Naringin-Sensitive Protein Phosphorylation Pathways in the Regulation of Hepatocytic Autophagy

by

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NARINGIN (5,7,4'-trihydroxyflavanone-7-neohesperidoside)
is a flavanone isolated from the grapefruit peel
as the bitter principle.

I dedicate this work to both
my daughter Christina,
and my family!

LIST OF CONTENT

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Finally, I would like to thank all my friends and my whole family for their patient and incentive support during almost a decade, especially my understanding and loving wife. Eventually, it has now been made possible for me to answer your universal question: "When are you going to finish your doctor's degree?" However, I still do not have any uncomplicated answer or reason for why it had to take this long time.

Some times, "reason" apparently goes beyond any scientific perception or logical cause!

Oslo, August 4th 2004.

Michael T.N. Møller

PREFACE

When I started on this project, our primary aim was to identify naringin-sensitive protein kinases that were hypothesized to participate in the regulation of hepatocytic autophagy. The general idea was, that this non-toxic flavonoid, isolated as the bitter principle from grapefruit, had exhibited promising autophagy-protective characteristics towards the diarrhetic shellfish toxin, okadaic acid (Gordon et al., 1995). As with many environmental toxins, okadaic acid exerts its toxic effects through overphosphorylation of cellular proteins by specifically inhibiting PP2A in low doses (Cohen et al., 1990). In the process of understanding of how cancer cells alter their complicated regulation of protein synthesis versus degradation in favor of cell growth, autophagy plays an important role in this finely tuned balance (Alva et al., 2004), as being responsible for the sequestration and transport of pieces of cytoplasm, with their constituent organelles and macromolecules, to the lysosomes for degradation (Ericsson, 1969).

Choosing a proteomic approach, we used a newly established two-dimensional PAGE separation technique in our laboratory to identify naringin-sensitive, okadaic acid-induced phosphoproteins, by means of monitoring the ratio of radioactively labeled proteins, and subsequent amino acid identification through classical N-terminal Edman degradation. After an exceptionally laborious mass screening, we were finally able to identify one naringin-sensitive, okadaic acid-induced phosphoprotein as GNMT (paper I), which is a key regulator of the cellular SAM/SAH ratio in methionine metabolism. Later we were able to identify a downstream metabolite, homocysteine, as a potent inhibitor of autophagy (paper II). In the presence of adenosine, homocysteine exhibited synergistic autophagy-inhibitory effects in isolated rat hepatocytes, suggesting that this metabolite interferes with the regulation of autophagy possibly by inducing intracellular ATP-depletion, but to our surprise without any subsequent activation of AMP-activated protein kinase (AMPK).

As amino acids induce strong autophagy-suppression along with an activation of S6K1 and subsequent phosphorylation of S6, we speculated that homocysteine also exerted an activation of this enzyme, which would suggest a mechanism for the homocysteine-induced suppression on autophagy. However, homocysteine had no effect on S6K1 (paper II). Nonetheless, we did discover that several autophagy-suppressive agent activated S6K1 in a naringin-sensitive manner mimicking the effects observed on autophagy (paper IV). We perused previous reports, which hypothesized that amino acid-induced suppression of autophagy were mediated through the activation of S6K1 and S6 (Blommaart et al., 1995; Blommaart et al., 1997b; van Sluijters et al., 2000) and continued, therefore, our research on these proteins. However, since only the amino acids were rapamycin-sensitive with regard to autophagy-suppression, we concluded that S6 had no relevance for the regulation

of autophagy. Although, tail phosphorylation of S6K1 at Thr^{421} and Ser^{424}, might still be considered to be involved in a Thr^{389}-indenpendt regulatory pathway (paper IV-V).

Since AICAR suppress the activating phosphorylation induced by amino acids, we continued to look into the upstream regulation of S6K1 (paper III and V). Because this autophagy-suppressive drug (Samari and Seglen, 1998) were a well-known activator of AMP-activated protein kinase, AMPK (Corton et al., 1995; Henin et al., 1996), we suspected that it could participate in the upstream regulation of S6K1. Phosphospecific antibodies against AMPKα at Thr^{172} had just become commercially available in late 2001, and we were now able not only to confirm that AMPK phosphorylation was induced by AICAR, but also by all tested autophagy-suppressive toxins. Since these drugs share similar naringin-antagonistic characteristics on both AMPK and autophagy-suppression, AMPK is now hypothesized as one key candidate protein kinase that acts as a mediator of hepatocytic autophagy regulation.

During cellular stress (e.g. heat shock, metabolic poisoning, hypoxia etc.) AMPK is activated by elevated AMP concentrations. As the ATP/AMP ratio drops below a certain level, AMPK shuts down energy-consuming processes and thus serves to maintain a finely tuned balance between anabolism and catabolism. Furthermore, under sustained stress, AMPK activates other stress-inducible proteins like SEK1 and JNK. Indeed, we found that these proteins were activated in a similar naringin-sensitive manner as observed for S6K1 and AMPK (paper III). As a working hypothesis, we now speculate that AMPK may well mediate autophagy-regulation through a pathway that involves AMPKK (LKB1)/AMPK/SEK1/JNK. We also show that this pathway intercepts with S6K1 in a Thr^{389}-independent manner, suggesting that S6K1 exhibits other functions beyond the regulation of S6.

We have previously observed that toxin-induced autophagy-suppression is accompanied by hepatocellular cytoskeletal disruption and apoptosis. In paper III we demonstrate that besides keratin (Blankson et al., 2000; Blankson et al., 1999), plectin also undergoes a naringin-sensitive phosphorylation. These cytoskeletal proteins could possibly take part in the autophagy regulation, perhaps by serving as a structural and supporting scaffold for the involved protein kinases and phosphatases. Based on PP2A or PP1-specificity we, finally, suggest a dual mechanism for algal toxin-induced hepatocytic apoptosis. Low doses of okadaic acid or microcystin induce a slow, naringin-sensitive apoptosis, reflecting the involvement of PP2A, whereas a rapid toxin-induced apoptosis may be mediated through PP1 in a naringin-insensitive manner.

ABBREVIATIONS

MA	3-methyladenine
5-CH_3-THF	5-methyltetrahydrofolate
5-CH_3-THF-Glu_5	5-methyltetrahydrofolate pentaglutamate
AA-mix	amino acid mixture
ACC	acetyl-CoA carboxylase
ACS	associated with SNF1 complex
Ado	adenosine
AdoHcy/SAH	S-adenosylhomocysteine
AdoMet/SAM	S-adenosylmethionine
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMPK kinase (also identified as LKB1 that exists as a complex with two accessory subunits, MO25α/β and STRADα/β)
ATP	adenosine triphosphate
ASK1	apoptosis signal-regulating kinase 1
BHMT	betaine-homocysteine methyltransferase
CA	calyculin A
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBS	cystathionine β-synthase domain
Cdks	cyclin-dependent kinases
CN	cantharidin
DB-cAMP	N6,2'-O-dibutyryl cyclic adenosine monophosphate
dCF	2'-deoxycoformycin (pentostatin®)
eIF-4E	eukaryotic initiation factor 4E
Erk1/2	extracellular signal-regulated kinases 1 and 2
4E-BP1	eIF-4E-binding protein-1
GEE	glutathione ethyl ester
Glu	glutamate
GMP	guanosine monophosphate
GNMT	glycine N-methyltransferase
GPP	glutamate-dependent type-2A protein phosphatase
GS	glycogen synthase
Gx	glutamine or glutamic acid
HCy	homocysteine
HMG	hydroxymethylglutaryl-CoA
HMGR	hydroxymethylglutaryl-CoA reductase
HPLC	high-performance liquid chromatography
HSL	hormone-sensitive lipase
ITu	5-iodotubercidin
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
K8, K18	cytokeratin 8, cytokeratin 18
KIS	kinase interaction sequence
LDH	lactate dehydrogenase
LKB1	interacting protein-1 (a serine-threonine protein kinase)
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinase
MAT	methionine adenosyltransferase (synonym with SAMS)
MC	microcystin-LR
Met	methionine

MLK	mixed-lineage group of MAP kinase kinase kinases
mTOR	mammalian target of rapamycin
mTORK	mTOR kinase
N^6-MPR	N^6-mercaptopurine riboside
NA/NAR	naringin (5,7,4'-trihydroxyflavanone 7-neohesperidoside)
NAC	*N*-acetylcysteine
ND	no data
NR / NS	naringin-resistant /naringin-sensitive
PAGE	polyacrylamide gel electrophoresis
PDK	PtdIns(3,4)P_2/(3,4,5)P_3-dependent kinases
PI3K	phosphatidylinositol 3'-kinase
PIKK	phosphoinositide kinase related kinases
PIP2/PIP3	phosphatidylinositol 3,4-triphosphate / phosphatidylinositol 3,4,5-triphosphate
PK	phosphorylase kinase
PKA	cyclic AMP-dependent protein kinase
PKB	protein kinase B
PNS	post nuclear supernatant
PP1	type-1 protein phosphatase
PP2A	type-2A protein phosphatase
PP	protein phosphatase
PPM	protein phosphatases with specifity towards phosphoserine/ threonine residues (Mg^{2+}-dependent)
PPP	protein phosphatases with specifity towards phosphoserine/ threonine residues
PtdIns	phosphatidylinositol.
PTP	protein phosphatases with specifity towards phosphotyrosine residues
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PVDF	polyvinylidene difluoride
OA	okadaic acid
Raptor	regulatory associated protein of mTOR
Ras	an abbreviation that originated from *rat sarcoma*, a low-molecular-weight GDP/GTP-binding guanine triphosphatase (GTPase)
Rheb	Ras ortholog enriched in brain
RSK	p90 ribosomal S6 protein kinase
S6	small ribosomal protein S6
S6K	S6 kinase
SAMS	S-adenosyl methionine synthetase (synonym with MAT)
SAPK	stress-activated protein kinase
SB	SmithKline Beecham inhibitors (e.g. SB203580 inhibitor of p38-MAPKs)
SEK1	stress-activated protein kinase/extra cellular signal-regulated kinase 1
Ser / S	serine
SNF	sucrose non-fermenting
TA	tautomycin
TCA	trichloroacetic acid
THF	tetrahydrofolate
Thr / T	threonine
TLC	thin layer chromatography
TOS	TOR signaling
Tyr / Y	tyrosine
Tyrphostins	inhibitors of tyrosine protein kinases
Wort. / WT	wortmannin
ZMP	5-aminoimidazole-4-carboxamide ribotide

LIST OF PUBLICTIONS

All of the work presented in this thesis is the product of the collaboration with other Ph.D students or undergraduate students, and other scientific staff. The final paper does not always reflect the complete scientific contribution to the projects, In paper III to V e.g. the western blot data embraces more than 3,000 polyacrylamide gels, and in paper I several hundreds of two-dimensional gels. Furthermore, it is not always easy to exactly evaluate the work of each contributor in the presented work. In paper I-III and V we therefore, have considered *the first two authors to have contributed with equal amounts of work (§),* The position as first author, therefore, often merely reflects who initiated the work – or who originated as first author in the early drafts (e.g. paper III and V).

ARTICLES INCLUDED IN THIS THESIS

I **Møller[§], M.T.N**., Samari[§], H.R., Fengsrud, M, Strømhaug, P.E., Østvold, A.C. and Seglen, P.O. Okadaic acid-induced, naringin-sensitive phosphorylation of glycine N-methyltransferase in isolated rat hepatocytes. *Biochem. J. (2003)* ***373****: 505-513.*

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INTRODUCTION

Common to all cells is a fine-tuned balance between protein synthesis and protein turnover. Both involve several strictly and continuously controlled mechanisms. As cells grow, the balance shifts in favour of a net accumulation of proteins. This can be accomplished by an increased rate of protein synthesis as well as by a decreased rate of protein degradation. For the scope of this thesis, we focus mainly on protein degradation. Protein degradation may be divided into two major classes, non-lysosomal and lysosomal. Non-lysosomal degradation is predominantly responsible for the degradation of *short-lived* or abnormal and misfolded proteins (Goldberg, 1990; Klausner and Sitia, 1990; Pontremoli and Melloni, 1986). However, most important for the degradation of *long-lived* proteins and other macromolecules are the lysosomes (de Duve et al., 1955; de Duve and Wattiaux, 1966), The lysosomes represent the final stage in the degradation process, and receive material via different pathways. Of most interest for this thesis is *autophagy,* a basically non-selective process that delivers whole cytoplasm and organelles to the lysosomes.

AUTOPHAGY

In virtually all mammalian cells, autophagy is operative as a process that sequesters and transports pieces of cytoplasm, with their constituent organelles and macromolecules, to the lysosomes for degradation (Ericsson, 1969). Phagophores, composed of protein-poor, single or multiple membrane cisternae initiate the process by enclosing cytoplasm into vacuoles called autophagosomes (Ericsson, 1969; Fengsrud et al., 2000; Fengsrud et al., 1995; Seglen, 1987; Strømhaug et al., 1998). Intermediary acidic vesicles, called amphisomes, are then formed as the autophagosomes fuse with early or late endosomes (Berg et al., 1998; Gordon and Seglen, 1988; Høyvik et al., 1991; Strømhaug and Seglen, 1993). Finally, the amphisomes fuse with the lysosomes, where the autophagocytosed material is degraded intralysosomally to small-molecular weight components that can be re-utilized by the cell. However, this comes with some sacrifice, as the autophagic machinery is energy-dependent, both at the sequestration step and during the fusion step(s) of autophagosomes with other organelles (Reunanen and Nykanen, 1988). The initial sequestration step is particularly sensitive to intracellular ATP concentrations (Plomp et al., 1989; Plomp et al., 1987). The autophagic pathway may account for as much as 80% of the total intracellular protein degradation in rat hepatocytes under conditions of amino acid depletion (Seglen, 1987). However, under normal situations, the autophagy would not be so predominant in the overall protein degradation. Hepatocytic autophagy is subdued to strict regulation in

order to reflect the role of the liver as a supplier of e.g. amino acids to other cells during starvation. Therefore, important *in vivo* regulators of autophagy include amino acids (Kovacs et al., 1981; Seglen and Gordon, 1984; Woodside and Mortimore, 1972) and the hormones glucagon and insulin (Schworer and Mortimore, 1979). Autophagy has also been reported to participate in cell differentiations and embryogenesis (Glaumann et al., 1981). Evidently, complex regulation mechanisms must be involved in the regulation of autophagy. Other known regulators include protein phosphatase type 2A (Holen et al., 1993), phospholipids and lipid kinases (Blommaart et al., 1997c; Petiot et al., 2000), cyclic nucleotides (Holen et al., 1991; Holen et al., 1996), purines (Kovacs et al., 1998; Seglen and Gordon, 1982), trimeric GTPases (Blommaart et al., 1997c; Ogier-Denis et al., 1995; Petiot et al., 2000), and calcium (Gordon et al., 1993), Most of these affect the initial sequestration step. More recently, the role of several protein kinases has been meticulously mapped to unveil the complexity of autophagic regulation. Ca^{2+}/calmodulin-dependent protein kinase II (Holen et al., 1992; Holen et al., 1993), S6 kinase (Blommaart et al., 1995), stress-activated protein kinases (Cheng et al., 2000; Ruud Larsen et al., 2002; Xi et al., 2001) and AMP-activated protein kinase (Arico et al., 2001; Dubbelhuis and Meijer, 2002; Ruud Larsen et al., 2002; van Sluijters et al., 2000) are some of the candidates brought to attention.

INHIBITORS AND ACTIVATORS: BASIC TOOLS FOR THE STUDY OF AUTOPHAGY

In this thesis, we have used a variety protein and lipid kinase inhibitors/activators and protein phosphatase inhibitors in conjunction with numerous antagonists, in order to map the complexity of hepatocytic autophagy regulation.

Protein phosphatase inhibitors

There are three to four times fewer genes encoding protein phosphatases than protein kinases in, e.g., Saccharomyces cerevisiae (Costanzo et al., 2000) and Drosophila melanogaster (Adams et al., 2000). However, the variety of protein phosphatases is nevertheless high, since catalytic subunits often associate with different regulatory subunits to form distinct holoenzymes. Protein phosphatases have traditionally been classified into three families based on amino acid sequence identity and similarity of three-dimensional structures, whereas all known protein kinases appear to descend from one primordial gene. These families include one (PTP) with specifity towards phosphotyrosine residues (Fischer et al., 1991; Pot and Dixon, 1992), and two with specifity towards phosphoserine and phospho-threonine residues (Cohen, 1989), designated as PPP and PPM (Mg^{2+}-dependent). Dual

specifity does exist among some of the PTP protein phosphatases. However, it has recently become evident that the protein phosphatase family is even more diverse. In mammals, a novel family with specifity towards phosphohistidine residues has been identified (Zolnierowicz and Bollen, 2000), and it is likely that there may exist even more unidentified families or sub-families. Protein phosphatase type C (PP2C) and pyruvate dehydrogenase are classified as part of the PPM-family, whereas PP1, PP2A and PP2B belong to the PPP-family. Biochemically, the serine/threonine phosphatases (PPP and PPM) have further been classified as group I or II based on the relative activity against the α- and β-subunit of phosphorylase-kinase in conjunction with inhibitors II and I. Phosphatases with inhibitor-resistant activity towards the α-subunit are denoted type 2, while those with inhibitor-sensitive activity towards the β-subunit are denoted type 1.

We employed a variety of naturally occurring cell-permeable inhibitors of serine/threonine-specific protein phosphatases (okadaic acid (OA), microcystin-LR (MC), calyculin A (CA), tautomycin (TA) and cantharidin (CN)) in the mapping of protein phosphorylation pathways in rat hepatocytes. None of these inhibitors are absolutely specific, and they all show some inhibitory effect on both PP1 and PP2A (see table I). As discussed in paper III-V they all seem to fall into one of two groups, with naringin-sensitive (NS) or naringin-resistant (NR) effects, respectively, on protein phosphorylation (Strømhaug et al., 1997), autophagy suppression (Gordon et al., 1995), apoptosis or disruption of the hepatocytic plectin (Ruud Larsen et al., 2002) and keratin networks (Blankson et al., 1995; Blankson et al., 1999) (cf. also the *Naringin* paragraph on page 18). Low doses of the naringin-sensitive inhibitors OA or MC effectively inhibit PP2A with little or no effect on PP1. Increasingly higher doses also gradually inhibit PP1, along with a diminishing naringin antagonism.

