours. Cells were lysed in CEB at indicated time points equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab.

cells we next studied the kinetics of 17 kDa induction in Ba/F3 cells. In the experiment shown, induction of 17 kDa RAGE was detected after 6 hours of IL-3 depletion (Figure 9.6), well before the onset of apoptosis and further increased at later time points.

We were also interested in whether addition of exogenous methylglyoxal (MG) could influence the formation of 17 kDa RAGE. MG is a very potent glycating agent of proteins and the most important precursor of advanced glycation endproducts (AGEs) (48,49). RAGE is known to be upregulated in the presence of its ligands, providing a positive feedback loop when activated (50,51). Moreover, it has been shown that exogenous MG is strongly synergistic with the depletion of IL-3 in the induction of apoptosis in Ba/F3 cells (Audiffret et al., submitted, see also chapter 6). IL-3 depletion-induced generation of 17 kDa RAGE was already observed after 3 hours upon addition of exogenous MG (Figure 9.6). Furthermore, the level of 17 kDa RAGE, induced by IL-3 depletion, was substantially higher than in the absence of exogenous MG.

17 kDa RAGE is also generated in Hek293 cells

To corroborate our findings in Ba/F3 cells we tested if 17 kDa RAGE could also be detected in other cell lines. Hek293 cells were therefore transiently transfected with a vector expressing $_{FL}$RAGE and cells were depleted of serum 24 hours after transfection. Cells were lysed 24 hours after depletion and analyzed by immunoblotting with C-t-Ab (Figure 9.7.A). In control cells and mock transfected cells, the 17 kDa RAGE could not be detected. However, when cells were transfected with $_{FL}$RAGE and cultured in the absence of serum, the formation of 17 kDa RAGE was induced. This shows that the 17 kDa RAGE is not restricted to a specific cell line or species (mouse and human), hereby suggesting induction of 17 kDa RAGE is a general mechanism. In addition, 17 kDa RAGE was also observed in the mouse β-cell line MIN6 (Toepoel et al., unpublished results).

Several mechanisms can be responsible for the formation of 17 kDa RAGE, the two most plausible are alternative splicing and proteolytic cleavage. To examine these

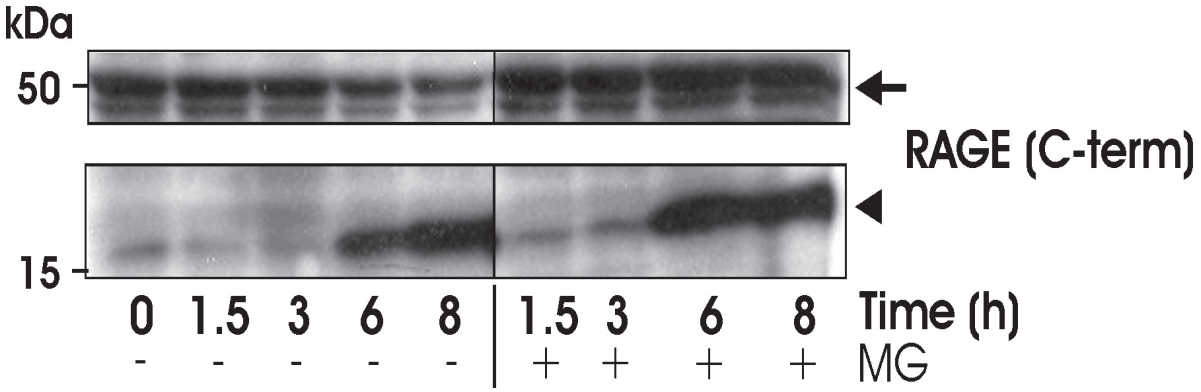

Figure 9.6. 17kD RAGE induction by exogenous MG.
G_1 arrest was induced by depletion of IL-3 and exogenous MG was added. Cells were lysed in CEB after indicated time intervals, equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab.

mechanisms, Hek293 cells were transiently transfected with vectors containing the cDNA encoding C-terminal flagged RAGE and N-terminal flagged RAGE (designated RAGE-C-flag and N-flag-RAGE respectively). As expected, analysis of immunoblots with an antibody directed against the flag-tag showed high levels of overexpression of RAGE with either construct. However, the generation of 17 kDa RAGE could be observed using an antibody raised against the flag-tag, only when this tag was attached at the C-terminus of RAGE, hereby validating the observations made with the C-terminus directed anti-RAGE antibody (Figure 9.7.B). Most isoforms described to date have been reported to result from alternative splicing and posses different C-termini (reviewed in (40,41)). However, since 17 kDa RAGE can also be generated by overexpression of RAGE using a cDNA, alternative splicing can be excluded.

In the lysates of the cells transfected with N-flag-RAGE an additional band could be observed with a molecular mass of approximately 45 kDa (Figure 9.7.B, arrowhead). This band could very well be the remainder of the cleaved RAGE. The molecular weight differs from the band seen near the 75 kDa marker, which probably represents the glyosylated full length flag-tagged RAGE, and the difference in molecular weight could be explained by the removal of the 17 kDa RAGE part. This band was absent in the lysates of cells transfected with the RAGE-C-flag, endorsing that this isoform had an alternative C-terminus, but an intact N-terminus. Several reports indicate the involvement of protein cleavage in the formation of a soluble form of RAGE in mice (43,52). Moreover, recently it has been reported that limited proteolysis of RAGE with trypsin also gives rise to low molecular weight bands with comparable migratory properties (47), hereby further emphasizing the hypothesized proteolytic cleavage of RAGE. It should be noted however that 17 kDa was present even when serum was not depleted.