Table I
In vitro IC_{50}-values for some inhibitors against PP1 and PP2A

The table lists published *in vitro* IC_{50} values for inhibition of protein phosphatases PP1 or PP2A by microcystin-LR (MacKintosh et al., 1990), okadaic acid (Holmes et al., 1990), calyculin A (Ishihara et al., 1989), tautomycin (Hori et al., 1991; Suganuma et al., 1992) and cantharidin (Honkanen, 1993; Li et al., 1993). ‡The last column indicates naringin-sensitive (NS) or naringin-resistant (NR) effects on protein phosphorylation, autophagy suppression, apoptosis or disruption of the hepatocytic plectin and keratin networks.

Inhibitor	**Source**	**IC_{50}**	**IC_{50}**	**Selectivity**	**‡**
		PP2A	**PP1**	**^{PP2A} / $_{PP1}$**	
Microcystin-LR	Cyanobacteria	40 pM	1.7 nM	42.5	NS
Okadaic acid	*Dinoflagellates*	0.1 nM	10-15 nM	100-150	NS
Calyculin A	Marine sponges	0.5-1 nM	2 nM	2-4	NR
Tautomycin	*Streptomyces spiroventrillatus*	10 nM	1 nM	0.1	NR
Cantharidin	Blister beetles	40 nM	473 nM	11.8	NR

Thus, the propensity of protein phosphatase inhibitors to be antagonized by naringin would seem to correlate with the inhibition of PP2A as indicated by the $^{PP2A}/_{PP1}$ sensitivity

ratio. A certain threshold value of this ratio (between 20 and 40) appears to distinguish quite well between inhibitors with predominantly naringin-sensitive effects (OA, MC) and those with predominantly naringin-resistant effects (CA, TA and CN) on intact cells (Blankson et al., 1999).

Drug-targeted protein and lipid kinases implicated in autophagy

• *Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)* In several thorough studies, large numbers of known protein kinase inhibitors were screened for their okadaic acid-antagonistic ability on autophagy suppression. Four potent CaMKII inhibitors: KT-5926, KT-5823, KN-62 (Holen et al., 1993) and KN-93 (our unpublished data) have been identified as okadaic acid antagonists. Both KN-62 and the more water-soluble KN-93 are purportedly very specific inhibitors of CaMKII (Sumi et al., 1991; Tokumitsu et al., 1990), indicating that CaMKII might be involved in the regulation of autophagy. However, the significance of these findings is debatable, as the inhibitors have also been reported to affect proteins other than CaMKII. For example, KN-93 seems to directly inhibit IP(3) reseptor-1 (Smyth et al., 2002) and the G-protein signaling cascade (Yue and Sanborn, 2001), independently of its effect on CaMKII.

• *Phosphatidylinositol 3'-kinase (PI3K)* 3-methyladenine (3MA) (Seglen and Gordon, 1982), wortmannin (WT) and LY294002 all inhibit autophagy as well as PI3K (Blommaart et al., 1997a; Strømhaug et al., 1997). The PIK3 family is classified into three classes on the basis of their *in vivo* lipid substrate specificity, structure and likely mode of regulation (Zvelebil et al., 1996). $PI3K^{ClassI}$ members (110-130kDa proteins) phosphorylate PtdIns, PtdIns(4)*P* and PtdIns(4,5)P_2, although PtdIns(4,5)P_2 is the most likely *in vivo* substrate. All mammalian PI3Ks from this class interact with active GTP-bound Ras and are activated by (Scr-like) tyrosine kinases. $PI3K^{ClassII}$ members (>200 kDa proteins) only phosphorylate PtdIns and PtdIns(4)*P* (Domin et al., 1997) and are scantily sensitive to WT and LY294002 (Virbasius et al., 1996); their activity is under the regulation of G-protein βγ-subunits and Ras. The substrate specificity of $PI3K^{ClassIII}$ members is restricted to PtdIns; their activity is constitutively activated.

In mammalian cells, $PI3K^{ClassIII}$ associates with a p150 membrane adaptor protein that recruits the kinase to the membrane and stimulates the lipid kinase activity (Panaretou et al., 1997). The membrane recycling possibly controls *de novo* autophagosome formation. PtdIns(3)*P* produced from $PI3K^{ClassIII}$ stimulates the autophagic pathway. Both WT and 3MA may interrupt this pathway by the specific inhibition of $PI3K^{ClassIII}$. On the contrary, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 produced from $PI3K^{ClassI}$ inhibits the autophagy pathway

in HT-29 cells (Kadowaki et al., 1996; Petiot et al., 2000). The downstream signaling pathway from PI3K^{ClassI} involves PtdIns(3,4)P_2/(3,4,5)P_3-dependent kinases like PDK and AKT (Burgering and Coffer, 1995), various protein kinase C isoforms (Akimoto et al., 1996; Nakanishi et al., 1993) and p70S6 kinase (Blommaart et al., 1997a; Romanelli et al., 1999). (The involvement of S6 kinase in autophagy regulation is further discussed in the "*S6 and S6 kinase(S6K)"* section on page 24.)

• *Cyclin-dependent kinases (Cdks)* The Cdk inhibitor, olomoucine, strongly inhibits autophagy (Strømhaug et al., 1997), suggesting that Cdks may be involved in the autophagic process. Cdks exert a major and essential function in cell growth control, but they have also been reported to play a role in apoptosis (Meikrantz and Schlegel, 1996). Since long-lasting autophagic activity has been show to correlate with apoptotic death in cultured hepatocytes (Schwarze and Seglen, 1985), a regulation of both processes by Cdks would not seem to be entirely unreasonable.

• *p38 mitogen-activated protein kinase (p38-MAPK)* SB203580 was originally developed as a specific inhibitor of p38-MAPK(α) and p38-MAPK(β) (Cuenda et al., 1995; Lee et al., 1994). However, SB203580 has also been reported to inhibit, at high concentrations, the two stress-activated protein kinase/c-Jun NH_2-terminal-kinase (SAPK/JNK) isoforms of 52 and 54 kDa in human cardiac cells (Clerk and Sugden, 1998). As shown in papers III and V, JNK appears to be one of several kinases acting downstream of AMPKK/AMPK, and we have unpublished data that show that SB203580 exhibits an antagonistic effect on AICAR-suppressed autophagy in isolated rat hepatocytes.

• *Tyrosine protein kinases and phosphotyrosine protein phosphatases* Based on substrate-competitive capabilities, several inhibitors of the EGFR have been developed and designated as tyrosine-protein kinase inhibitors (tyrphostins) (Gazit et al., 1989). Several tyrphostins, however, also inhibit epidermal growth factor-independent tyrosine protein kinases. A comprehensive survey has unveiled that tyrphostins can inhibit the initial sequestration step of autophagy as well as several individual steps in the endocytic pathway (Holen et al., 1995). For example, tyrphostin 46 specifically inhibits autophagy, whereas tyrphostin 1 (actually a poor EGFR inhibitor, cf. (Gazit et al., 1989; Wijetunge et al., 1992) and tyrphostin 51 inhibit an early endocytic step, and tyrphostin 25 a late endocytic step. Quercetin and genistein (Ogawara et al., 1986) are other tyrosine kinase inhibitors that suppress autophagy (Fosse et al., 1995). However, genistein has a dual effect, low doses being capable of antagonizing okadaic acid-induced suppression of auto-

phagy (Holen et al., 1992). Vanadate, a nonspecific inhibitor of tyrosine protein phosphatases, unexpectedly inhibits hepatocytic autophagy (Fosse et al., 1995) and protein degradation (Seglen and Gordon, 1981), but has also been shown to antagonize the okadaic acid-induced disruption of hepatocytic plectin and keratin networks (Blankson et al., 1999). Interference with intracellular protein tyrosine phosphorylation would thus seem to have complex, and relatively unpredictable, effects on autophagy.

• *Cyclic AMP-dependent protein kinase (PKA)* The cyclic nucleotide analogues dibutyryl-cAMP (DB-cAMP) and dibutyryl-cGMP significantly suppress autophagic sequestration in isolated rat hepatocytes (Holen et al., 1991). Similarly, administration of theophylline, a non-specific inhibitor of phosphodiesterases, prevents intracellular degradation of cAMP and cGMP and inhibits autophagy. However, use of deacetylforskolin and atriopeptin, activators of adenylate- and guanylate-cyclase, respectively, suggests that the inhibition of autophagy is solely caused by elevated levels of cAMP, and not by cGMP. KT5720, a specific inhibitor of PKA, substantially antagonizes the autophagy-suppressive effect of DB-cAMP, thus suggesting a role for PKA in the regulation of autophagy (Holen et al., 1996). It may be relevant in this connection that PKA has been reported to phosphorylate the ribosomal protein S6 (Blommaart et al., 1995), thus providing a parallel to the stimulation of S6 phosphorylation and inhibition of autophagy by amino acids (see the *"S6 and S6 kinase (S6K)"* section on page 24).

• *AMP-dependent protein kinase (AMPK)* 5-aminoimidazole-4-carboxamide riboside (AICAR) activates AMPK (Corton et al., 1995; Henin et al., 1996) after intracellular phosphorylation to 5-aminoimidazole-4-carboxamide ribotide (ZMP) by adenosine kinase (Baggott et al., 1986; Sabina et al., 1985), and is a strong inhibitor of autophagy in rat hepatocytes (Samari and Seglen, 1998). Administration of AICAR to rat hepatocytes also causes inactivation of another known AMPK target, i.e., 3-hydroxy-3-methylglutaryl-CoA-reductase, and almost total cessation of two of the known target pathways, i.e., fatty acid and sterol synthesis (Henin et al., 1995). AICAR-induced autophagy suppression is completely abolished (Samari and Seglen, 1998) by the adenosine kinase inhibitor 5-iodotubercidin (ITu) (Henderson et al., 1972). The N^6-thiolated adenosine analogue N^6-mercaptopurine *(N^6-MPR)* also inhibits autophagy in an ITu-sensitive manner, and is suggested to inhibit AMPK in isolated rat hepatocytes via the adenosine kinase product, N^6-MRP 5´monophosphate (Samari and Seglen, 1998). *Adenosine* exhibits the same autophagy-suppressive and ITu-sensitive capabilities as AICAR and N^6-MPR, its effect being strongly potentiated (Kovacs et al., 1998) by the adenosine deaminase inhibitor, 2'-

deoxycoformycin/pentostatin (dCF) (Henderson et al., 1977), owing to rapid increases in the intracellular levels of adenosine and, subsequently, of AMP (Hardie and Carling, 1997). However, adenosine *plus* dCF is also known to elevate the hepatocytic levels of S-adenosylmethionine and S-adenosylhomocysteine (Helland and Ueland, 1982; Hoffman et al., 1980), suggesting that some involvement of *methionine metabolism* (see page 21) ought to be considered. (The AMPK pathway and its function is further discussed in the *"AMP-activated protein kinase (AMPK)"* section on page 29.)

Inhibitors and activators of autophagy

• *Amino acids* Besides providing a mechanism for the continuous renewal of cellular proteins, RNA and organelles, a major function of autophagic degradation is to supply amino acids that can be reutilized, or used for energy generation, by other cells. The main *in vivo* regulators of autophagy are generally believed to be the amino acids, working in collaboration with the pancreatic hormones, insulin and glucagon. Upon amino acid depletion, hepatocytes immediately activate the autophagic degradation pathway (Schworer et al., 1981). Similarly, high concentrations of amino acids strongly inhibit hepatocytic autophagy (Kovacs et al., 1981; Woodside and Mortimore, 1972). In hypoosmotic media, cell swelling increases the sensitivity of autophagic proteolysis to inhibition by low concentrations of amino acids (Meijer et al., 1993). It has been reported that a membrane-bound receptor could be responsible for mediating the amino acid signal (Miotto et al., 1994), and that GTP-binding proteins are required in macroautophagic vacuole formation (Kadowaki et al., 1994). The section *"S6 and S6 kinase (S6K)"* further describe the interaction with hormones and the mTOR pathway (page 24-29).

• *Hormones* In the intact liver, insulin inhibits (Pfeifer, 1978) and glucagon stimulates autophagy (Schworer and Mortimore, 1979). In isolated rat hepatocytes, these hormones have little effect on their own, but autophagy suppression by intermediate levels of amino acids is potentiated by insulin but antagonized by glucagon (Poli et al., 1981). The fascinating cooperation between amino acids and insulin is not only seen in hepatocytes, but also in various insulin-sensitive cells such as rat pancreatic β-cells (Xu et al., 1998), rat adipocytes (Fox et al., 1998) and human HT-29 intestinal cells (Ogier-Denis et al., 1997). Amino acids stimulate insulin production in the pancreatic β-cells, and also promote β-cell proliferation via intracellular signaling.

Naringin: An autophagy-preserving, toxin-antagonistic flavonoid

In order to probe the mechanisms by which the various autophagy inhibitors influence autophagy, several substances have been screened for possible antagonistic (autophagy-preserving) effects. As discussed above, several protein kinase inhibitors possess this ability (e.g. KN-62/KN-93, KT5720 and SB203580). However, tyrosine protein kinase inhibitors mostly suppress autophagy rather than antagonize the effect of e.g. okadaic acid (Holen et al., 1995). One exception is the isoflavone genistein (Fig. 1), which has been reported to inhibit tyrosine protein kinases (Akiyama et al., 1987), serine/threonine protein kinases like S6 kinase (Linassier et al., 1990) and cAMP-dependent protein kinase (Jinsart et al., 1992) as well as histidine protein kinases (Huang et al., 1992). Genistein was found to inhibit hepatocytic autophagy at high concentrations, but at low concentrations it antagonized okadaic acid, thereby preserving autophagy (Holen et al., 1992). In a search for genistein analogues with a more pure autophagy-protective effect, large numbers of other flavonoids were screened in a comprehensive study (Gordon et al., 1995). Prunin (5,7,4'-trihydroxyflavanone-7-glucoside), neohesperidin (5,7,3',4'-tetramethoxyflavanone-7-neohesperidoside), neoeriocitrin (5,7,3',4'-tetrahydoxyflavanone-7-neohesperidoside) and naringin (5,7,4'-trihydroxyflavanone-7-neohesperidoside) were found to effectively antagonize the okadaic acid inhibition of autophagy, with little or no effect by themselves. The most potent of the protective flavonoids was naringin (Sanskrit: *naringi* for orange; (Hoffmann, 1879), which is the bitter principle from grapefruit, and thus a very inexpensive and easily available compound. For these reasons, naringin has been extensively used to study the involvement of protein phosphorylation in the regulation of autophagy.

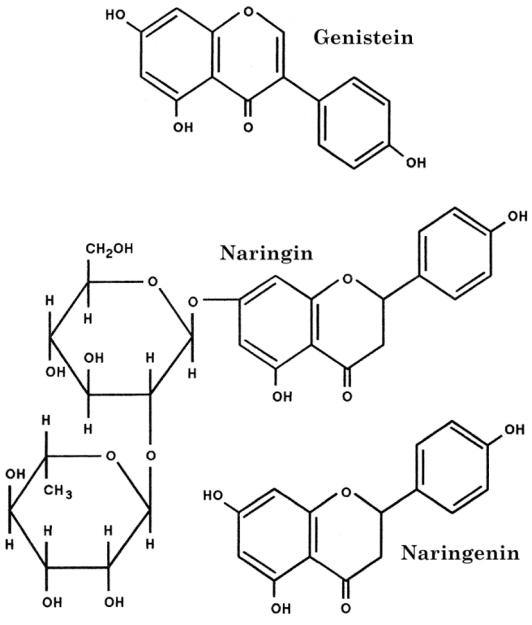

Fig. 1. **Structures of flavonoids.** From the top, the isoflavone genistein (5,7,4'-trihydroxyisoflavone): the flavanones naringin (5,7,4'-trihydroxyflavanone-7-neohesperidoside) and naringenin (5,7,4'-trihydroxyflavanone).

As shown in papers III and V, naringin antagonizes okadaic acid-induced activation of AMP-activated protein kinase (AMPK). However, further work has to be done to determine the precise molecular mechanism by which naringin keeps AMPK inactive. Interestingly, naringenin (5,7,4'-trihydroxyflavanone), the aglycone of naringin, does not possess any okadaic acid-antagonistic ability (it is, on the contrary, strongly autophagy-suppressive), indicating that glycosylation in the 7-position of the flavonoid is necessary for its protective function (Gordon et al., 1995). Naringin, like many other flavonoids, has structural similarities to ATP. This may suggest that the inhibitory effect of naringin could be caused by competition with ATP for an active site on an enzyme (Graziani et al., 1983).

THE CYTOSKELETON

Besides the autophagy-inhibitory effect of okadaic acid, severe damage and disruption of the hepatocytic cytoskeletal network occurs (Holen et al., 1992). Furthermore, okadaic acid increases the overall intermediate filament phosphorylation (Eriksson et al., 1992; Yatsunami et al., 1991). Several inhibitors of CaMKII (K-225a, KT5926 and KN-62) prevent the cytoskeleton-disruptive effect of okadaic acid (Blankson et al., 1995; Seglen et al., 1996)). Immunocytochemical methods and fluorescence microscopy have revealed that the okadaic acid-induced damage is limited to the cytokeratin intermediate filament and plectin networks (including the bile canalicular sheaths), with little or no effect on microtubuli or microfilaments (apart from the actin filaments in the bile canalicular sheaths). Concentrations of vinblastine or cytochalasin D that disrupt microtubuli and actin microfilaments, respectively, thus have no effect on autophagy (Blankson et al., 1995; Seglen et al., 1996). However, vinblastine does inhibit a relatively late step in the autophagic process, thereby causing accumulation of prelysosomal autophagic vacuoles (Seglen, 1987).

Okadaic acid induces overphosphorylation of both keratin K8/K18 and plectin. An equivalent effect on keratin phosphorylation and distortion of the keratin intermediate filament and plectin networks occurs also with the protein phosphatase inhibitors, microcystin-LR (MC), calyculin A (CA) (Takuma et al., 1993) and tautomycin (TA) (Blankson et al., 2000; Blankson et al., 1999; Ohta et al., 1992). Genistein, prunin and naringin offer prominent protection against okadaic acid-induced disruption of the keratin intermediate filament network (Blankson et al., 1995) and to some lesser extent of the plectin network (Ruud Larsen et al., 2002). Furthermore, naringin antagonizes both okadaic acid- and microcystin-induced phosphorylation of keratin K8/K18 and plectin, but has no effect on calyculin- or tautomycin-induced phosphorylation (Blankson et al., 2000; Blankson et al., 1999). This would indicate an involvement of both a type 2A protein phosphatase (PP2A)

and a type 1 protein phosphatase (PP1) in maintaining the integrity of the hepatocytic intermediate cytoskeleton, with naringin-sensitivity restricted to the PP2A effects. Autophagy may, consequently, depend on both an intact intermediate filament organization and on a naringin-sensitive protein kinase.

The cytoskeletal effects of okadaic acid and naringin are not universal, but depend on cell type as well as on the biological environment. For example, intragastrically administered naringin failed to protect against okadaic acid effects on the cytoskeleton of colonocytes *in vivo*, whereas the nonspecific protein kinase inhibitor K-225a significantly reduced the extent of colonocytic keratin fragmentation (Berven et al., 2001). Furthermore, although intravenously (but not intragastrically) administered okadaic acid induced a naringin-sensitive disruption of hepatic bile canalicular sheaths *in vivo*, the toxin failed to disrupt the general hepatocytic keratin network (Berven et al., 2001). Possibly, the cell-to-cell or cell-to-matrix contacts in the intact tissue exert a stabilizing and protective effect on the general keratin network.

The relationship between autophagy and the cytoskeleton remains obscure, but a role for keratin or plectin in the sequestration process would seem possible (Blankson et al., 1995; Seglen et al., 1996). One may, e.g., envisage a keratin/plectin scaffold serving as support for autophagic sequestration; if so, an okadaic acid-induced overphosphorylation of these proteins could cause scaffold collapse and cessation of the sequestration process. An involvement of intermediate filaments in autophagic protein degradation has also been reported in HTC cells (Earl et al., 1987).

TOXIN-INDUCED, NARINGIN-SENSITIVE APOPTOSIS

Okadaic acid (OA) and microcystin-LR (MC) induce apoptotic cell death in isolated rat hepatocytes (Boe et al., 1991). The *slow apoptosis* induced by low doses of OA or MC is completely prevented by naringin, but not by other flavonoids like genistein, apiin, neohesperidin or PD-98059 (Blankson et al., 2000). As described in the previous section, low doses of OA induce early phosphorylation of keratin and subsequent disintegration of the keratin intermediate filament network, without affecting the microtubular and microfilamentous networks. High doses of OA or MC cause, in addition, extensive cytoskeletal rearrangements with surface blebbing and chromatin hyper condensation, and elicit a *rapid apoptosis* that is naringin-resistant (Blankson et al., 2000; Fladmark et al., 1999; Ruud Larsen et al., 2002). Two distinct apoptotic mechanisms would thus seem to operate: one regulated by PP2A (a naringin-sensitive, slow apoptosis), the other by PP1 (a naringin-resistant, rapid apoptosis). See figure 2.

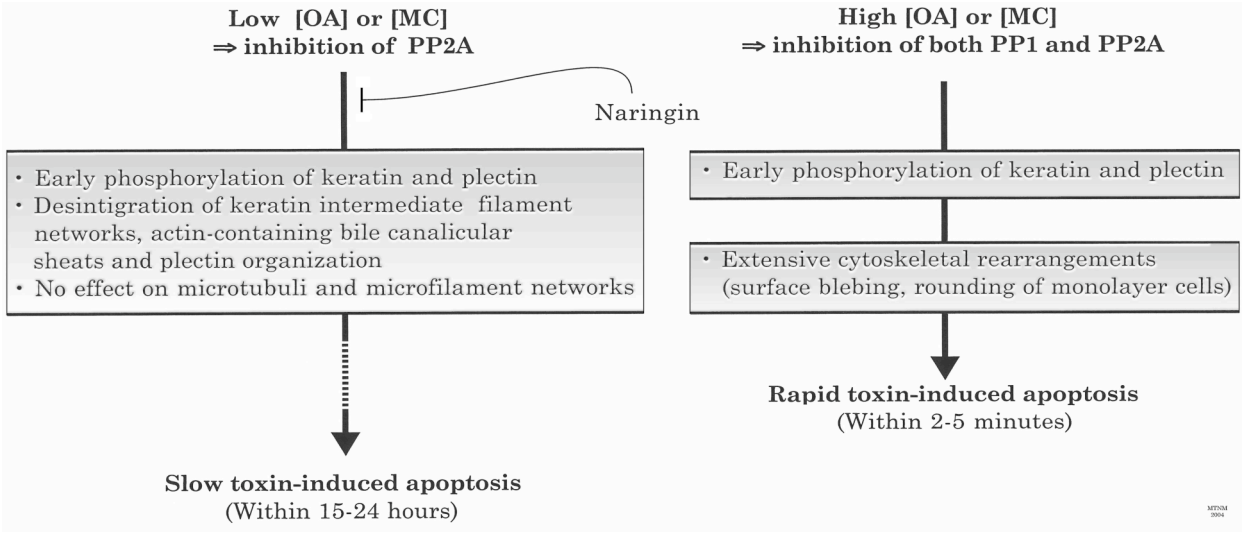

Fig. 2. **Dual mechanism for algal toxin-induced hepatocytic apoptosis.**· Low doses of okadaic acid (OA) or microcystin-LR (MC) inhibit PP2A, with subsequent naringin-sensitive slow apoptosis. High doses of toxin inhibit both PP1 and PP2A, with subsequent naringin-resistant rapid apoptosis.

Interestingly, in human and rat hepatoma cells, naringin had no cytoprotective, anti-apoptotic effect (Blankson et al., 2000). Furthermore, OA-induced cell death in human CA3 hippocampal neurons was prevented by the flavonoid PD-98059, but not by naringin (Runden et al., 1998). Both PD-98059 and naringin failed to protect against the spontaneous apoptosis undergone by freshly isolated colonocytes (Berven et al., 2001). Thus, the cytoprotective, anti-apoptotic effect of naringin appears to be quite cell-specific, suggesting interesting therapeutic possibilities.

It has been proposed that the keratin system may participate in the regulation of anti-apoptotic signaling in human vascular endothelial cells (Miao et al., 1997). In human epithelial cells (SNG-M) and mouse parietal endodermal cells (HR-9), however, this relationship seems less obvious, as type I keratins (K18 and K19) are commonly cleaved secondarily to the activation of apoptotic proteinases of the caspase family (Caulin et al., 1997; Ku et al., 1997). Accordingly, it is still uncertain whether apoptotic cell death and cytoskeletal disruption are causally connected or just parallel effects of algal toxins.

METABOLIC REGULATION OF METHIONINE FLOW: CORRELATION WITH AUTOPHAGY

Methionine is an essential sulfuric amino acid, and its metabolic products are involved in multiple fundamental biological processes. Besides protein synthesis, methionine is crucial for the synthesis of S-adenosylmethionine (SAM) and in the formation of the polyamines spermine and spermidine. Both methionine adenosyltransferase (MAT) and protein synthesis compete for available methionine. Every tissue possesses the key enzymes

needed for the methionine cycle (Fig. 3). However, due to two unique hepatic isoforms of MAT, liver cells can synthesize more SAM in response to excessive methionine than other cells. SAM has a very reactive methyl group that makes it an important biological methylating agent in transmethylation reactions, yielding SAH as one end product. It is the second most widely used enzyme substrate after ATP (Cantoni, 1975). With the known exception of glycine N-methyltransferase (GNMT), SAH acts as a competitive inhibitor of most, if not all, of the methyltransferases; its continuous removal is, therefore, necessary for methylation to proceed. Adenosylhomocysteinase reversibly hydrolyzes SAH to adenosine (Ado) and homocysteine (Hcy). However, since the equilibrium of this reaction favors SAH synthesis (de la Haba and Cantoni, 1959), the removal of the products is crucial. Hcy can bind to proteins, and is also readily exported to the extracellular compartment (Greenberg et al., 1989; Hoffman et al., 1980); (Finkelstein et al., 1971; Svardal et al., 1986).

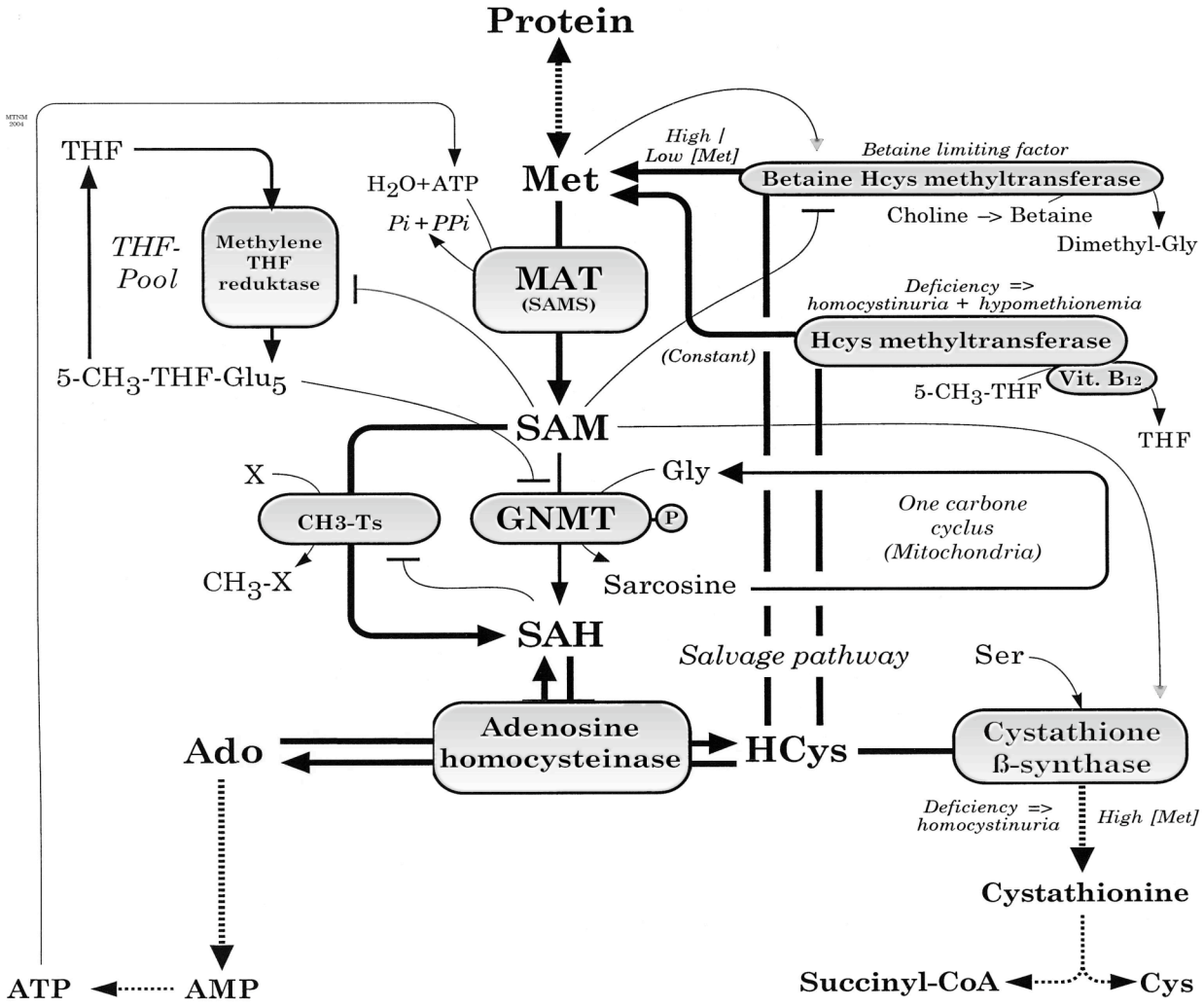

Fig. 3. **Metabolism of methionine.** Open arrowhead, activation; filled arrowhead, metabolic flow; arrow bar, inhibition; dashed line, metabolic flow via several intermediate steps. MAT/SAMS, methionine-adenine-adenosyltransferase/S-adenosyl-methionine-synthetase; GNMT, glycine-N-methyl-transferase; CH3-Ts, methyltransferases; X, substrate for methyltransferases; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcys, homocysteine; THF, tetrahydrofolate.

In contrast to SAH, Hcy can be taken up by liver and other cells where it serves as substrate for three different enzymes. The pyridoxal-dependent heme enzyme cystathione-β-synthase (Kery et al., 1994) catalyzes the only reaction that irreversibly removes Hcy, with the formation of cystathionine (which in liver and kidney cells is further metabolized to cysteine by cystathionase). Through the "salvage pathway", the homocysteinyl moiety is conserved for the resynthesis of methionine by two parallel enzymatic reactions. In the first reaction, unique to liver and kidney cells, cytosolic betaine-homocysteine methyltransferase (BHMT) utilizes betaine as the methyl donor in a reaction that is essential for the oxidative catabolism of choline. BHMT is inhibited by SAM in a feedback regulation serving to maintain the carefully balanced intracellular SAM/SAH ratio, whereas both low and high levels of methionine activate the enzyme in a poorly understood biphasic regulation (Finkelstein et al., 1982). The enzyme also helps to metabolize excessive amounts of betaine and Hcy. In the second "salvage" reaction, common to all tissues, the vitamin B_{12}-dependent enzyme Hcy-methyltransferase utilizes 5-methyl-THF as methyl donor in the resynthesis of methionine. This reaction is important for the recycling of methyl groups (transferred by the enzyme methylene-THF-reductase) from the one-carbon (THF) pool.

To prevent a build-up of SAM, cytosolic GNMT continuously monitors and regulates the SAM/SAH ratio, and so serves to dispose of excess methyl groups by methylation of glycine to sarcosine. Thus, GNMT functions as a main regulator of the cellular methylation capacity. However, SAM can also be routed back to methionine through several intermediate steps that take part in the synthesis of spermine and spermidine, involving the enzymes S-adenosylmethionine decarboxylase, spermidine synthetase and methylthioadenosine phosphorylase (pathway not shown). Despite their biological importance, the synthesis of polyamines may only use a small fraction of the SAM available (Mudd and Poole, 1975).

Unlike other methyltransferases, GNMT (Blumenstein and Williams, 1960) is an abundant homotetrameric folate-binding protein (Ogawa and Fujioka, 1982) that is allegedly activated by phosphorylation (Wagner et al., 1989) and specifically inhibited by 5-methyl-tetra-hydrofolate pentaglutamate (5-CH_3-THF-Glu_5) (Wagner et al., 1985). Furthermore, okadaic acid-induced phosphorylation of GNMT is naringin-sensitive in isolated rat hepatocytes (paper I). As the balance of SAM/SAH shifts in favor of SAM, GNMT will cooperatively bind its substrate to activate the enzyme. Phosphorylation seems to facilitate further binding of SAM (Wagner et al., 1989), at the same time as SAM itself inhibits methylene-THF-reductase and thus reduces the formation of the inhibitor 5-CH_3-THF-Glu_5. Furthermore, SAM will activate cystathione-β-synthase in liver cells to catabolize excessive Hcy. As the level of SAM gradually falls, 5-CH_3-THF-Glu_5 once more, inhibits GNMT, and Hcy enters the salvage pathway.

Interestingly, *in vivo* suppression of autophagy by ethionine administration in male rats has been reported (Ohshita, 2000). Ethionine, an ethyl analog of methionine, is known to cause a marked decrease of hepatocytic ATP (Shull et al., 1966) and GTP levels (Puddu et al., 1967), owing to a trapping of adenosine moieties through formation of S-adenosyl-methionine. As discussed in the *"Autophagy"* section on page 11, the initial steps in the autophagic pathway are particularly sensitive to intracellular ATP concentrations (Plomp et al., 1989; Plomp et al., 1987). Subsequent administration of both adenine and methionine reverses the ethionine suppression of autophagy (Ohshita, 2000), indicating that the suppression probably is caused by ethionine-induced ATP depletion. Restriction of excessive SAM accumulation by GNMT may thus conceivably help to maintain cellular ATP levels through conservation of adenosine. The inhibition of autophagy by Hcy shown in paper II was accompanied by a depletion of cellular ATP, consistent with adenosine trapping in SAH (catalyzed by adenosine homocysteinase) and SAM (due to SAH inhibition of SAM-consuming methyltransferases). However, since added adenosine potentiated rather than antagonized the inhibition of autophagy by Hcy, mechanisms additional to the depletion of adenosine and ATP would seem to be involved. Although no role in autophagy could be found for any of the tested metabolites related to Hcy metabolism (paper II), enzymes involved in methyl metabolism, e.g., BHMT, could well perform a dual function. The previously described binding of BHMT degradation products to lysosomal membranes (Ueno et al., 1999) appears to be part of the normal autophagic-lysosomal degradation of this enzyme (Furuya et al., 2001), but in addition to this, our laboratory has recently found a novel BHMT isoform that binds specifically to autophagosomal membranes (A. Øverbye, M. Lunde Sneve and M. Fengsrud, unpublished results). If this protein performs an autophagic function, it could well be subject to regulation by Hcy, the substrate of its parent enzyme. Several other autophagosomal membrane-binding proteins have also been identified in our laboratory, but none of them have yet been shown to take part in the regulation of autophagy.