Inhibition of caspases partially inhibits the generation of 17 kDa RAGE

Our data excludes alternative splicing as a mechanism to generate 17 kDa RAGE and suggest the involvement of proteolytic cleavage. Proteolytic cleavage was subsequently further explored by using several specific inhibitors. Initial experiments with different protease inhibitors showed no effects (data not shown). Since IL-3 starvation of Ba/F3 cells also leads to apoptosis, we sought to determine if the inhibition of caspase activation could inhibit formation of 17 kDa RAGE. When Ba/F3 cells were starved of IL-3, 17 kDa RAGE formation was induced, as observed by analysis of lysates using immunoblotting with C-t-Ab. In the presence of the pan-caspase inhibitor Z-VAD-FMK however generation of 17 kDa RAGE was partially inhibited (Figure 9.8.A, arrow). Other isoforms, such as the 50 kDa from were unaffected (Figure 9.8.A, arrowhead). Subsequently Hek293 cells were transiently transfected with pCAGGS-RAGE-C-flag and depleted of serum in the presence of Z-VAD-FMK. As observed by immunoblotting with an antibody against flag, inhibition of

caspase activation led to a decrease in 17 kDa RAGE (Figure 9.8.B). Formation of 17 kDa RAGE was inhibited by Z-VAD-FMK to the same extent in Hek293 cells as in Ba/F3 cells.

Interestingly, in the modelsystem with RAGE overexpression in Hek293, a strong induction of 17 kDa was observed, even in the presence of serum (Figure 9.7.B and 9.8.B).

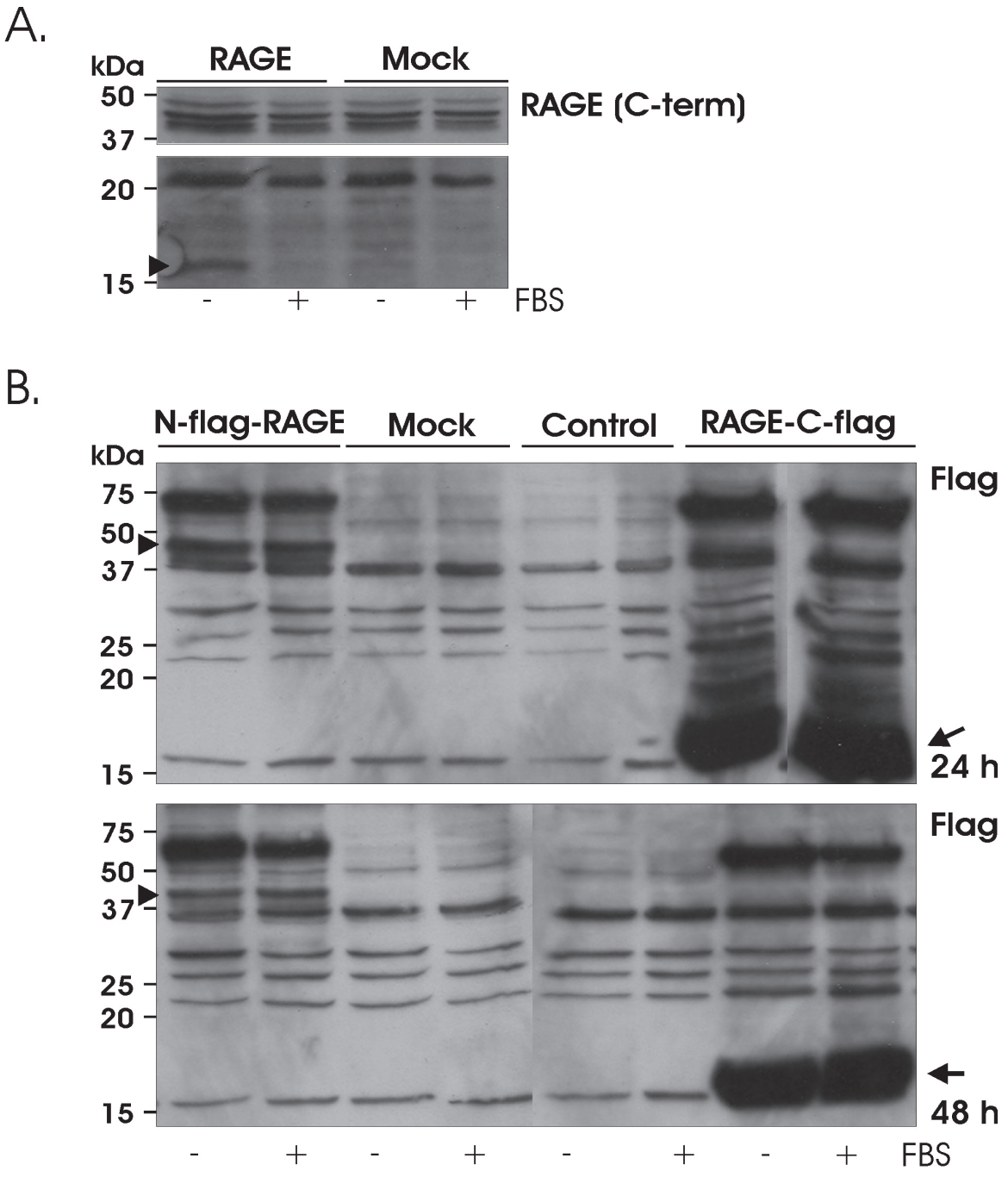

Figure 9.7. Generation of17 kD RAGE in Hek293 cells.
A. Presence of 17 kDa RAGE in Hek293 cells. Hek293 cells were transfected with expression vectors containing full length RAGE. After 24 hours, transfected cells were serum-starved over night. Lysates were made in CEB and equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-t-Ab.
B. Formation of 17 kDa RAGE in Hek293 cells. Hek293 cells were transfected with expression vectors containing either N-flag-RAGE or RAGE-C-flag. After 24 hours, transfected cells were serum-starved for 1 or 2 days. Lysates were made in CEB and equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using an anti-flag antibody (Sigma Aldrich).

A possible hypothesis would be it is induced by the G_1 arrest caused by contact inhibition (53-56). In the presence of serum, however, addition of Z-VAD-FMK led to a strong rise in the level of 17 kDa RAGE.