S6 AND S6 KINASE (S6K)

S6 is one of 33 proteins that together with the 18S rRNA constitute the mature 40S ribosomal subunit. S6 is a highly evolutionary conserved phosphoprotein that is identical in rat (Chan and Wool, 1988; Wettenhall et al., 1988), mouse (Lalanne et al., 1987) and man (Heinze et al., 1988; Lott and Mackie, 1988). It has been shown that phosphorylation of the ribosomal subunit S6 promotes ribosomal binding of a special subclass (5'TOP) of mRNAs that contain an oligopyrimidine tract at their 5' transcriptional start site (Jefferies et al.,

1994). These mRNAs encode ribosomal proteins and translation factors; their increased expression thus serves to enhance the cellular protein synthesis capacity (Nielsen et al., 1982). S6 is sequentially phosphorylated at five serine sites: Ser^{235}, Ser^{236}, Ser^{240}, Ser^{244} and Ser^{247} (Bandi et al., 1993; Ferrari et al., 1991; Krieg et al., 1988) by two very homologous S6-kinases termed S6K1 (Ballou et al., 1988b) and S6K2 (Shima et al., 1998).

S6K1 is expressed from one mRNA transcript, but alternative translational start sites generates two isoforms (Volarevic and Thomas, 2001): a long form, and a 23 amino acids shorter form, respectively termed $S6K^{p85}$ and $S6K^{p70}$ (Banerjee et al., 1990; Grove et al., 1991; Kozma et al., 1990; Reinhard et al., 1992). A nuclear localization signal within the N-terminal domain of $S6K^{p85}$ explains why this protein is found exclusively in the nucleus, as opposed to the largely cytosolic $S6K^{p70}$ (Reinhard et al., 1994). The ubiquitously expressed S6K2 (synonymous with $S6K^{p70beta}$, (Gout et al., 1998) also has a nuclear localization signal (Saitoh et al., 1998). S6K2 shares 67% identity with S6K1 (Saitoh et al., 1998). Compensatory up-regulation of the S6K2 transcript has been found in S6K1-mutated mouse, suggesting overlapping and counterbalancing roles for these enzymes (Shima et al., 1998). All S6Ks share similar structure and domain characteristics consisting of an N-terminal acidic domain, a highly conserved serine/threonine kinase catalytic domain and a regulatory carboxy-terminal tail with an autoinhibitory sequence, similar to the motif surrounding phosphorylation sites in S6 (Banerjee et al., 1990; Ferrari et al., 1992). See figure 4.

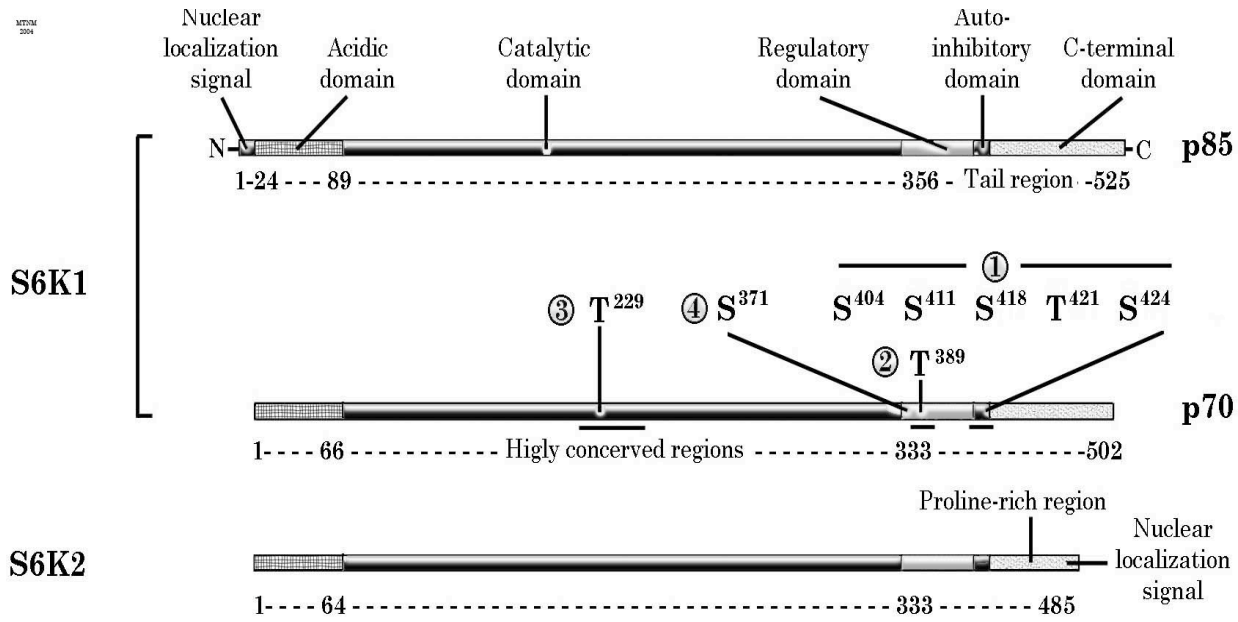

Fig. 4. **A proposed model for the structure and functional domains of S6K.** Encircled numbers (①-④) on S6K1 p70 indicate the suggested sequential phosphorylation of at least seven specific regulatory sites that activate the enzyme. In the inactive state, the acidic and C-terminal domains interact. The first phosphorylation step facilitates the phosphorylation of Thr^{389} by mTOR, which disrupt the domain interactions and allow the subsequent phosphorylation of Thr^{229} by PDK1. The final activation step involves the phosphorylation of Thr^{371} by members in the PKC family (PKCλ and PKCζ). Modified and redrawn from (Volarevic and Thomas, 2001).

The function of S6K2 and the functional significance of the differential subcellular localization of the two S6K1 isoforms are still poorly understood. S6K1 and S6K2 appear to differ in terms of activation mechanisms, as recent reports have suggested that S6K2, but not S61, is regulated by a mechanism that involves MEKs and perhaps ERKs (Wang et al., 2001). Considerably more is known about the activation and regulation of the S6K1s.

In the dephosphorylated and inactive state, S6K1 loops back or folds due to interactions between the C-terminal and acidic domains. Activation of S6K1 is suggested to depend on the sequential phosphorylation of at least seven specific regulatory sites (Pullen and Thomas, 1997). The initial activation step is thought to occur through the phosphorylation of Ser^{404}, Ser^{411}, Ser^{418}, Thr^{421} and Ser^{424} located in the *tail region*; these phosphorylations facilitate the mitogen-induced, rapamycin-sensitive phosphorylation (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992) of Thr^{389} by mTOR (Dennis et al., 1996) that disrupts the domain interactions (Dennis et al., 1998). The final activation steps involve mitogen-induced phosphorylation at Thr^{229} by PDK1 (Alessi et al., 1998; Pullen et al., 1998) and at Thr^{371} by an atypical PKC family kinase (PKCλ or PKCζ) that is PI3K-regulated and phorbol ester-insensitive (Moser et al., 1997; Romanelli et al., 1999). However, it should be noted that the exact order of the phosphorylation events in the *in vivo* activation of S6K1 is still debated, and the involved protein kinases have not yet been conclusively identified.

In rat hepatocytes, insulin, amino acids, cAMP, metabolic stress and AMP all take part in the fine-tuned regulation of either 5′TOR mRNA translation or general translation in response to the various stimuli. See figure 5 on next page. Amino acids and insulin not only inhibit autophagy (Kovacs et al., 1981; Woodside and Mortimore, 1972), but they also induce a rapamycin-sensitive mTOR-mediated phosphorylation of S6K and S6 (Blommaart et al., 1995; Luiken et al., 1994). Insulin activates the wortmannin/LY294002-sensitive $PI3kinase^{classI}$ (Blommaart et al., 1997a; Strømhaug et al., 1997) through the insulin receptor, producing PtdIns(3,4,5)P_3 (page 17 and 14) which recruits PKB (protein kinase B/Akt) to the membrane, where it is activated by the constitutively active PDK1 by phosphorylation at Thr^{308} and Ser^{473} (Vanhaesebroeck and Alessi, 2000). In the presence of amino acids, PKB either directly activates mTOR by phosphorylating Thr^{2448}, or by inducing structural changes in mTOR that facilitate phosphorylation by a still unidentified mTOR kinase(s) (mTORK) (Krause et al., 2002a; Nave et al., 1999). Some activation of mTOR is also achieved with administration of amino acids alone (Blommaart et al., 1995; Luiken et al., 1994), but not with PKB alone (Nave et al., 1999). In rat hepatocytes, it has been suggested that high concentrations of amino acids activate $S6K1^{p70}$ independently of PKB (Krause et al., 2002b). Activated mTOR has at least three functions: to inactivate 4E-BP1 by phosphorylating Thr^{37} (Gingras et al., 1999; Hara et al., 1998), to participate in the activation of

S6K1 by phosphorylating Thr^{389} (Dennis et al., 1996) and to phosphorylate and inactivate PP2A (Begum and Ragolia, 1996). PP2A selectively dephosphorylates S6K (Ballou et al., 1988a), whereas PP1 selectively dephosphorylates S6 (Olivier et al., 1988). Inactivation of PP2A will prevent dephosphorylation of PKB (Meier et al., 1998; Millward et al., 1999), 4E-BP1 (Nanahoshi et al., 1998) and S6K1, and therefore enhance the effect of amino acids and insulin. PDK1 also has numerous roles, as a non-membrane-bound cytosolic kinase that phosphorylates S6K1^{p70} at Thr^{229} (Pullen et al., 1998) and activates the atypical PKCs (Romanelli et al., 1999). Thus, amino acid- and insulin-mediated activation of mTOR both activates *general protein synthesis* through dissociation of eIF-4E (a 7-methyl-guanosine mRNA cap-binding protein) from 4E-BP1, and *5´TOR-mRNA translation* through phospho-S6.

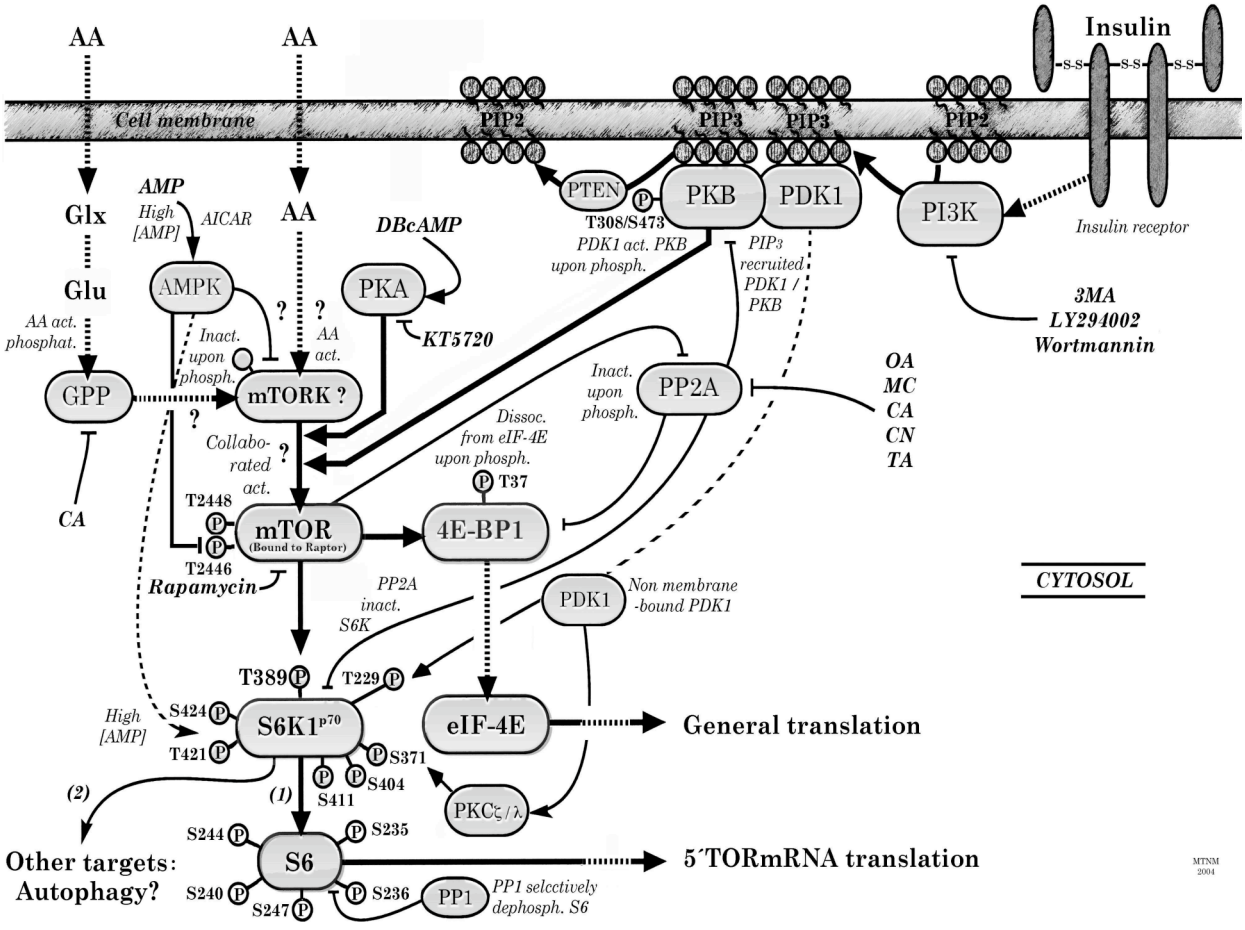

Fig. 5. **Model for the interactions between insulin- and amino acid-dependent signaling in the mTOR-mediated regulation of 5´mRNA translation and general protein synthesis**. Targets of (1) Thr^{389}-dependent and (2) Thr^{389}-inde-pendent S6K activation are indicated. See main text for detailed description of the model. (It should be noted, however, certain details in this figure are just recently debated e.g. mTORK and negative control of mTOR activation by AMPK-mediated phosphorylation at Thr2426: See the main text and *"Discussion"* section!) GP, glutamate-dependent protein phosphatase; Glx, glutamine or glutamic acid; PKA, cAMP-dependent protein kinase, mTOR, mammalian target of rapamycin; mTORK, proposed mTOR kinase(s); AMPK, AMP-activated protein kinase; PKB, protein kinase B; PDK1, phosphoinositide-dependent kinase 1; PTEN, phosphatase and tensin homologue on chromosome 10; PI3K, phosphatidylinositol 3-kinase; eIF-4E, eukaryotic initiation factor 4E; 4E-BP1, eIF-4E-binding protein 1. The AA/insulin-induced PI3K/mTORK/mTOR/ S6K/S6 pathway for the mTOR-mediated protein translation is based on (van Sluijters et al., 2000), and the GPP/AMPK/ mTORK pathway on the work of (Krause et al., 2002a) and various other studies as indicated in the main text.

In isolated rat hepatocytes, the PKA activator DBcAMP mimics the effect of amino acids in causing a rapamycin-sensitive, PKB-independent S6 phosphorylation by S6K), and a KT5720-sensitive suppression of autophagy in isolated hepatocytes. In adipocytes, a forskolin-induced increase in cellular cAMP instead antagonizes the effect of insulin and amino acids (Scott and Lawrence, 1998), suggesting that the role of PKA in the regulation of mTOR and S6K is highly cell type-dependent (Blommaart et al., 1995).

AICAR-activated AMPK mediates a naringin-sensitive tail phosphorylation of S6K1^{p70}, but no subsequent phosphorylation of S6 is observed, apparently because AICAR prevents the mTOR-mediated phosphorylation of S6K^{p70} at Thr^{389} (paper III; see also the "*AMP-activated protein kinase (AMPK)"* section on page 29). Additionally, it has been reported that both AICAR and amino acid withdrawal significantly reduce the phosphorylation of 4E-BP1 at Thr^{37}, S6K1 at Thr^{389} (Bolster et al., 2002) and PKB at Ser^{473}/Ser^{308} in rat skeletal muscle (Winder, 2001) and in HEK293/CHO-IR cells (Hara et al., 1998). Whether AMPK phosphorylates S6K1 at Thr^{421}/Ser^{424} directly or through intermediate steps is not yet known. However, it does seem less likely that AMPK inactivates mTOR directly because S6K1 inactivation by AICAR is slower than by rapamycin (Krause et al., 2002a).

mTOR is regulatorily associated to the 150 kDa binding protein Raptor (regulatory associated protein of mTOR) (Hara et al., 2002), which serves as a scaffold protein for the binding of the mTOR-raptor complex to 4EBP1 and S6K1 through their respective TOS (TOR signaling) motifs (Nojima et al., 2003; Schalm et al., 2003). The complex promotes the downstream phosphorylations in nutrient stimulated mammalian cells. However, it has been reported that binding of mLST8/GL to the mTOR kinase domain is necessary for mTOR to form a nutrient-sensitive interaction with raptor (Kim et al., 2003). Several new proteins have been identified to take part in the complex upstream regulation of the mTOR-S6K-S6 signaling pathway. The protein products of the tumor suppressor genes tuberous sclerosis TSC complex 1 and 2 form a protein complex (TSC1–TSC2) that inhibit the mTOR signaling pathway (Marygold and Leevers, 2002) and the activation of the small GTPase Rheb (Ras ortholog enriched in brain) (Kim et al., 2002; Loewith et al., 2002) that participates in the rapamycin-sensitive phosphorylation of S6K1 (Manning and Cantley, 2003). The significance of Rheb has been demonstrated in cells where the protein has been over expressed and prevents the rapid dephosphorylation and inactivation of S6K1 caused by amino acid withdrawal. Rapamycin completely block the Rheb-induced activation of S6K1 and over expression of TSC1 and TSC2 is sufficient to decrease the phosphorylation of both S6K1 and 4EBP1 (Manning and Cantley, 2003; Marygold and Leevers, 2002). However, it has not yet been shown whether the mTOR interacting proteins TSC1/TSC2 and/or Reheb actually binds to the mTOR-raptor complex.

Activation of mTOR involves several phosphorylation sites (Cheng et al., 2004; Scott et al., 1998), and at least two of these sites are autophosphorylated. The autokinase activity towards at least one these (Ser^{2481}) is furthermore, rapamycin-resistant (Peterson et al., 2000) and LY294002/wortmannin-sensitive at higher doses (Brunn et al., 1996) reflecting the structural homology to PI3K and other members of the large protein family termed PIKKs (phosphoinositide kinase related kinases). Increased phosphorylation of mTOR at Ser^{2448} is associated with insulin stimulation (Scott and Lawrence, 1998), and it has been reported that mTOR can be phosphorylated directly by PKB (Nave et al., 1999), although it is debatable whether this actually takes place *in vivo* (Scott and Lawrence, 1998). It is thus likely that other protein kinases may be involved in the insulin/amino acid-stimulated activation of mTOR (confer with the *"Discussion"* section: page 47-52). The activity of an mTOR kinase could well be regulated by phosphorylation and dephosphorylation, and it has been suggested that the amino acid signaling pathway leading to $S6K^{p70}$ activation could include a glutamate-activated protein phosphatase (Hara et al., 1998; Peterson et al., 1999). Depletion of glutamine and asparigine causes inactivation of $S6K1^{p70}$ in Jurkat cells (Iiboshi et al., 1999), possibly due to inactivation of this phosphatase. A calyculin A-sensitive glutamate-dependent protein phosphatase type 2A (GPP) has been suggested as a link between AMPK and mTOR kinase activation in rat hepatocytes (Krause et al., 2002a). However, our data suggest (paper IV) that calyculin A primarily activates $S6K1^{p70}$ at Thr^{421}/Ser^{424} rather than at the mTOR-sensitive site Thr^{389}. Calyculin A might also be expected to prevent dephosphorylation of S6K1 by inactivation of PP2A.

The ability of AICAR to induce an AMPK-mediated (naringin-sensitive) phosphorylation of S6K1 at Thr^{421}/Ser^{424} while simultaneously suppressing the mTOR-mediated phosphorylation at Thr^{389} (naringin-insensitively and thus probably independently of AMPK) generates a uniquely phosphorylated S6K1 species that is inactive in S6 phosphorylation, but which may have other biological effects. Mediation of AICAR/AMPK-induced autophagy suppression (Krause et al., 2002a) is one possibility (paper V), phosphorylation of the stress-activated protein kinases SEK1 and JNK (paper III) is another.

AMP-ACTIVATED PROTEIN KINASE

The first original reports of AMPK date back to 1973, when it was found that a soluble fraction from rat liver caused a time-dependent inactivation and phosphorylation of microsomal HMG-CoA-reductase (Beg et al., 1973) and acetyl-CoA-carboxylase (Carlson and Kim, 1973) in the presence of ATP. The responsible protein kinases were individually designated as HMG-CoA-reductase kinase (Beg et al., 1978; Gil et al., 1980; Ingebritsen et al., 1978; Keith et al., 1979) and acetyl-CoA-carboxylase-3 (Carling and Hardie, 1986)

respectively. Both HMG-CoA-reductase-kinase (Ferrer et al., 1985) and acetyl-CoA-carboxylase-3 (Carling et al., 1987) were reported to be stimulated by AMP, and when it in 1988 became clear that these kinases were identical, they were renamed AMP-activated protein kinase (AMPK) (Munday et al., 1988; Sim and Hardie, 1988). Recently, AMPK has also more accurately been referred to as AMP-*dependent* protein kinase, although, studies dated back to 1978 and 1980 had already suggested that ADP and AMP inactivated HMG-CoA-reductase (Ingebritsen et al., 1978) and acetyl-CoA-carboxylase (Yeh et al., 1980) in crude microsomal systems, it was discovered that these enzymes were subjected to regulation through an "energy charge" sensing mechanism, which monitors the phosphorylation state of cellular ATP, ADP and AMP.

Purification of AMPK from rat hepatocytes has shown that it consists of three subunits (Carling et al., 1989; Davies et al., 1994) referred to as 63 kDa α, 38 kDa β and 35 kDa γ, which all co-purify on an affinity column with Sepharose-bound ATP (Davies et al., 1994). The catalytic capability is confined to the N-terminal domain of the α-subunit (Carling et al., 1989), whereas the two others subunits offer regulatory, structural and stabilizing functions for AMPK. Autoinhibitory (Crute et al., 1998) and activation (Stapleton et al., 1996) domains are located proximal to the catalytic site of the α-subunit. Assembly of both the α- and γ-subunits depend on binding to conserved KIS- and ASC-domains on the β-subunit (Thornton et al., 1997). In mammalian cells, there are two α-subunits (α_1, α_2), two β-subunits (β_1, β_2) and three γ-subunits (γ_1, γ_2, γ_3), all encoded by different genes. High levels of α_2 are, e.g., found in rat and porcine hepatocytes, skeletal and cardiac muscle (Gao et al., 1995; Stapleton et al., 1996; Verhoeven et al., 1995), as opposed to the more widely distributed α_1, although high levels of α_1 are only found in testis (Stapleton et al., 1996). Nevertheless, the more active α_1-subunit accounts for about 90% of the total AMPK-activity in rat hepatocytes. The noncatalytic γ_2 and γ_3 subunit isoforms are predominantly found in brain, distinct from the γ_1 isoform found in hepatocytes. Thus, a potentially large subfamily of heterometric (αβγ) AMPKs, based on various combinations of the three AMPK subunits, may be present, with different isoform profiles in different cell types (Cheung et al., 2000; Stapleton et al., 1997; Thornton et al., 1998).

The mammalian AMPK subunits show striking similarities to the gene products from the yeast *Saccharomyces cerevisiae*. The AMPK α-subunit is about 60% identical to Snf1p (Mitchelhill et al., 1994), the γ-subunit 35% identical to Snf4p (Carling et al., 1994) and the β-subunit is related to a family with three gene products Snip1p, Snip2p and Gal83p (Gao et al., 1996; Stapleton et al., 1994; Woods et al., 1996). SNF1 (sucrose non-fermenting; the term applied to the enzymatically active complex between Snf1p, Snp4p and possible other additional subunits) and AMPK both phosphorylate acetyl-CoA-carboxylase (Mitchelhill et

al., 1994; Woods et al., 1994), and appear to be enzymes that have been conserved since the common ancestor of mammalian and yeast diverged. In yeast, starvation activates SNF1 and inactivates acetyl-CoA-carboxylase; however, AMP does not regulate the enzyme directly despite the correlation between energy (glucose) depletion and SNF1 activation (Wilson et al., 1996). AMP is thought, instead, to regulate a putative upstream kinase. Suggested models for glucose-regulated protein interactions within the yeast SNF1 protein kinase complex (Jiang and Carlson, 1996; Jiang and Carlson, 1997) have been fundamental for the interpretation of the analogous interaction of AMPK subunits. Recently, Tos3p, Pak1p and Elm1p has been identified as three upstream kinases that activate the SNF1 protein kinase *in vivo* (Hong et al., 2003).

It has been proposed that binding of AMP to four tandem repeated CBS-domains (Bateman, 1997) on the γ-subunit (Cheung et al., 2000) elicit steric alterations that impede the autoinhibitory α segment and thus facilitate subsequent activation by phosphorylation at Thr^{172} by the upstream AMPK kinase (AMPKK) (Hawley et al., 1996), as illustrated in figure 6.

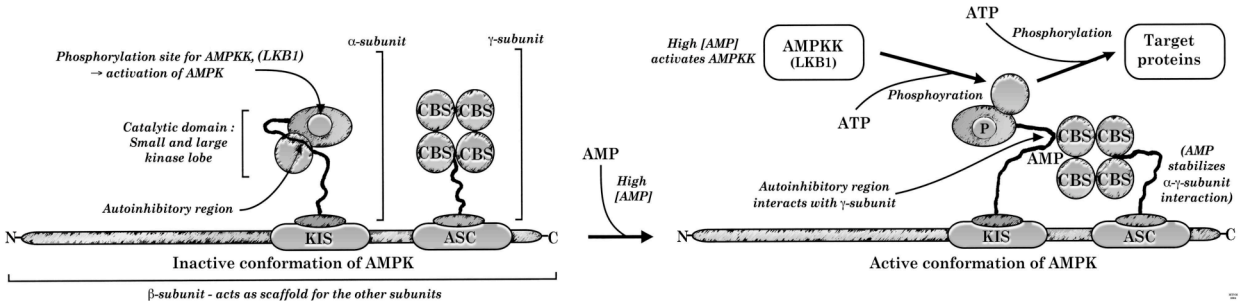

Fig. 6. **Model for the assembly and interactions of the α-, β- and γ-subunits of AMPK in the inactive and active conformation.** At high levels of cytosolic AMP, the nucleotide will bind to the α- and γ-subunits, forcing a conformation change that facilitates subsequent phosphorylation and thus activation of the catalytic site on AMPK. In the active state with AMP bound, an inactivation by the autoinhibitory region or by dephosphorylation is sterically prevented. As [AMP] falls, AMP again dissociates from AMPK forcing a conformational change that exposes the catalytic domain for both dephosphorylation and auto inhibition, which inactivate the enzyme. CBS, cystathionine β-synthase domain; KIS, kinase interaction sequence; ASC, association with SNF1 complex. Redrawn and modified from (Cheung et al., 2000).

High cytosolic levels of AMP also inhibit subsequent dephosphorylation and deactivation of AMPK by a protein phosphatase type-2C (PP2C) (Davies et al., 1995). Therefore, AMP will activate AMPK much in accordance with the classical allosteric Monod/Wyman/Changeux model (Monod et al., 1965), with AMP promoting the transition from the tight state (T) to the relaxed state (R). In the R-state, the autoinhibitory region displaces from the catalytic domain on the α-subunits and interacts instead with the γ-subunit. AMP both stabilizes the α-γ-subunit interaction and exposes the Thr^{172} for phosphorylation by AMPKK (LKB1) (Crute et al., 1998). As the concentration of AMP drops, AMP again dissociates from AMPK, promoting the reverse transition back to the T-state, in which PP2C is able to dephosphorylate and deactivate AMPK. See figure 7.

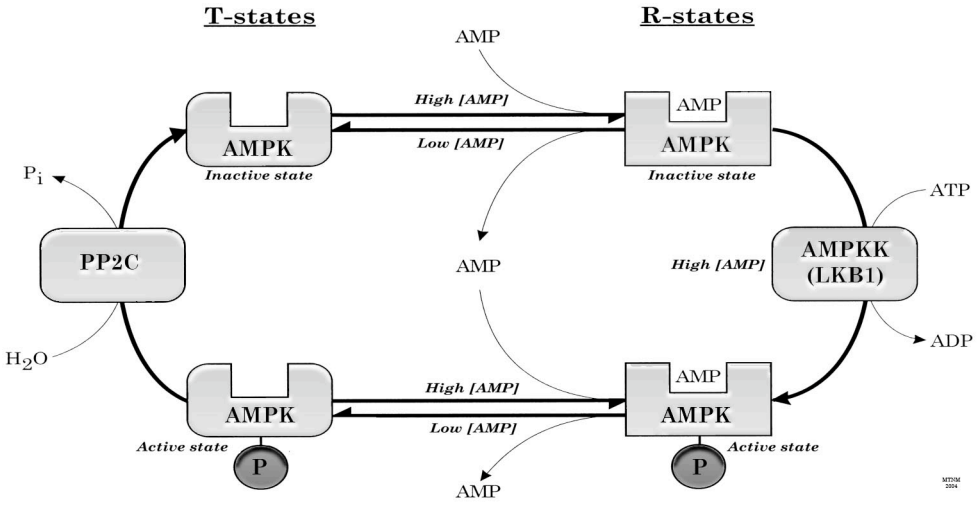

Fig. 7. **Proposed classical Monod/Wyman/Changeux model (Monod et al., 1965) for the allosteric regulation of AMPK by AMP and AMPKK (i.e. LKB1:STRAD:MO25).** As the cellular [AMP] increases, AMP binds to AMPK in the T-state ($AMPK^{T\text{-}state}$) and induces a conformational change to the R-state ($AMPK^{R\text{-}state}$). AMPKK is able to phosphorylate $AMPK^{R\text{-}state}$, and thus activate AMPK. Similarly, as the cellular [AMP] drops, AMP dissociates from $AMPK^{R\text{-}state}$ and induces a conformational change back to $AMPK^{T\text{-}state}$. In this state, PP2C is able to deactivate AMPK by dephosphorylation. AMPK is only active in the phosphorylated state. AMP also activates AMPKK, although it is not subjected to phosphorylation. Redrawn from (Hardie and Carling, 1997; Hardie and Hawley, 2001).

The exact location of the AMP-binding site within the α-γ-subunit complex remained unknown until recently, although mutations in the CBS-domain (Cystathionine Beta Synthase) of human cystathionine-β-synthase (i.e. homocystinuria) had indicated that similar domains in AMPK might be involved in the allosteric binding of AMP (Kluijtmans et al., 1996). Binding of ATP to a pair of CBS-domains in the γ_1-subunit has now been reported to act as an intrasteric inhibitor by linking the α and β subunit such that enzyme activity in this state is depressed by preventing phosphorylation of Thr^{172} and by exposing the site for dephosphorylation (Adams et al., 2004). Both ATP and AMP compete for the same allosteric regulatory site. However, AMP activates the enzyme by making Thr^{172} more readily phosphorylated. It has been suggested that the structure of the allosteric binding domain for AMP in both AMPK and cystathionine β synthase, should be named "Bateman modules"(Adams et al., 2004; Bateman, 1997).

Recently, AMPK kinase was identified as the previously described serine-threonine protein kinase LKB1 (interacting protein-1) (Lizcano et al., 2004; Woods et al., 2003a), which is mutated in patients with an autosomal dominantly inherited cancer syndrome predisposing to multiple benign and malignant tumors, termed Peutz-Jeghers syndrome (Hemminki et al., 1998; Jenne et al., 1998). LKB1 functions as a tumor suppressor perhaps by controlling cell polarity and exists as a complex with two accessory subunits, MO25α/β and STRADα/β. LKB1 is known to be phosphorylated at Ser^{431} by the p90 ribosomal S6 protein kinase (RSK) and cyclic AMP-activated protein kinase (PKA) (Collins et al., 2000; Sapkota et al., 2001). However, it has been reported that as many as eight residues on LKB1 are phosphorylated. It has been suggested that LKB1 affects the level of PTEN (a

lipid phosphatase metabolizing the phosphatidylinositol 3,4,5-triphosphate, PIP_3 – cf. figure 5 on page 27), which controls proliferation and survival of cells through the second messenger PIP_3 (Cantley, 2002). Even though that LKB1 possesses a nuclear localization signal at its N-terminal region, and is mainly localized in the nucleus (Sapkota et al., 2002; Smith et al., 1999), a small fraction is also found in the cytoplasm (Nezu et al., 1999; Tiainen et al., 2002) where it may play an important role in mediating its tumor suppressor properties.

A continuously increasing number of identified new targets for AMPK are emerging in the literature. With the recently available Thr^{172} phosphate-specific antibody, the impact and importance of AMPK as a major metabolic regulator and energy gauge has become increasingly evident. In response to severe stress that e.g. affects the ATP-synthase and respiratory chain in the mitochondria, activated AMPK not only phosphorylates HMG-CoA-reductase and acetyl-CoA-carboxylase, but also other enzymes that are crucial for the swift and fine-tuned balance between anabolism and catabolism. See figure 8.

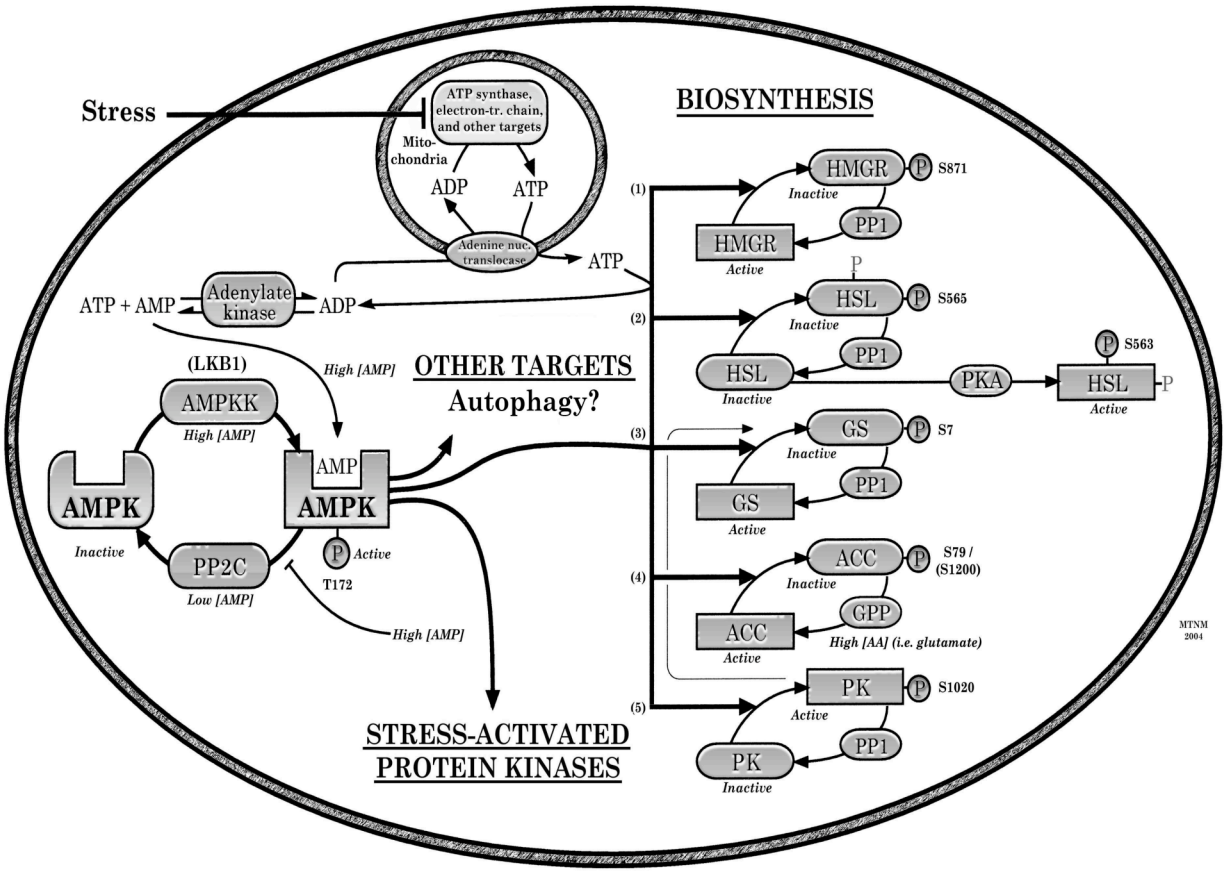

Fig. 8. **Targets for AMPK.** Cellular stress (e.g. heat shock, metabolic poisoning, hypoxia etc.) may cause an ATP depletion in the mitochondria, which increases the cytosolic AMP level by the action of adenylate kinase, thus activating AMPK. Main targets apart from the stress-activated protein kinases: (1) HMG-CoA reductase, (2) hormone-sensitive lipase, (3) glycogen synthase, (4) acetyl-CoA carboxylase and (5) phosphorylase kinase. GPP; glutamate-dependent type-2A protein phosphatase; PP1, protein phosphatase type 1. Modified and redrawn from (Corton et al., 1994; Hardie and Carling, 1997).