Analysis of posttranslational modification of RAGE during the cell cycle using 2-dimensional gel electrophoresis

In order to further examine the possible involvement of RAGE in the cell cycle we also analyzed whether posttranslational modification of RAGE occurs during the cell cycle, using 2-dimensional gel electrophoresis (2-DE). The cell cycle of Ba/F3 cultures was synchronised in the G_1 phase by IL-3 depletion over night and subsequently released from this block by re-addition of IL-3. Cells were lysed at different time points after the re-addition of IL-3, when cells were in different phases of the cell cycle.

Immunoblots are shown in Figure 9.9.A which were detected with C-t-Ab. Although the most acidic isoform remains constant, the displayed isoforms strongly alternate. The upper panel shows lysates of a Ba/F3 culture in G_1. Three isoforms can be distinguished at this time, the two most acidic however are not well separated and appear almost fused. In the S phase, the middle spot disappeared, and seems to have merged with the most acidic spot. In the G_2/M phase the pattern of the two most acidic isoforms strongly resembles the pattern in G_1, but the basic isoform had vanished. Early in the G_1 phase of the following cycle, the most basic isoform reappears and in late G_1 two new spots arise, more basic than the basic spot observed so far. The newly formed basic spots then seem to shift again towards the more acidic side during the S phase.

To date the only posttranslational modification described for RAGE is glycosylation (43-45). Differences in glycosylation have however been shown to result in differences in molecular weight, which are not observed here. Unpublished data from Salliau et al. suggest phosphorylation might play a part in the alternating pattern observed. The short cytoplasmic domain of RAGE contains 2 predicted sites for phosphorylation, and when RAGE was purified from metabolically labeled L929 cells, specific spots were observed on autoradiographs from 2D gels (Salliau et al., unpublished data). Since these patterns observed on 2-dimensional electrophoresis strongly resemble the patterns observed for GLO1 and arise analogous with the modifications of GLO1 (see chapter 6 and data not shown), it is conceivable that phosphorylation indeed is responsible for at least part of the modifications observed.

To further substantiate the cell cycle-dependent modification of RAGE, we then investigated if the induction of G_1 arrest by the depletion of IL-3 also induced alterations in the pattern of displayed isoforms of RAGE (Figure 9.9.B). We depleted Ba/F3 cells of IL-3

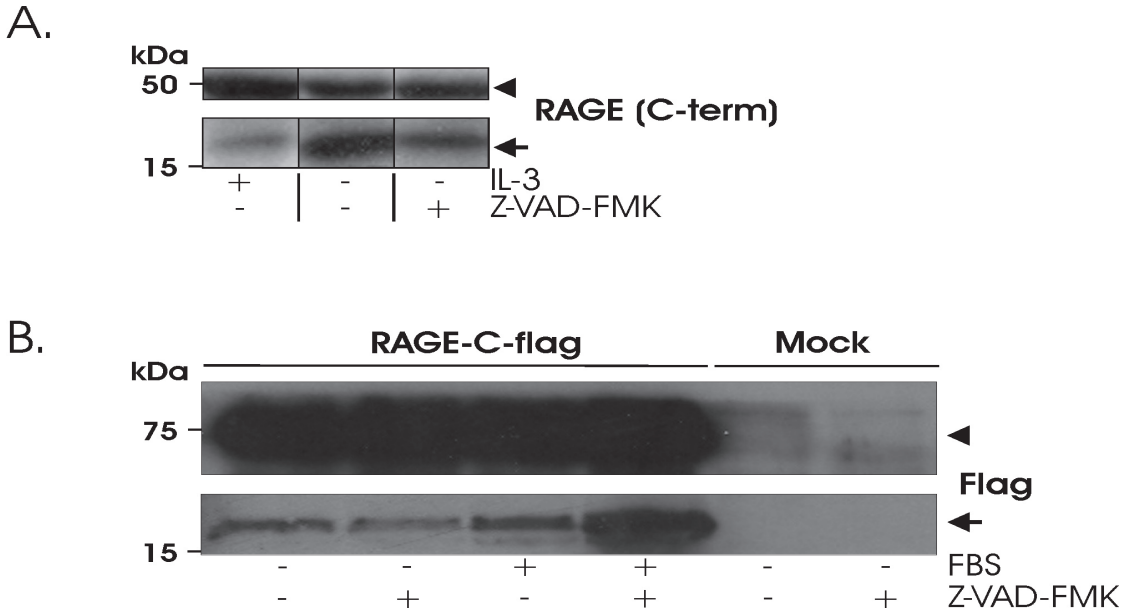

Fig. 9.8. Inhibition of the generation of 17 kDa RAGE.
A. 17kDa RAGE generation in Ba/F3 cells. Ba/F3 cells were IL-3-depleted and Z-VAD-FMK (20 μM) was added. After 24 hours, cells were lysed in CEB and equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using the C-terminus-directed anti-RAGE antibody. **B.** Generation of 17 kDa in Hek293 cells. Hek293 cells were transfected with expression vectors containing RAGE-C-flag. After 24 hours, transfected cells were serum-starved over night and Z-VAD-FMK (20 μM) was added. Lysates were made in CEB and equal protein concentrations were separated by SDS-PAGE and transferred onto PVDF using electrotransfer. Expression of different isoforms of RAGE was analysed using an anti-flag antibody.

and lysates were made after the indicated time intervals. Asynchronously growing Ba/F3 cells displayed four isoforms, the pattern showed strong similarities with the pattern seen in late G_1 (Figure 9.9.A), but a fourth spot was also detected, situated between the most acidic isoform and the duplet of more basic isoforms. After 1 and 3 hours the second most acidic isoform vanishes and the most basic spot shits towards the more basic side. After 7 hours of IL-3 starvation only three isoforms remain and the observed pattern strongly resembles the pattern observed in late G_1.

Discussion

Activation of RAGE is not completely understood and knowledge is only fragmented. RAGE expression is high during development, repressed in mature tissues, and upregulated again in the course of several pathologies such as in different cancers, so RAGE signaling has often been involved in the regulation of cell cycle (3). The involvement of RAGE in proliferation however seems rather dualistic, since RAGE activation has been shown to be involved in proliferation (9,23,26,30,31) as well as in growth arrest and differentiation (32,36-38,57,58), two processes that are consecutive and mutually exclusive.