In adipocytes, AMPK phosphorylates hormone-sensitive lipase (HSL) at Ser^{565} (Garton et al., 1988), thus preventing activation of the enzyme by phosphorylation at the competing site (Ser^{563}) by cAMP-dependent protein kinase (Garton et al., 1988). In muscle, glycogen-synthase (GS) is inactivated directly by phosphorylation by AMPK and indirectly by AMPK-activated phosphorylase-kinase (PK) (Carling and Hardie, 1989). To preserve the ATP level during stress (e.g. heat shock, metabolic poisoning, hypoxia etc.), AMPK inhibits the synthesis of fatty acids, sterols/isoprenoids (Corton et al., 1994; Corton et al., 1995) and triglycerides (Muoio et al., 1999), while stimulating glucose uptake (Fryer et al., 2000; Kurth-Kraczek et al., 1999) and fatty acid oxidation in skeletal muscle and liver (Merrill et al., 1997; Velasco et al., 1997). However, it has also been reported that AMPK is activated in muscle during exercise (Winder and Hardie, 1996) and that AMPK efficiently inhibits protein synthesis (i.e., 5′TOR-mRNA translation and general protein synthesis; cf. With the *"S6 and S6 kinase (S6K)"* section on page 24-29) by preventing amino acid-dependent, rapamycin-sensitive mTOR-mediated phosphorylation on the ribosomal protein S6 in rat hepatocytes (Dubbelhuis and Meijer, 2002). Surprisingly, AICAR not only activates AMPK (refer to page 16) with subsequent phosphorylation of S6-kinase at Thr^{421}/Ser^{424}, but also inhibits mTOR-mediated phosphorylation at Thr^{389}, which is essential for S6 phosphorylation (paper IV). The inhibitory effect of AMPK on protein synthesis is much more forceful than what is achieved with rapamycin, indicating the participation of signaling pathways other than through mTOR. In paper IV-V we suggest the involvement of AMPK in the negative, naringin-sensitive regulation of autophagy, involving the stress-activated kinases SEK1 and JNK.

STERSS-ACTIVATED PROTEIN KINASES (SAPKs)

The stress-activated kinases is a subfamily of the mitogen-activated protein kinase (MAPK) super family and consists of two distinct classes in mammalian cells: the *c-Jun N-terminal kinases (JNK)* and the *p38 MAPKs* (Garrington and Johnson, 1999; Ip and Davis, 1998). They are activated in response to cellular stress such as irradiation, heat shock, osmotic imbalance, DNA damage, as well as by inflammatory cytokines (Ip and Davis, 1998; Minden and Karin, 1997). All MAPK family members are activated by the phosphorylation of a threonine and a tyrosine residue lying in a Thr-X-Tyr motif, catalyzed by a dual specificity kinase termed MAPK kinase (MKK or MEK). See figure 9.

JNK is phosphorylated and activated by two upstream MKKs, termed MKK4 (SEK1; JNKK1; SKK1) and MKK7 (SEK2; JNKK2; SKK4) (Derijard et al., 1995; Sanchez et al., 1994; Tournier et al., 1997)). There are several isoforms of JNK, the JNK-group of kinases being encoded by three genes (jnk1, jnk2 and jnk3). These genes are alternatively spliced

to give four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms (Carboni et al., 1997). The activation of JNKs leads to the phosphorylation and activation of several nuclear transcription factors such as ATF2 (activating transcription factor 2), Elk-1 and c-Jun, which, e.g., bind to the *c-jun* promoter, resulting in induction of *c-jun* expression (Cavigelli et al., 1995; Gupta et al., 1995; Sanchez et al., 1994). JNK is also known to be required for embryonic morphogenesis and to take part in the regulation of cell proliferation and apoptosis. Interestingly, it has been reported that the tissue distributions of MKK4 and MKK7 in the mouse differ, as indicated by particularly high expression of MKK4-mRNA and very low levels of MKK7-mRNA in hepatocytes (Nishina et al., 1999).

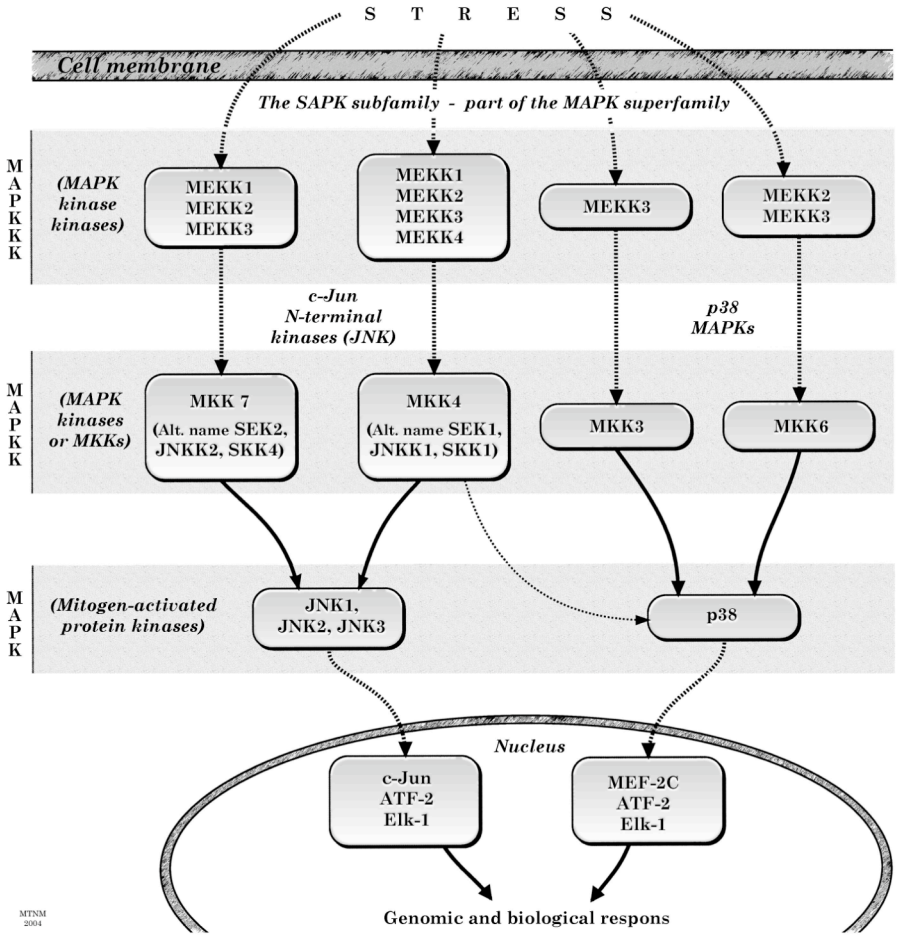

Fig. 9. **Simplified diagram of the stress-activated protein kinase (SAPK) signaling pathways in mammalian cells.** See main text for detailed description of the signaling pathway. Partially based on (Weston and Davis, 2002; Weston et al., 2002).

MKK7 functions as a specific activator of JNK. In contrast, MKK4 also phosphorylates p38 *in vitro* and in p38-overexpressing mammalian cells (Derijard et al., 1995; Lin et al., 1995), but an actual role for MKK4 in the normal activation of p38 is debated. On the basis of current evidence, it seems more likely that two other MKK family members, MKK3 and MKK6, usually mediate the activation of p38 (Raingeaud et al., 1996).

Mammalian scaffold proteins most likely organize the MAPK signaling pathways in response to specific stimuli (Whitmarsh et al., 1998). Examples of these complexes include the JNK-interacting protein (JIP1, JIP2, JIP3) group that binds to JNK, MKK7 and the mixed-lineage group of MAP kinase kinase kinases (MLKs) as well as to ASK2, MEKK2, MEKK4 and TAK1, and facilitate signal transmission by the protein kinase cascade (Kelkar et al., 2000; Whitmarsh et al., 1998; Yasuda et al., 1999). The assembly of the JNK module by scaffold proteins does not function to amplify signals, but is rather thought to facilitate the activation of JNK. It seems likely that the JIP group of scaffold proteins represents only one of several groups of proteins that assemble a functional JNK signaling module. It has been suggested that e.g. the actin-binding protein filament may function as a putative scaffold for the assembly of a cytokine receptor module that activates JNK (Marti et al., 1997).

Protein kinase B (PKB) seems to play an important role in SAPK regulation. In human 293T cells it has been shown that insulin-mediated activation of PKB suppresses the SEK1/JNK signaling pathway by phosphorylating SEK1 at Ser^{78}, and that the inhibitory action of insulin on SEK1 or JNK1 activation can be abolished by the phosphatidylinositol 3-kinase inhibitor LY294002 (Park et al., 2002). In intact cells, activated PKB physically interacts with and phosphorylates SEK1 at Ser^{78}, thereby suppressing SEK1-mediated signaling. Activated PKB has also been shown to phosphorylate and inhibit ASK1 (apoptosis signal-regulating kinase 1), a MAPKKK that stimulates JNK and p38 MAPK signaling pathways (Kim et al., 2001).

Activation of JNK and p38 is known to induce apoptosis in numerous cell types. We have previously shown that okadaic acid-induced apoptosis in rat hepatocytes is naringin-sensitive, most likely by involving the AMPKK/AMPK signaling pathway (Blankson et al., 2000; Blankson et al., 1999), and that the AICAR-mediated naringin-sensitive activation of AMPK causes phosphorylation and activation of JNK (paper III). An involvement of AMPK in the JNK activation is supported by the observation that sustained (>10h) activation of AMPK by AICAR in human FTO2B hepatoma cells induces JNK activation, caspase-3 activation and apoptosis (Meisse et al., 2002). However, during drastic ATP depletion, e.g., through anoxia, AMPK activation is transient and promotes necrosis rather than apoptosis (Marsin et al., 2000). Iodotubercidin completely blocks the AICAR-induced activation of AMPK, JNK and caspase-3 by inhibiting adenosine kinase and thus ZMP-formation (Henderson et al., 1972). The JNK inhibitor, dicoumarol (Krause et al., 2001), does not affect AMPK activity, but prevents JNK and caspase-3 activation as well as apoptosis, indicating that JNK is downstream of AMPK in the hepatocellular apoptosis pathway (Meisse et al., 2002). Similarly, JNK inhibitors might be helpful in establishing a relation-

ship between AICAR-induced autophagy suppression and JNK activation. We have found that high concentrations of the p38/JNK inhibitor SB-203580 (Clerk and Sugden, 1998) antagonize both the autophagy-suppressive effect of AICAR and its activation of the SEK1/JNK pathway (unpublished data*)*. Although p38 MAPK and MKK3 have also been reported to be activated by AICAR in clone 9 cells (Xi et al., 2001) and by okadaic acid in rat hepatocytes (paper III), we find that the p38 phosphorylation is naringin-insensitive and thus unlikely to be mediated through AMPK activation.

The mitogen-activated protein kinase (MAPK) super family also consists of the closely SAPK-related MAP kinases Erk1 and Erk2. However, despite the fact that the Erk1/2 inhibitor PD98059 has been reported to suppress amino acid starvation-induced autophagy in HT-29 intestinal cells (Ogier-Denis et al., 2000), PD98059 has no autophagy-suppressive effect alone in rat hepatocytes, nor does it antagonize okadaic acid- or amino acid-induced inhibition of autophagy (our unpublished data). Therefore, the involvement of MAPKs in autophagy regulation seems to be tissue-specific.

SUMMARY OF THE PAPERS

PAPER I: Okadaic acid-induced, naringin-sensitive phosphorylation of glycine-N-methyltransferase in isolated rat hepatocytes

By mass screening two-dimensional gels, we found that several proteins were overphosphorylated in cells treated with the protein phosphatase inhibitor okadaic acid, and that the phosphorylation of some of these could be antagonized by treatment with the flavonoid naringin. One protein was identified as rat glycine N-methyltransferase (GNMT). GNMT is an abundant cytosolic protein that is known to have a key role in monitoring the S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH) ratio and hence the cellular methylation capacity. However, phosphorylation of GNMT did not alter the basal levels of SAM and SAH. Similarly, both AICAR, an activator of hepatocytic AMP-activated protein kinase and dibutyryl-cAMP, an activator of cAMP-dependent kinase induced GNMT phosphorylation without any effect on the intracellular SAM/SAH ratio. Therefore, phosphorylation does not seem to substantially alter the activity of GNMT with respect to its assumed function in the regulation of methyl metabolism (Wagner et al., 1989), but may modify some as yet unknown function of the protein.

PAPER II: Suppression of hepatocellular autophagy and ATP levels by homocysteine

In this paper we show that the amino acid L-homocysteine induced a dose-dependent suppression of autophagy in isolated rat hepatocytes. The suppression was increased by adenosine, which also acted synergistically with homocysteine to accumulate high levels of SAH, and to a lesser extent, of SAM. Comparable increases in SAM and SAH were induced by methionine plus adenosine, but methionine had no inhibitory effect on autophagy. Similarly, no autophagy suppression was seen with other metabolites related to homocysteine metabolism, ruling out that any of these mediate the effect of homocysteine. Although homocysteine is a much stronger autophagy suppressant than any other individual amino acid, it did not stimulate phosphorylation of the ribosomal protein S6, unlike other amino acids. ATP measurements revealed that homocysteine caused a dose-dependent decrease in the intracellular level of ATP, and thus possibly hamper ATP-dependent processes such as

autophagy. However, we did not see any activation of the AMP-activated protein kinase (AMPK), suggesting that homocysteine may inhibit autophagy by means of ATP depletion, independently of this enzyme.

PAPER III: Naringin-sensitive phosphorylation of plectin, a cytoskeletal cross-linking protein, in isolated rat hepatocytes

In this work we identified plectin as a toxin- and naringin-sensitive phosphoprotein, the phosphorylation of which could possibly be involved in toxin-induced hepatocellular cytoskeletal disruption. Use of various protein phosphatase inhibitors showed that calyculin A, tautomycin, cantharidin, microcystin-LR, and okadaic acid all induced plectin phosphorylation, but only the effects of the latter two were naringin-sensitive, at doses that inhibited type 2A protein phosphatase (PP2A) but not type 1 phosphatase. However, high doses of okadaic acid induced a naringin-resistant phosphorylation, indicating that both PP1 and PP2A may be involved in plectin phosphorylation/ dephosphorylation, like in the case of keratin phosphorylation (Blankson et al., 2000; Blankson et al., 1999). In this paper we also tried to identify the naringin-sensitive kinase. CaMKII has been considered as one candidate, but was shown not to be affected directly by naringin *in vitro,* nor did naringin have any effect on PKA or PKC. However, we identified a number of other naringin-sensitive protein kinases. Particularly interesting, we found that AMPK phosphorylation by AMPK kinase in the presence of AICAR or okadaic acid was antagonized by naringin. AMPK is a sensitive sensor of energy charge and metabolic stress that is suggested to be involved in activation of the stress-activated protein kinase p38 (Romanelli et al., 1999). We found two other stress-activated kinases, SEK1 and JNK, showed naringin-sensitive phosphorylation, thus qualifying as downstream mediators of an AMPKK/AMPK signaling cascade. Furthermore, a naringin-sensitive tail phosphorylation of S6 kinase (at Thr^{421}/ Ser^{424}) might be part of a putative AMPKK(LKB1)/AMPK/SEK1 /JNK/S6K signaling pathway. However, we also found that AICAR suppressed S6 kinase phosphorylation at Thr^{389}, thereby preventing S6 phosphorylation. Thus, if AICAR-induced phosphorylation of S6 kinase is involved in the regulation of autophagy, there is no further signaling through S6. This may suggest a second, hitherto unknown function of S6 kinase, which is independent of the amino acid-induced signaling through rapamycin-sensitive mTOR (Blommaart et al., 1995).