Three major isoforms of RAGE have been described (reviewed in (40)). However, additional splice variants and isoforms keep on being discovered, sometimes with a high

specificity for certain tissues (41,42). Hence, the outcome of RAGE signaling is most likely highly tissue-specific. Moreover, it is clear that effects mediated by RAGE are not so much the result of one specific isoform of RAGE, but of a concerted interplay of the different isoforms and the relative availability of different ligands. This prompted us to study which different isoforms are present in Ba/F3 cells and whether these were regulated in a cell cycle dependent manner. Although we did not detect a difference in total RAGE expression on the membrane, RAGE expression was indeed strongly modulated throughout the cell cycle. We were able to correlate different forms of RAGE to different phases in the cell cycle and we observed modulation of these isoforms at phase transitions.

However, given the high molecular weight of the isoforms described, the observed effects can be disputable. The high molecular weight isoforms of RAGE observed are hypothesized to be oligomers or complexes containing RAGE. Such complexation and oligomerization has been described for RAGE (43,47,59). Moreover, the specificity of the antibodies we used was demonstrated earlier (Salliau et al., submitted). Our hypothesis is further emphasized by the observation of high molecular weight bands when flag-tagged RAGE was overexpressed in Hek293 cells. Immunoblotting with an antibody raised against

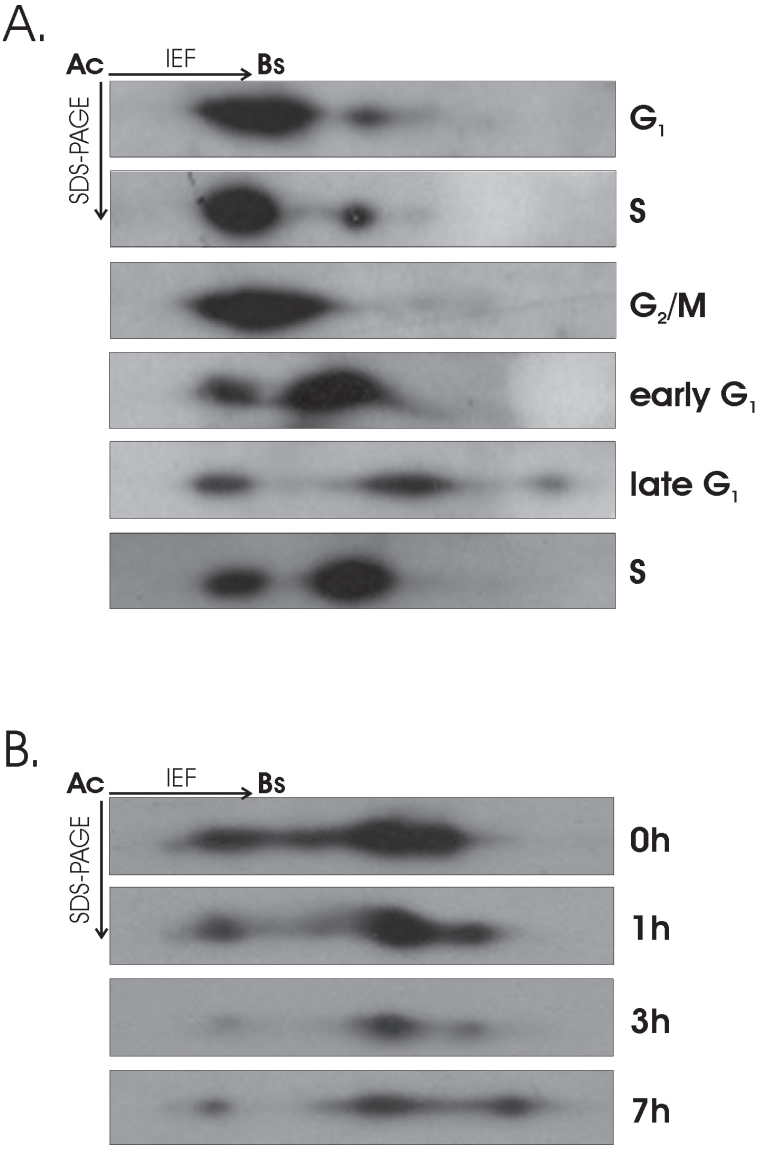

Figure 9.9. RAGE is modified during the cell cycle and IL-3 withdrawal-induced G_1 arrest.
A. RAGE is posttranslationally modified during the cell cycle. Ba/F3 cells were synchronized by IL-3 starvation over night. Cell cycle was induced by readdition of IL-3. Samples were taken in several phases of the cell cycle. Cells were lysed in CEB and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with C-t-Ab. **B.** RAGE is posttranslationally modified during IL-3 withdrawal-induced G_1 arrest. Ba/F3 cells were depleted of IL-3, cells were lysed in CEB at the indicated time points and after ethanol precipitation resuspended in 2-DE lysis buffer. Protein concentrations were determined and 200 µg of the lysates was loaded for separation by 2-dimensional gel electrophoresis (pH 4-7). Representative western blots of 2-DE gels developed with C-t-Ab.

the flag-tag also showed high molecular weight bands (data not shown).

We also demonstrated the presence of a novel low molecular weight isoform of RAGE. This isoform is strongly induced during G_1 arrest, but was present at several time points in the S phase as well. This isoform was not restricted to one specific cell type, since it could be observed in three different cell lines and two different species, mouse and human. This low molecular weight from of RAGE was most likely a product of proteolytic processing of FLRAGE since it also was formed upon overexpression from a C-terminally tagged RAGE, expressed from a cDNA, hereby excluding splicing. The precise mechanism could not be clarified yet. However, addition of MG, a cytostatic and cytotoxic compound which exerts part of its activity in a RAGE-dependent manner (60), strongly enhanced formation of this isoform.