PAPER IV: Toxin-induced Tail Phosphorylation of Hepatocellular S6 Kinase: Evidence for a Dual Involvement of the AMP-activated Protein Kinase Regulation

In this work, we demonstrate the that phosphatase-inhibitory toxins, okadaic acid, calyculin A, cantharidin and tautomycin all induce phosphorylation of S6 kinase at Thr^{421}/Ser^{424} in the tail region, but not at Thr^{389}. Furthermore, the AMPK-activating drug AICAR, achieved the same effect on S6 kinase. In contrast to calyculin A, cantharidin and tautomycin, the effects of AICAR, okadaic acid and microcystin were sensitive to the plant flavonoid naringin, mimicking their effects on AMPK. The naringin-sensitive and naringin-resistant mechanisms are thought to reflect the differential effects of the toxins on the inhibition of protein phosphatase PP2A and PP1 respectively (Paper III; Ruud Larsen et al., 2002). Amino acids activate S6 kinase and induce S6 phosphorylation in a rapamycin-sensitive manner by involving mTOR. As a paradox, the toxins induced a rapamycin-resistant S6 phosphorylation at $Ser^{235}/Ser^{236}/Ser^{240}/Ser^{244}$ without activating S6 kinase, suggesting activation of another S6-phosphorylating enzyme than S6K/mTOR. This is supported by the observation that AICAR antagonizes the effect of amino acids by preventing S6 kinase phosphorylation at Thr^{389} in a naringin-sensitive manner. Although S6 kinase is thus not involved in an AMPK-mediated phosphorylation of S6, it cannot be ruled out that an enzyme related to S6K1 could be the responsible kinase. One candidate would be S6 kinase 2, which differs sufficiently from S6 kinase to escape detection by phospho Thr^{389} antibody.

PAPER V Stimulation of Hepatocytic AMP-Activated Protein Kinase by Okadaic Acid and other Autophagy-suppressive Toxins.

AICAR and several phosphatase-inhibitory toxins has previously been shown to suppress hepatocytic autophagy (Holen et al., 1993; Samari and Seglen, 1998). I this study we show that okadaic acid, microcystin LR, calyculin A, cantharidin and tautomycin all induce strong dose-dependent AMPK phosphorylation on Thr^{172} as well on multiple other phosphatase-sensitive sites on AMPKα. The phosphorylation correlate with AMPK activity *in situ* as measured by the AMPK-dependent phosphorylation of acetyl CoA carboxylase at Ser^{79}. Our data thus suggest that AMPK not only mediates the autophagy suppressive effect of AICAR, but also the effects of toxins. AICAR, okadaic acid and microcystin LR

induce a naringin-sensitive activation of AMPK, in contrast to the other toxins. Apparently, the activation of AMPK is influenced by both a naringin-sensitive and a naringin-resistant mechanism involving protein phosphatases PP2A and PP1, respectively. Such differential naringin sensitivity was also observed in our previous study of toxin-induced plectin phosphorylation (Paper III; Ruud Larsen et al., 2002).

METHODOLOGIGAL CONSIDERATIONS

Cells

All experiments were done with freshly isolated hepatocytes (from male rats fasted for 18 h), prepared by two-step collagenase perfusion (Seglen, 1976). Cell preparations from one single rat provides us with large amounts of primary cells, making it possible to perform relative comprehensive experiments (or several experiments under identical conditions) in a short time. Compared to cultured cells, the primary hepatocytes would also be expected to behave more like normal cells in intact animals. However, isolation of the hepatocytes does upset the *in situ* cell polarity, and disrupts the tightly regulated cell-to-cell interactions. As a proxy for *in vivo* studies, isolated cells thus have altered characteristics, which should be taken carefully into consideration.

Incubation of cells

Isolated hepatocytes were incubated in glass tubes in a water bath at 37˚C as 300 µl (100 mg/ml) cells in suspension buffer (Seglen, 1976) and 100 µl of isotonic neutral additions. To insure sufficient oxygenation of the highly concentrated cell suspension, tubes were agitated relatively violently, usually for one hour. However, for measuring autophagy rates, the cells were incubated for two hours to make sure that enough lactate dehydrogenase (LHD) had accumulated in autophagic vacuoles.

For studies of cellular signaling pathways, even one hour of incubation could be considered a relative long time. Most protein modifications, such as phosphorylations, probably stabilize within a few minutes. However, in situations where cells were treated with several inhibitors and/or antagonists, we found it appropriate to ensure that the cells had sufficient time to take up the additives. All chemicals were added at the same time (before incubation, while keeping the cells at 4˚C.

Sample preparation of whole cell lysate and cytosol

In most experiments, we used samples prepared as whole cell lysates or as cytosolic fractions. Samples for two-dimensional gel electrophoresis or Western blotting were routinely prepared without any addition of protease inhibitors, since many of these inhibitors are reactive substances that may interfere with subsequent analyses. However, it cannot be ruled out that some proteins may be particularly vulnerable to protease activity; we therefore regularly checked the effect of a protease inhibitor cocktail. In Paper III this was an issue with the preparation of plectin, which is susceptible to proteolysis. However, we found that the addition of protease inhibitors did not significantly improve the plectin samples.

Polyacrylamide gel electrophoresis

In order to separate proteins on one- or two-dimensional polyacrylamide gels, we had to consider both protein solubility and other limitations of the methods. The solubility is dependent on several protein characteristics, e.g., size and charge. Adjusting the ionic strength and pH in the sample buffer was usually sufficient to overcome solubility problems. However, in Paper III the high molecular weight plectin precipitated within the polyacrylamide gels, and we therefore had to modify the method. Urea (6 M) was included in the lysis buffer and in all subsequent solutions. Furthermore, we used 5 % acrylamide gels with 6 M urea in both the stacking and resolving gels. To prevent proteins from precipitating in the stacking gels, the electrophoresis was run at low voltage. Also, gels used for Western blotting were transferred to nitrocellulose membranes in the absence of methanol. Except for the plectin studies, standard conditions were applied using either 10 or 15 % polyacrylamide gels.

Limited availability of gel strips for isoelectric focusing prevented us from separating proteins with low or high pI (<3 and >10) on two-dimensional gels. Therefore, screening for proteins involved in autophagy would selectively exclude, e.g., highly basic transcription factors. Although some separation was initially done with gels containing acetic acid and urea (Spiker, 1980) or Hepes and histidine (Paulson et al., 1992), these studies were not pursued. We did find, however, that, e.g., histone $H1_0$ was phosphorylated by okadaic acid in a naringin-sensitive manner (Strømhaug et al., 1997).

Characterization of Proteins

For the identification and sequencing of proteins, we have employed both N-terminal Edman degradation and MALDI-TOF mass spectrometry. The latter method has only just recently become available in our laboratory. Therefore, in Paper I we initially used the more laborious N-terminal Edman degradation procedure to identify glycine *N*-methyl transferase (GNMT) (Fernandez et al., 1994). Since the N-terminal amino group of GNMT is blocked by acetylation (Ogawa et al., 1987), we had to digest the enzyme in-gel with trypsin prior to sequencing of the tryptic peptides. Typically, by pooling proteins from huge numbers of two-dimensional gels, after brief visualization with 1 M KCl (Bergman and Jornvall, 1987; Nelles and Bamburg, 1976) we were able to obtain an amino acid sequence in one out of two trials. Later we confirmed the identity of GNMT with our own mass spectrometer.

In all subsequent protein characterization we relied mainly on mass spectrometry for rapid identification of proteins. Thus, the use of mass spectrometry has enabled us, on a routine basis, to screen two-dimensional gels for large numbers of proteins in a way that previously was impossible (Abbud et al., 2000).

[^{32}P]-Protein labeling

For the *in vivo* labeling of proteins with [^{32}P]-orthophosphate in Paper I, we made some modifications to the incubation protocol. Isolated hepatocytes were incubated in glass tubes with only one third of the usual amount of cells (30 mg/ml) to reduce the requirement of radioactive isotopes and to obtain higher incorporation rates. Therefore, we had to do additional dose-response experiments to adjust the concentrations of okadaic acid and naringin needed. This was done by measuring the sequestration rate of LDH under the same standard conditions (data not shown) as used for all other similar experiments presented in the subsequent papers. We found that the inhibitory effect of 30 nM okadaic acid on autophagy was sufficiently antagonized by 100 μM naringin. Although this is a somewhat lower concentration of okadaic acid than usual (50 nM), we did not find that the dose of naringin had any significant influence on the autophagy itself. Also, we assured that the addition of orthophosphate did not affect the autophagy rate.

Lately, as phosphospecific antibodies have become available towards a broad spectrum of proteins, we gradually were able to avoid the hazardous work with radioactive isotopes. This is evident in paper IV-V, where we used a variety of recently available antibodies to identify complete signaling pathways. However, for the initial mass screening of proteins subjected to dynamic phosphorylation and dephosphorylation, we still find radioactive labeling superior and more sensitive – typically in conjunction with the separation of complex protein fractions on two-dimensional polyacrylamide gels. In paper III, we were, nevertheless, able to use an anti-phosphothreonine antibody to successfully detect dynamic phosphorylation of plectin in diluted polyacrylamide gels. Therefore, in some cases, these site-nonspecific antibodies may be excellent alternatives to radioactive isotopes when phospho-specific antibodies are not available for the protein of interest.

Phosphopeptide and amino acid mapping

The phosphopeptide and amino acid mappings were done with an apparatus that was self-constructed with a two-chamber tray system for electrophoresis. It is based on the separation of peptides/amino acids by thin-layer chromatography on silica plastic sheet using electrophoresis in the first dimension and traditional chromatography in the second dimension. We found that it was more suitable than commercial available devices, allowing us to obtain an uncomplicated and excellent separation in the first dimension.

DISCUSSION OF THE RESULTS

The aim of this study has been to obtain new knowledge about the regulation of mammalian autophagy, using isolated rat hepatocytes as a model system. Various autophagy inhibitors in conjunction with their antagonists have been important tools to unravel some of the complicated protein phosphorylation signaling pathways that constitute the regulation. In particular, we wanted to emphasize the importance of the toxin antagonist naringin, which we now realize has major influence on a variety of cellular functions such as the rigidity of the cytoskeleton (paper III), stress-activated signaling pathways (paper III and V), protein synthesis (paper IV) and autophagy.

This thesis has provided new and more detailed insights into several signaling pathways that are thought to be involved in the regulation of autophagy. Furthermore, our observations emphasize the importance of an intact cytoskeletal network. Finally, but most importantly, we have identified one naringin-sensitive protein kinase (AMPK) that may well be one of the primary targets of this drug, and which may intercept with other protein kinases involved in the regulation of protein synthesis (S6K1).

Tail-specific phosphorylation of SK61 at Thr^{421} and Ser^{424} is mediated through two distinct mechanisms

We have shown that AICAR-induced phosphorylation at Thr^{421} and Ser^{424} is unaffected by 3MA (unpublished data), rapamycin and wortmannin (paper IV). Consequently, AMPK causes S6K1 phosphorylation independently of the mTOR pathway, which also is consistent with our observations that amino acid-induced S6K1 activation in fact is naringin-resistant. Thus, it seems possible that the naringin-sensitive and mTOR-independent phosphorylation of these sites could assign S6K1 to new Thr^{389}-independent functions as e.g. to take part in the regulation of autophagy. Amino acid-induced, mTOR-mediated S6K1 activation serves to inhibit autophagy by a mechanism that involves S6K1 phosphorylation at Thr^{421}, Ser^{424} and Thr^{389}, whereas naringin-sensitive, AMPK-mediated S6K1 phosphorylation only engages Thr^{421} and Ser^{424}, suggesting that tail phosphorylation alone could be sufficient to inhibit autophagy during e.g. cellular stress, hypoxia, heat shock etc. In such a perspective, the complex hormone-regulated PI3K-mediated S6K1 phosphorylation could play an assisting role in the S6K1 activation rather than to regulate autophagy. It should be stressed, however, that under ordinary *in vivo* conditions with more normal amino acid concentrations, hormone-modulation does have a significant role in the mTOR pathway. By serving two distinct phosphorylation pathways, S6K1 may perform a dual

role as a crosspoint effector of the fine-tuned regulation of S6 phosphorylation and autophagy when ample nutrition is available, and act as major switch that turns off the ATP-incinerating autophagy machinery and cellular growth during severe cellular stress characterized by rapidly increased AMP-levels. Indeed, in HEK29 cells it has been reported that the mTOR pathway is additionally influenced by the intracellular concentration of ATP (Dennis et al., 2001). This would suggest a mechanism where AMPK indirectly inactivates the mTOR pathway and directly inhibits autophagy through the tail phosphorylation of S6K1 when the cellular concentration of AMP is high, whereas ATP activates the pathway in a rapamycin-sensitive manner as the ATP/AMP level rises in favor of ATP. Our data (paper III-IV) would support such a mechanism as the strong suppressive effect of AICAR on both autophagy and protein synthesis principally parallels the mTOR-independent phosphorylation of S6K1 at Thr^{421} and Ser^{424}, while specifically abolishing the phosphorylation at Thr^{389}. However, the significance of S6K1 in the regulation of autophagy still remains uncertain.

AICAR - and toxin-induced AMPK activation is possibly linked to the negative regulation of mTOR

Both autophagy and protein translation is dynamically regulated in response to different cellular needs. During times of cellular growth, protein translation is up regulated, and reduced when cells lack nutrients or amino acids. Nutrient-regulated rapamycin-sensitive PKB-mediated phosphorylation of mTOR at Ser^{2448} has previously been described (Nave et al., 1999). Amino acids stimulate this phosphorylation, as do insulin or mitogens through activation of PKB (Avruch et al., 2001) - c.f. with figure 5 on page 27. Activation of mTOR has been associated with the inactivation of 4E-BP1 (Gingras et al., 1999; Hara et al., 1998), the activation of S6K1 (Dennis et al., 1996) and the inactivation of PP2A (Begum and Ragolia, 1996), hereby enhancing the cellular protein synthesis capacity (Nielsen et al., 1982). As the mTOR pathway is influenced by the intracellular concentration of ATP, independent of the abundance of amino acids, mTOR has also been suggested to serve a homeostatic ATP sensor (Dennis et al., 2001). However, the regulatory effect on mTOR is more likely linked to the activity of AMPK that directly responding to change in the cellular balance of ATP/AMP. It has been suggested that AMPK provides an overriding switch linking S6K1 regulation to cellular energy metabolism (Kimura et al., 2003). In paper III/V we indeed demonstrate that AICAR, an activator of AMPK (Corton et al., 1995; Henin et al., 1996; Samari and Seglen, 1998), in a naringin-sensitive manner prevents amino acid-induced S6K1 phosphorylation at Thr^{389}, a site known to be associated with activation of

the enzyme (Dennis et al., 1996; Dennis et al., 1998). A clue as to how AMPK regulates the mTOR and S6K1 activity may come from a recent report that identifies a novel phosphorylation site on mTOR at Thr^{2446}, which serves as a negative regulator of mTOR activity (Cheng et al., 2004). The phosphorylation at Ser^{2448} is directly related to amino acid and nutrient status (Nave et al., 1999; Reynolds et al., 2002). Conditions that increase phosphorylation at Thr^{2446} tend to decrease phosphorylation at Ser^{2448} and vice versa. Thus, phosphorylation at Thr^{2446} and Ser^{2448} are mutually exclusive, indicating that these sites may act as switches that integrate the counteracting signals of growth factors and nutrient deprivation.

Toxin-induced, naringin-sensitive phosphorylation of S6K1 and S6 is restricted to protein phosphatase type 2A inhibitors

PP1 and PP2A are essential for the regulation of S6K and S6 activity, by selectively dephosphorylating S6 (Olivier et al., 1988) and S6K (Ballou et al., 1988a) respectively. By screening several protein phosphatase inhibitors, we have shown that they, based on naringin-sensitivity, can be divided into two groups: naringin-sensitive (NS) and naringin-resistant (NR). (Cf. with table I on page 13.) They all induce overphosphorylation of S6K1 and S6, but only the specific PP2A inhibitors are naringin-sensitive.
Paradoxically, although AMPK can be dephosphorylated at Thr^{172} by mixtures of PP2A and PP1 in cell extracts (Woods et al., 2003b), the dephosphorylation apparently seems to be carried out entirely by PP2C in intact cells (Davies et al., 1995), suggesting that the toxins most likely exert an indirect effect on AMPK, possibly through PP2C. In fact, it has been reported that PP2Cα is a phosphoprotein subjected to toxin-sensitive dephosphorylation (Kobayashi et al., 1998).

Naringin-sensitive and naringin-resistant toxin-induced activation of AMPK: Activation most likely involves multiple phosphorylation sites

Administration of low okadaic acid or microcystin doses, sufficient to selectively inhibit PP2A, induces naringin-sensitive AMKP phosphorylation at Thr^{172}, whereas higher doses also inhibit PP1 and induce additional phosphorylation on AMPK, as suggested by the appearance of additional low-mobility bands on SDS PAGE (paper V). Increasing AICAR concentrations induce a similar stepwise multi-phosphorylation of AMPK, whereas the PP1-inhibitory (naringin-resistant) toxins tend to induce early multi-phosphorylation. Based on structural similarities with ATP (Graziani et al., 1983), naringin may under normal condi-

tions in which only Thr^{172} is phosphorylated, compete directly with AMP or AICAR. As additional phosphorylation sites are engaged, naringin-binding could be hampered. Alternatively, naringin could also disrupt the AMP/AICAR-sensitive interaction between the α and β subunits, rather than impose a direct competitive inhibition on AMPK. (Cf. figure 6 on page 31.) Differential protein phosphatase specifity towards various regulatory sites could explain the existence of both a PP2A-associated, naringin-sensitive, and PP1-associated, naringin-resistant mechanism for the activation of AMPK. In conclusion, paper V suggest that several phosphorylation sites may contributes to the activation of AMPK, thus implicating that several protein kinases besides LKB1 may be involved in the complex activation mechanism.

Suppression of hepatocytic autophagy by ATP depletion correlates with AICAR - or toxin-induced activation of AMPK

During situations with ATP depletion, hepatocytic autophagy is strongly repressed (Plomp et al., 1989; Plomp et al., 1987) reflecting that the autophagic machinery is sensitive to changes in the ATP/AMP balance. As AICAR or toxin-induced AMPK activation also suppresses autophagy, it would not be unexpected if this enzyme were partly responsible for suppression by any changes in the ATP imbalance. We have, furthermore, shown that all tested naringin-sensitive hepatocytic autophagy-suppressive drugs not only induce APMK activation, but also activate SEK1 and JNK (paper III), suggesting that under condition of transient stress, AMPK might be an upstream regulator of autophagy as well as a general mediator of cellular stress (paper V). Prolonged activation of AMPK also induces apoptosis in response to sustained stress (Meisse et al., 2002), or even necrosis during drastic ATP depletion (Marsin et al., 2000). A previous report has suggested that a stress-activated protein kinase (p38) may participate in the regulation of autophagy (vom Dahl et al., 2001). The JNK inhibitor, dicoumarol, prevents JNK activation and apoptosis, but without affecting AMPK activity, suggesting that JNK is downstream of AMPK (Meisse et al., 2002). In fact, we have observed that high concentrations of the p38/JNK inhibitor SB-203580 antagonize both the autophagy-suppressive effects of AICAR and the activation of the SEK1/JNK pathway (our unpublished data). Therefore, we propose as a working hypothesis, that an AMPKK(LKB1) /AMPK/SEK1/JNK pathway may be involved in autophagy regulation (paper III). However, further studies have to substantiate such a pathway in the regulation of hepatocytic autophagy.