Formation of 17 kDa RAGE upon IL-3 or serum starvation could partially be inhibited by inhibition of caspases. However, overexpression of full length RAGE could also strongly induce formation of 17 kDa RAGE, even in the presence of serum. This formation could not be inhibited by caspase inhibition, but addition of Z-VAD-FMK even seemed to enhance its formation, hereby seemingly contradicting the observations made in the depletion model system. Possibly different or redundant mechanism underlie these observations. RAGE could be processed in order to attenuate the strong signaling caused by the high levels of RAGE expression. Alternatively, the presence of the C-terminal flag-tag could interfere with the function of RAGE, leading to the breakdown of the receptor. The strong formation of 17 kDa RAGE in Hek293 cells in the presence of serum was not joined by an increase in apoptosis, suggesting that the presence of 17 kDa RAGE per se did not induce apoptosis.

The hypothesis that 17 kDa RAGE is formed by proteolysis is in line with earlier observations (43,52). Proteolytic cleavage of RAGE might be an important event in the regulation of RAGE signaling and it is conceivable that proteolytic processing of RAGE provides a negative regulation or a negative feedback loop in RAGE signaling. Either to inhibit prosurvival signaling or signaling towards proliferation, when IL-3 or serum is absent, or to downregulate RAGE signaling to prevent overactivation.

When high resolution 2-DE was used to study post translational modification of RAGE, our data further substantiate that RAGE is strongly susceptible to regulation during the progression of the cell cycle and when growth arrest is induced by IL-3 depletion. To date the only described posttranslational modification of RAGE is glycosylation (43-46). The different spots we observed could be differences in the level of glycosylation, since this would yield a different pI and deglycosylation would be able to account for the shifts observed. Glycosylation would however also alter the molecular weight of the isoforms, and deglycosylation would therefore not only induce a shift towards the basic side, but most likely also result in a faster migration in the second dimension. However, while we

were able to observe differences in pI, concomitant reductions in molecular weight could not be detected.

We observed that the shifts in pI strongly coincided with the modifications observed in GLO1 (see chapter 6 and data not shown). Furthermore, results from our lab showed that when L929 cells were metabolically labelled and stimulated with tumor necrosis factor (TNF), RAGE could be phosphorylated (Salliau, unpublished results). These observations suggest that the modifications shown are possibly phosphorylation and dephosphorylation.

In summary, the data shown here indicate a strong regulation of RAGE during the cell cycle. This regulation appears to take place at several levels. At a first level, we observed a strong correlation of certain isoforms with different cell cycle phases. This different expression could not be related to the expression of RAGE on the plasmamembrane, since the levels of membrane expression seemed to remain constant during the cell cycle. This could reflect the different requirement for signaling during different phases or a difference in the available ligands for RAGE. We also observed RAGE was regulated by proteolysis, a possible additional regulatory mechanism, or a negative feedback loop in RAGE signaling. Furthermore studies using 2-DE further show RAGE is posttranslationally modified, most likely phosphorylated. We therefore conclude that RAGE signaling is regulated at different levels during cell cycle and growth factor withdrawal-induced G_1 arrest and consequent apoptosis. This stresses the importance of RAGE signaling in the regulation of cell cycle and cell death and further emphasizes the complex nature of RAGE signaling. Our observations could also partially provide a basis for the dualistic nature role of RAGE signaling in proliferation and differentiation, and in survival and the induction of cell death.

Materials and Methods

Cell Lines and Cultures

The IL-3 dependent pro-B cell line Ba/F3 (61) was purchased from DSMZ. Ba/F3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen) supplemented with heat-inactivated FBS (10% v/v) (Cambrex), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and 5% conditioned medium from WEHI-3B cells as a source of mouse IL-3. Ba/F3 cells were cultured at 37°C in a humidified incubator under an 5% CO_2 atmosphere.

Hek293 cells, were cultured in DMEM with glutamax (Invitrogen) supplemented with heat-inactivated FBS (10% v/v), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) at 37°C in a humidified incubator under an 8% CO_2 atmosphere.

Cell stimulations

Ba/F3 cells were synchronized by washing exponentially growing cells 3 times in culture medium without conditioned WEHI-3B medium and resuspended in culture medium without conditioned WEHI-3B medium for 8 hours or over night. At the end of the synchronisation conditioned WEHI-3B medium (5% v/v) was readded. This time point was designated 0 hours.

For IL-3 depletion experiments, IL-3 was removed by washing exponentially growing Ba/F3 cells 3 times in culture medium without conditioned WEHI-3B medium and resuspended in culture medium without conditioned WEHI-3B medium.

Hek293 cells were depleted by washing them 3 times with culture medium without FBS and subsequently cultured in culture medium in the absence of FBS.

Hek293 cells were transiently transfected by Ca-phosphate-precipitation. Vectors used are pCAGGS vectors containing full length RAGE ($_{FL}$RAGE), N-terminally flag tagged full length RAGE (N-flag-RAGE), or C-terminally tagged full length RAGE (RAGE-C-flag). The vectors were kindly provided by Stefanie Salliau (VIB department of Medical Protein Research, Molecular and Metabolic Signaling Unit, Flanders Institute for Biotechnology, Faculty of Medicine and Health Sciences, Ghent University).

Chemicals and inhibitors used are nocodazole (0.8 µM; Sigma aaldrich) and Z-VAD-FMK (promega). Duration and concentrations used as described.

DNA distribution analysis

Cells were stained with propidium iodide (PI, Sigma) containing staining solution (62) and analyzed by a FACSCalibur flow cytometer ($488_{Ex}/590_{Em}$). Cell Quest software was used to analyze cell cycle distribution (FACS Calibur, Becton Dickinson) (62,63). Ten thousand cells were routinely analyzed.

Electrophoresis and immunoblotting

The cells were washed 3 times with ice-cold PBS buffer and lysed with cytosol extraction buffer (10 mM Tris-HCl pH7.4, 50 mM EDTA pH8.0, 25 mM NaCl, 0,7% TritonX-100, 100 mM PMSF, 1 tablet Complete Protease Inhibitor Cocktail (Roche)/50 ml cytosol extraction buffer) or modified radioimmune precipitation assay buffer (200 mM NaCl, 50 mM Tris-HCl (pH 8), 0.05% SDS, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM Na_3VO_4, 1 mM NaF, 20 mM β-glycerophosphate, and Complete Protease Inhibitor Cocktail). Cell lysates were cleared by centrifugation (14,000 x g). Protein concentrations

were determined using Bradford method. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with the desired antibodies followed by ECL-based detection (Amersham Pharmacia Biotech). Quantification of protein bands was performed using Genetools software (Westburg).

Preparation of cytosolic and nuclear fractions

Nuclear and cytoplasmic extracts were prepared as previously described with small modifications (64). In brief, 10 X 10^6 Ba/F3 cells were synchronized and after indicated time intervals collected. After harvesting and washing the cells 3 times with ice-cold PBS, the cells were lysed in 200 µl buffer A (10 mM HEPES, pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 200 mM sucrose, 1 tablet Complete Protease Inhibitor Cocktail/50 ml buffer A, 0.5% NP-40). The lysates were incubated 15 min on ice and pelleted at 11,000 x *g* for 10 min and the cytoplasmic supernatant was transferred to a new tube. The remaining nuclear pellets were washed twice with buffer A and resuspended in 150 µL buffer B (20 mM HEPES, pH 8.0, 20% (vol/vol) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 tablet Complete Protease Inhibitor Cocktail/50 ml, 1% NP-40). These extracts were subsequently passed 1 time through a 18 G needle and 3 to 4 times through a 27 G needle and centrifuged 30 sec at 14,000 3 *g*. The pelleted debris was discarded, while the nuclear extract and cytosolic supernatants were used for immunoblot analysis.

2-Dimensional Gel Electrophoresis

Isoelectric focusing was carried out on 18 cm IPG strips, pH 4-7 (GE Healthcare) according to the manufacturer's instructions. For the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE, 12%).

Surface staining of RAGE

1X 10^6 Ba/F3 cells were washed 3 times in ice-cold FACS buffer (PBS, 1% FBS, 0.5 mM EDTA) and incubated with a polyclonal antibody raised against the N-terminus of RAGE (H-300, Santa Cruz Biotechnology) for 20 min. Subsequently cells were washed twice in FACS buffer and incubated with a secondary Alexa R488 conjugated anti-rabbit antibody (Molecular Probes) for 20 min. Cells were then washed and immediately measured and analysed on FACS.

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Part III:
General Conclusions

General conclusions

The ubiquitous and highly conserved nature of the glyoxalase system suggests a fundamental role in life. Indeed, the glyoxalase system has been linked to several cellular processes including cell proliferation and growth, the assembly of microtubules and vesicle transport (1,2). Still, the glyoxalase is a rather complex enzyme system for merely a detoxification reaction. Moreover, several other enzyme systems are also capable of detoxifying α-oxoaldehydes. These observations indicate the glyoxalase system may have a more profound physiological function. In the 1960s Nobel prize laureate Albert Szent-Györgyi hypothesized that the glyoxalase system exerts a regulatory role in cell division. Ever since then, several investigators reported a strong correlation between high levels of expression and high activity of glyoxalase I, and strong cell division. However, the exact mechanistic link has yet to be established (3).

In chapters 6 and 8, we describe a potential involvement of phosphorylated glyoxalase I in growth factor-depletion induced G_1 arrest and consequent apoptosis, and the regulation of the cell cycle. Previous research in our group showed for the first time that glyoxalase I can be phosphorylated in mammalian cells (4). Earlier findings in yeast by Inoue and his colleagues around 1990 showed that treatment with mating factor induces phosphorylation of glyoxalase I and alters glyoxalase I activity (5,6). Mating factors induce several responses in yeast, and one thereof is a cell cycle arrest in G_1 (7). As described in chapter 6, in the hematopoietic cell line Ba/F3 the induction of G_1 arrest by IL-3 depletion strongly induced multiple phosphorylation of glyoxalase I. Also during the cell cycle, glyoxalase was transiently phosphorylated. In the course of our study it was also reported that treatment of rice leafs with gibberellin, a regulator of plant growth, induced phosphorylation of glyoxalase I (8). Taken together, our data and the observations made by others in yeast and rice leafs, strongly suggest either an involvement of phosphorylated glyoxalase I in the regulation of the cell cycle or at least the necessity for cells to phosphorylate glyoxalase I during the cell cycle. However, the precise biological function of phosphorylated glyoxalase I remains to be determined. Although in yeast (5) and plants (9) phosphorylation of glyoxalase I has been reported to be accompanied by an increase in glyoxalase activity, this does not seem to be the case in mammalian cells ((4) and chapters 6 and 8). This might be explained in part by the fact that some plants express a novel plant-specific type of glyoxalase (10,11). As a consequence, our data rule out the possibility that, in mammalian cells, phosphorylation of glyoxalase I occurs in order to allow the cells to cope with higher levels of methylglyoxal, caused by a cell cycle-dependent increase of glycolysis and/or the increased oxidative stress. Together with cell division, growth factors dynamically regulate the glycolysis. Growth factor withdrawal on the other hand, leads to a decrease in glycolysis. It is therefore conceivable that growth factors link the regulation of

the glyoxalase system to the regulation of metabolism.

Studies with kinase inhibitors and *in vitro* kinase assays revealed the involvement of several kinases in pathways leading to the phosphorylation of glyoxalase I. Studies using kinase inhibitors showed the involvement of GSK-3β in phosphorylation of glyoxalase I upon IL-3 starvation and the involvement of the PI3K/Akt/mTOR pathway in cell cycle dependent phosphorylation of glyoxalase I. On the other hand, *in vitro* studies pointed out that CaMKII can directly phosphorylate glyoxalase I. However, unlike inhibition of GSK-3β, inhibition of CaMKII, could not inhibit G_1 arrest and apoptosis upon IL-3 depletion in Ba/F3 cells. On the other hand, in other cell signaling pathways, phosphorylation of GLO1 could be inhibited by pharmacological inhibition of CaMKII (Rondas et al., in preparation). These data indicate that glyoxalase I is a direct physiological target of CaMKII. Interestingly, multiple phosphorylations can be induced on glyoxalase I and several kinases and signaling pathways have been shown to be involved in the phosphorylation of glyoxalase I. It is therefore conceivable that multiple kinases can directly phosphorylate glyoxalase I *in vivo*, or that kinases can substitute for one another in the phosphorylation of glyoxalase I, in case the physiological kinase is impaired. Such mechanisms have been described e.g. for the phosphorylation of GSK-3β (12,13). Alternatively, several pools of glyoxalase I might exist, and different pools might be regulated by various signaling pathways and might be involved in different cellular processes. This might explain how phosphorylation does not affect the detoxification of methylglyoxal. It might be also possible that depending on the site in glyoxalase I that is phosphorylated that effect on the enzyme and thus also the biological response can be different.

Concomitant with the phosphorylation of glyoxalase I, the formation of specific methylglyoxal-derived advanced glycation endproducts (MG-AGEs) could be observed. Currently we do not know the exact relationship between the phosphorylation of glyoxalase I, and the formation of MG-AGEs. One could speculate that formation of MG-AGEs is an indirect consequence of a phosphorylation-induced inhibition of glyoxalase I. However, this was shown not to be the case. Furthermore, phosphorylation of glyoxalase I could not be correlated with alterations in glyoxalase I activity. In yeast and plants, however, where phosphorylation was accompanied by an increase in activity of glyoxalase I (5,9).Quite interestingly, when phosphorylation of glyoxalase I was inhibited, also formation of specific MG-AGEs decreased, similar to the events in TNF-induced necrosis. These observations suggest that also in our cell system phosphorylated glyoxalase I and formation of MG-AGEs are on the same pathway.

The specific targets for methylglyoxal-modification are currently unknown. Although methylglyoxal-modification has been considered to be involved in development of several pathologies, such as vascular complications in diabetes, it’s becoming more apparent that

methylglyoxal-modification of proteins could be of physiological importance as well (14,15). The presence of methylglyoxal is proportional to the rate of glycolytic flux. Since many cellular processes and signaling pathways influence glycolysis, methylglyoxal levels and methylglyoxal-modification of specific proteins could function as a sentinel for the metabolic state of cells. It is tempting to speculate that phosphorylated glyoxalase I is involved in the formation of these specific MG-AGEs.

Several mechanisms have been described of how MG-modification on proteins exerts its effect. Reaction of methylglyoxal with amino acids generates reactive oxygen species (16), which can subsequently activate several signaling pathways. Furthermore, methylglyoxal has been reported to covalently modify specific residues of several proteins, hereby regulating their function (14,15,17,18). Another possibility is through the activation of the receptor for advanced glycation endproducts (RAGE). Many signaling pathways have been reported to emanate from RAGE (see chapter 3.3). Although RAGE is expressed in every cell line studied so far (19), under physiological conditions expression levels are low, and RAGE is strongly upregulated under pathological conditions (20). When RAGE was studied throughout the cell cycle, we found a strong regulation of the different isoforms. These differences could be related to specific cell cycle phases and alterations in the expression levels coincided with transitions between phases. In spite of the strong regulation of the different isoforms of RAGE, no alteration in expression of RAGE at the plasmamembrane was measured. We also discovered a new proteolytic cleaved isoform of RAGE, 17 kDa RAGE, which was induced upon IL-3 depletion-induced G_1 arrest. We therefore hypothesize that RAGE signaling could be involved in proliferation or survival. The proteolytic processing of RAGE could thus provide a mechanism for cells to attenuate RAGE signaling. Alternatively, the 17 kDa RAGE itself could exert another function in G_1 arrest or the induction of apoptosis.

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Part IV:
Acknowledgements

Dankuwel…

… en dan nu het stukje dat iedereen leest. Het dankwoord. Op zich misschien goed dat iedereen het leest, want het is belangrijk. Ik heb ondertussen ontdekt hoe moeilijk doctoreren is en hoeveel een mens bijleert in die periode…en dan is er nog het wetenschappelijke luik ook. Ik besef dan ook dat onderstaande personen bijzonder belangrijk zijn geweest bij het tot stand komen van dit werkje en dat deze woordjes niet toerijkend zullen zijn.

Eerst en vooral wil ik Prof Katia Vancompernolle bedanken. Dankuwel Katia. Ik heb van jou de kans gekregen om dit onderzoek te starten en gaandeweg heb je me ook op andere vlakken kansen gegeven en dingen aangereikt. Op de werkvloer verbaasden je enthousiasme en de manier waarop je positief tegen verschillende uitdagingen aan bleef kijken me keer op keer.

Ook vakgroepvoorzitter Prof. Joël Vanderkerckhove zou ik hard willen bedanken voor de steun tijdens het doctoraat en voor de kans om op het toch wel mooie instituut Rommelaere te werken.

Ook naar mijn co-promotor Prof. Guy Haegeman gaat mijn dank uit. Zonder hem zou dit doctoraat heel wat moeilijker tot stand zijn gekomen.

Een persoon die het mogelijks niet verwacht om bedankt te worden (maar ze zal daar nog wel achter komen) is mevr. Lisa Duchâtelet. Mijn interesse in wetenschap werd voor een zeer groot stuk door haar bepaald, en ze heeft er geen idee van (werkelijk niet, maar ook daar zal ze nog wel achter komen) hoe hard ze me beïnvloed heeft.

Ik was niet de enige die voor Katia werkte. Ik heb gedurende de jaren enkele schitterende collega's gehad. De eerste die ik daar zag, en meteen ook degene die in hetzelfde bootje zit als ik, Stef. U was een fijne collega. Het was ook aangenaam om samen met jou ook deze laatste dingen te doorworstelen. Samen schrijven, elkaar onze nieuwste ontdekkingen van coreldraw aanleren, dat soort dingen….zelfs samen verdedigen binnenkort. Het was plezant. Vervolgens Virginie. Collega's als jij, dat ga ik niet meer vinden, denk ik. Bedankt voor de vele wetenschappelijke en menselijke gesprekken. Ik wens je nog veel succes bij het afronden van je phd en ik weet zeker dat de dingen die je erna gaat doen bijzonder spannend gaan zijn. Dieter, op het eind van de rit naast mezelf de enige man. Bedankt voor de vele babbels, de voetbalpraatjes, def jam fight for NY, je vermogen om te relativeren en gewoon een geweldig toffe collega te zijn. Nog veel succes met je doctoraat. Anneleen, ook jij was een ware sensatie om mee samen te werken. Inmiddels ben je ook met veel belangrijkere dingen bezig dan doctoreren. Ik hoop dat je bevalling binnenkort goed verloopt, en dat je een gezond kindje krijgt waar je veel van kan genieten. Mascha, de buitenlandse post-doc, ook jij bedankt voor de geweldige inbreng in

het labo…vooral de pindakaas met cashewnoten, ik kan je er niet genoeg voor bedanken. Ik wens je verder veel moed en sterkte momenteel. Ook Sigrid, van harte bedankt. Je harde, goede en verzorgde werk was ongelooflijk nuttig. Veel succes voor je nieuwe job. Het kan niet anders dan dat men daar content gaat zijn van jou. Ook de culinaire babbeltjes en dat soort dingen, het was plezant. Mathias, bedankt voor de vele keren dat je geholpen hebt wanneer we samen projecten moesten indienen, wanneer ik weer problemen had met computers…dat soort dingen. Ook Franky ben ik dankbaar.

Ik heb ook het ongelooflijke voorrecht gehad om een studente te mogen begeleiden tijdens een thesis. Een ongelooflijke ervaring. Niet alleen heeft ze bijgedragen tot een mooi deel van deze scriptie, ook de vele gesprekken over 'andromorphoseren van katten', 'de relatie tussen de lengte van de passen en de frequentie van het klikken van de hielen', en nog zoveel meer…een mens moet het een keer meemaken…veel succes nog tijdens jouw verdere wetenschappelijke loopbaan, maar vooral, geniet met volle teugen van het moederschap.

Werken op het Rommelaere werd vooral ook mogelijk gemaakt door Johan en Serge. Jullie leveren schitterend werk. Niet te onderschatten. Ook de mensen van het secretariaat Dirk en Veronique, en in een recent verleden Kristien, super hard bedankt. Ook jullie doen in alle bescheidenheid geweldig werk en het was fijn hoe hard jullie je best deden.

Eervolle vermeldingen gaan meteen ook naar Julie, bedankt voor de aanmoedigingen tijdens de laatste maanden en naar al die mensen die oprecht interesse toonden (Frank, Sofie, Prof. Broekaert, …) vaak kleine dingen, maar ze zijn wel belangrijk.

Deze scriptie zou niet tot stand zijn gekomen zonder Sylvie. Ik ben je voor veel dankbaar. Voor je steun op bepaalde zware momenten en je vriendschap, voor het nalezen van de scriptie en nog zoveel meer. Jij ook nog succes tijdens het laatste jaartje.

Een hartelijke dankuwel zeker ook voor Erika. Dank voor het nalezen van delen van de thesis en mijn gebrekkig Engels bij te sturen. Jij en Tim (en Alister) hartelijk bedankt ook om gewoon vrienden te zijn.

Ik ben ook dankbaar voor de geweldige vrienden die ik heb. Ze hebben een grenzeloos geduld getoond en zijn nog steeds mijn vrienden, ook al heb ik niet veel tijd voor hen gehad de laatste maanden. Ik kan ze niet allen bedanken, maar enkele wil ik toch bij naam noemen. Tom, je bent me ongelooflijk dierbaar. Ik heb veel steun van je gekregen. Wim en Birgit, zo hard bedankt, voor de aanmoedigingen en zo, en voor de vriendschap. Wim, ook ongelooflijk bedankt dat je iets later dit jaar ook mijn getuige wil zijn. Jan en Joke, vooral de regelmatige smsjes met aanmoedigingen de laatste maanden van Joke. Maarten en Hanna, voor het geduld. A special thanks goes to my dear friend Maete. If anything good came out of this phd, it's my friendship with you. Thanks for the support. Thanks

for taking me to Spain. Thanks for so much more. Cindy, wanneer beginnen we met die cocktailboot?

Een speciale vermelding ook voor een speciaal iemand…Gawein. Ook al zit je nu aan't andere eind van deze mooie planeet, ik heb veel aan je gehad en zal blij zijn wanneer je weer terug in't land bent.

Ik ben nu ongetwijfeld belangrijke mensen aan het vergeten…sorry daarvoor, dat wil niet zeggen dat ik minder dankbaar ben. Ik ben wel zeker dat ik de beste vriendenkring aller tijden heb.

Papa en Brigitte, Mama en Staf, ook naar jullie gaat een ongelooflijke dankuwel uit. Jullie hebben mij altijd gesteund, voor me gebeden, jullie hebben altijd interesse getoond, zijn er altijd voor mij geweest en hebben jezelf vaak weggecijferd voor mij. Zonder jullie zou ik dit niet gekund hebben. Had ik dit niet uitgehouden. Ik hou veel van jullie. Ook dank u aan Luc en Nelly (ook papa en mama). Bedankt voor de interesse en steun.

Ook mijn zussen en broers ongelooflijk veel dank voor de steun, de interesse en het begrip.

Ten slotte Ann. Bedankt voor je liefde en geduld. Bedankt dat je er bent, en dank je dat je mijn vrouw wil zijn. Ik hou van je.

Nochtans zal ik juichen in de Here,
Jubelen in de God van mijn heil.