Autophagy-suppressive toxins disrupt the integrity of the cytoskeleton network

Our studies with toxin-induced hepatocellular cytoskeletal disruption and apoptosis, has revealed that plectin (paper III) and keratin (Blankson et al., 2000; Blankson et al., 1999) are subjected to dynamic phosphorylation which apparently employ two different phosphorylation/dephosphorylation mechanisms: a naringin-sensitive, PP2A-regulated mechanism, and a naringin-resistant, PP1-regulated mechanism. Whereas the PP1-regulated mechanism has been shown to be involved in a rapid apoptotic process elicited by low concentrations of tautomycin, cantharidin and calyculin A, PP2A-regulated mechanism rather cause a slow apoptosis elicited by okadaic acid and microcystin. See table 1 on page 13. At higher dose, the toxins display less specific characteristics and produce an inhibitory effect on both the protein phosphatases.

The maintenance of specifity in intracellular signaling may not only depend on reversible phosphorylation, but also on enzyme regulation through the assembly of multiprotein complexes or enzymes anchored to a multivalent scaffold that is associated with e.g. cytoskeleton proteins (Pawson and Scott, 1997). Multiprotein complexes restrict access of kinases and phosphatases to particular microenvironments and/or specific proteins, and foster specificity of phosphorylation events by optimally positioning these enzymes to respond rapidly to cellular stimuli. Interactions between keratin, plectin and other cytoskeletal proteins are regulated by protein phosphorylation. Therefore, it would seem plausible that kinases upstream of plectin and keratin may participate in reversible phosphorylation mechanisms for the control of intracellular signaling cascades through alterations in the integrity of the cytoskeletal network.

Several protein kinases have been reported to phosphorylate the cytoskeleton. For example, in interphase cells, PKC-induced phosphorylation is involved in the plectin dissociation from vimentin, while PKA improves the binding (Foisner et al., 1991). Since we found that several specific inhibitors of PKC and PKA were unable to prevent okadaic acid-induced plectin phosphorylation, these kinases are most likely not involved. KN-62, a specific inhibitor of CaMK-II, presented 25% protection, suggesting that naringin might exert its okadaic acid antagonism through this kinase. However, when comparing the effect of naringin with the potent CaMK-II inhibitor KT-5926 (Mellgren et al., 1997) on CaMK-II-activity, it became obvious that the okadaic acid- and microcystin-antagonistic effect of naringin on plectin phosphorylation is unlikely to be due to a direct CaMK-II inhibition. At the present time, it is still uncertain which protein kinase(s) actually mediate the plectin and keratin phosphorylation. A clue may come from our observations in paper III -V where we, based on naringin-sensitive autophagy-suppressive phosphorylation

of AMPK, SEK1, JNK and S6K1, described a putative signaling pathway. Under cell-free conditions, both AMPK and S6K1 are capable of inducing keratin phosphorylation (Ku and Omary, 1994). Furthermore, S6K1 has been reported to participate in the regulation of the actin cytoskeleton (Berven et al., 2004), and AICAR to induce keratin phosphorylation in intact hepatocytes (Velasco et al., 1997).

The intracellular ratio of SAM/SAH is not influenced by phosphorylation of glycine N-methyltransferase (GNMT)

GNMT is a key regulator in the methionine metabolism, where it serves to continuously monitor and regulate the SAM/SAH ratio, and functions as a main regulator of the cellular methylation capacity. Unlike other methyltransferases, GNMT is allegly activated by phosphorylation (Wagner et al., 1989), and specifically inhibited by 5-methyltetrshydrofolate pentaglutamate (Wagner et al., 1985). However, we demonstrated that okadaic acid or AICAR-induced phosphorylation of GNMT had no effects on the basal levels of SAM or SAH (paper I). Furthermore, an accelerated SAM-SAH flux induced by the addition of methionine, is unaffected by okadaic acid. We conclude, therefore, that phosphorylation of GNMT apparently has no role in the regulation of the intracellular SAM/SAH ratio, but rather suggest other GNMT functions.

Homocysteine-mediated autophagy-suppression is most likely caused by ATP-depletion and without any AMPK activation or S6 phosphorylation

L-homocysteine is the most effective autophagy-suppressive amino acid in rat hepatocytes (paper II) and its effect is synergistically potentiated by the addition of adenosine in a process that induces a massive accumulation of SAH and to some slighter extent SAM. With the exception of homocysteine, none of the other metabolites in the methionine pathway were mediators of autophagy suppression, suggesting that homocysteine employed another regulatory pathway that conceivably is more similar to how other amino acids mediate their effect. However, homocysteine induced no phosphorylation on the ribosomal protein S6, but rather seemed to mediate a non-specific autophagy-suppression through a general cellular ATP-depletion, which does not activate AMPK. Since ATP-depletion and AMPK activation would be expected to correlate, our data suggest that ATP apparently has an ability to control autophagy independently of AMPK. This is somewhat surprising since such ATP depletion would be expected to evoke a general cellular stress, including AMPK activation. However, despite the fact that autophagy suppression probably is an

indirect consequence of the ATP depletion, it does make sense to suppress energy-requiring processes during extreme cellular stress. Homocysteine is known to block several anabolic processes in hepatocytes, including protein synthesis (Tinton and Buc-Calderon, 1995), and it would clearly be of interest to find out if this is effected through and ATP-sensitive regulatory mechanism other than the AMPK regulatory pathway.

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PAPER I

PAPER II

Suppression of Hepatocellular Autophagy and ATP Levels by Homocysteine

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Abstract

The amino acid L-homocysteine induced a dose-dependent suppression of autophagy (as measured by sequestration of the cytosolic enzyme LDH) in isolated rat hepatocytes, achieving an 80% inhibition at 4 mM. This makes homocysteine more effectively suppressive than any other individual autophagy-regulatory amino acid. The effect of homocysteine was potentiated by adenosine (in the presence of 2'-deoxycoformycin and 5-iodotubercicin to prevent adenosine deamination and phosphorylation, respectively). Homocysteine and adenosine acted synergistically to induce a massive accumulation of *S*-adenosylhomocysteine (AdoHcy) and, to a lesser extent, of *S*-adenosylmethionine (AdoMet). However, methionine, alone or in combination with adenosine, mimicked the effect of homocysteine on AdoHcy and AdoMet levels (and on the AdoHcy/AdoMet ratio) without much effect on autophagy, ruling out these metabolites as mediators of autophagy suppression. Other metabolites or drugs related to homocysteine metabolism, like cysteine (or its cell-permeable analogue, *N*-acetylcysteine), glutathione (or its cell-permeable analogue, glutathione ethyl ester), propargylglycine (an inhibitor of cystathionine γ-lyase), glycine or sarcosine (the precursor and product, respectively, of a methylation whereby AdoMet is converted to AdoHcy) similarly had little or no effect on autophagy. Homocysteine did not share the ability of an autophagy-suppressive amino acid mixture to stimulate the phosphorylation of ribosomal protein S6, but rather inhibited protein phosphorylation in general. ATP measurements revealed that homocysteine caused a dose-dependent decline in cellular ATP levels, which may account for its ability to inhibit ATP-dependent cellular processes like protein phosphorylation and autophagy. The ATP depletion was not accompanied by activation of the AMP-activated protein kinase, indicating that autophagic activity could be regulated by the cellular energy state independently of this enzyme.

1. Introduction

Autophagy is a process used by all mammalian cells to sequester and transport pieces of cytoplasm, with their constituent organelles and macromolecules, to the lysosomes for degradation [1]. The process is initiated by organelles called phagophores, composed of protein-poor, single or multiple membrane cisternae, which enclose cytoplasm into vacuoles called autophagosomes [2-5]. The autophagosomes can fuse with early or late endosomes to form intermediary, acidic vesicles called amphisomes, which later fuse with lysosomes [6-9]. The autophagocytosed material is then degraded intralysosomally to small–molecular weight components that can be reutilized by the cell. All steps in the autophagic pathway are energy-dependent, the initial sequestration step being particularly sensitive to intracellular ATP concentrations [10]. Under conditions of amino acid depletion, autophagy can be maximally activated to account for up to 80% of the total intracellular protein degradation in rat hepatocytes [2].

A variety of biological regulators of mammalian autophagy have been described [11]. These include hormones [12-14], amino acids [12,15,16], purines [14,17,18], calcium [19], cyclic nucleotides [20,21], phospholipids and lipid kinases [22-24], trimeric GTPases [23,25], protein phosphatase 2A [26] and several protein kinases [21,24,27,28], all acting at the initial sequestration step. In yeast, some 17 different genes/proteins have been found to be necessary for autophagy, including one protein kinase, Apg1/Aut3 [29,30]. About one-half of these yeast proteins are components of two protein conjugation systems that apparently serve to anchor the ubiquitin-like proteins, Apg12 and Apg8/Aut7, to a phagophore-like membranous organelle [31]. Mammalian homologues of Apg5, Apg6/beclin1, Apg8/LC3 and Apg12 have been identified [31,32]; in addition, several novel enzyme isoforms have been found to be selectively associated with rat liver phagophores [33]. It is, however, not clear how the signals from autophagy-regulatory metabolites, like amino acids, impinge on the phagophore-associated proteins to suppress the initiation of autophagy.

One of the most potent autophagy suppressants known, the protein phosphatase inhibitor, okadaic acid, is effectively antagonized by the flavonoid naringin, probably acting as an inhibitor of an autophagy-suppressive protein kinase [34]. In a search for okadaic acid- and naringin-sensitive phosphoproteins that might be involved in the regulation of autophagy, we found a cytosolic 32-kDa protein [24], later identified by mass spectrometry as glycine *N*-methyltransferase (EC 2.1.1.20), an enzyme believed to participate in the regulation of cellular methylation [35]. Upon investigating the possible relationship between autophagy and the intermediary methyl metabolism of isolated rat hepatocytes, we found the methionine precursor, L-homocysteine, to be a more effective suppressant of autophagy than any of the autophagy-regulatory amino acids previously studied, thus warranting an inquiry into its mechanism of action.

2. Materials and methods

2.1. Biochemicals

Leupeptin was purchased from Peptide Institue Inc. (Osaka, Japan), and 5-iodotubercidin from Research Biochemicals Inc. (Natick, MA, USA). 2'-Deoxycoformycin (pentostatin®) was a generous gift from Parke-Davis (Ann Arbor, MI, USA).

[1]These authors contributed equally to this work. [2]To whom correspondence should be addressed: Proteomics Section, Department of Cell Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Tel.: 47-22-93-59-47; Fax: 47-22-93-45-80; E-mail: per.seglen@labmed.uio.no. | ABBREVIATIONS AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; AMPK, AMP-activated protein kinase; CaMK-II, Ca^{2+}/calmodulin–dependent protein kinase II; DB-cAMP, N^6,2'-*O*-dibutyryladenosine 3',5'-monophosphate; GNMT, glycine *N*-methyltransferase; Hcy, homocysteine; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; PVDF, polyvinylidene difluoride; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; TLC, thin layer chromatography. | KEYWORDS rat, liver, hepatocyte, S-adenosylhomocysteine, S-adenosylmethionine, adenosine, ATP, AMP-activated protein kinase.

All other biochemicals were purchased from Sigma Chem. Co., St. Louis, MO, USA. Free L-homocysteine was freshly prepared for each experiment as described by Duerre and Miller [36]: 25 mg L-homocysteine thiolactone was dissolved in 300 µl 5 M NaOH in order to open the thiolactone ring; 1500 µl 1 M KH_2PO_4 was then added to neutralize the solution, and the pH was adjusted to 7.5.

2.2. Isolation and incubation of rat hepatocytes

Isolated hepatocytes were prepared from 18-h starved, 250-350 g male Wistar rats by two-step collagenase perfusion [37]. The hepatocytes were incubated, in shaking centrifuge tubes at 37°C, as 400 µl aliquots of cell suspension containing 30-35 mg cellular wet mass. The medium was suspension buffer [37] fortified with 15 mM pyruvate and 2 mM Mg^{2+}, i.e., an amino acid-free medium that induces maximal autophagy [15].

2.3. Measurement of autophagy

For autophagy measurements, 0.3 mM leupeptin was included in the medium during incubation of the cells (routinely for 2 h) to prevent intralysosomal degradation of autophagocytosed lactate dehydrogenase (LDH), used as an autophagy probe [38]. After incubation, the cells were washed twice in 10 % unbuffered, electrolyte-free sucrose, resuspended in 10 % unbuffered sucrose, and electro-disrupted by a single high-voltage pulse [16,39]. The disruptates were centrifuged through a metrizamide/sucrose cushion, and the amount of autophagically sequested LDH in the cytosol-free cell corpse sediment was measured and expressed as per cent of the total cellular LDH in the disruptate [38].

Cellular viability was measured as the cellular retention of LDH, this cytosolic enzyme being rapidly lost from cells with a damaged plasma membrane. The viability (% intact cells) after 2h of incubation was expressed as the percentage of LDH retained in sedimented hepatocytes relative to the amount at time zero.

2.4. HPLC analysis of S-adenosylmethionine, S-adenosylhomocysteine and sarcosine.

Intracellular *S*-adenosylmethionine (AdoMet; SAM) and *S*-adenosylhomocysteine (AdoHcy; SAH) levels were measured by reverse-phase HPLC essentially as described by She *et. al.* [40], using a Symmetry^{TM} C_{18} column (5 µm; 100 Å; 3.9 x 150 mm) and HPLC instrument system from Waters (Milford, Mass., USA). The equipment included a no. 486 tunable absorbance detector adjusted to 254 nm, a 717*plus* autosampler and pump, a 600E system controller, a multisolvent delivery system, and a Millennium^{32} chromatography manager for data analysis.

For the HPLC analysis, six incubated cell samples (about 200 mg cells) were pooled, the cells were sedimented (1,500 *g*; 5 min), resuspended in 300 µl 10% trichloroacetic acid and extracted for 15 min at 4°C. The suspension was centrifuged for 15 min at 3,700 *g*; the supernatant was then transferred to a new tube and stored at -20°C until the measurement day, when 200 µl was injected for analysis. All samples and solutions used in the HPLC system were filtered through a 0.22 µm Millipore filter prior to injection.

The mobile phase was an aqueous buffer containing 40 mM $NH_4H_2PO_4$, 8 mM 1-heptanesulfonic acid (sodium salt) and 18 % (v/v) methanol, pH 3.0 (adjusted by addition of HCl). Elution proceeded for 40 min at a constant flow rate (1 ml/min) with UV-detection at 254 nm. Between samples, the column was washed for 15 min at 1.5 ml/min with H_2O: methanol (1:1), and equilibrated for 5 min at 1.5 ml/min with the mobile phase buffer. The positions of AdoHcy and AdoMet were identified by co-elution with added standards.

Sarcosine was measured by HPLC according to the Waters AccQ-Tag amino acid derivatization method (Millipore Corp., Milford, MA, USA) in deproteinized hepatocyte extracts concentrated by freeze-drying. A Waters 486 detector was used for measurement of UV absorbance at 254 nm. Although a sarcosine standard was well resolved from the nearby peaks of histidine and ammonia, no intracellular sarcosine could be detected in isolated hepatocytes under any treatment conditions.

2.5. Immunoblotting

Washed cell samples (30-35 mg cells) were dissolved in one ml sodium dodecylsulfate (SDS) lysis buffer (0.4 % SDS, 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 20 mM Tris-HCl, pH 7.2), left for 30 min, and stored frozen overnight if necessary. After thawing, the samples were briefly sonicated, boiled for five min at 95°C, and cooled or frozen. The samples were mixed with an equal volume of SDS sample buffer (1 % SDS, 1 % IGEPAL CA-630, 5 mM sodium deoxycholate, 13 % glycerol, 10 % 2-mercaptoethanol, a few grains of bromophenol blue, 120 mM Tris-HCl, pH 7.2), the protein content was measured by the method of Bradford [41], and 10 µg protein was fractionated on a 10 % separating polyacrylamide gel (polymerized with 0.1 % SDS, 0.1 % ammonium persulfate, 0.04 % TEMED and 0.4 M Tris-HCl, pH 8.8) with a 5% stacking gel on top (0.1 % SDS, 0.1 % ammonium persulfate, 0.01 % TEMED and 0.13 M Tris-HCl, pH 6.8). Mini Protean equipment from BioRad (Munich, Germany) was used to run the gels.

The separated proteins were transferred from the gel onto a Nitropure nitrocellulose support membrane (Micron Separations Inc., West Borough, MA, USA), using a BioRad Semi-Dry Transblot apparatus and Towbin's transfer buffer (20 % methanol, 192 mM glycine and 25 mM Tris, pH 8.3). After blotting for 30 min at 15 V, the blots were blocked with 5 % non-fat dried milk in phosphate-buffered saline (PBS) + 0.1 % Tween 20 for 2h or overnight. The blots were then washed for 15 min in 10 ml PBS + 0.1 % Tween 20 on a rotating horizontal sample mixer to remove the dried milk; then incubated for one hour at room temperature with polyclonal rabbit antibodies against human ribosomal protein S6 (Ser235/236 or Ser240/244) or against Thr172-phosphorylated, AMP-activated protein kinase (Cell Signalling Technology Inc., Beverly, MA, USA). The blots were washed for 1 x 5 min and 3 x 15 min in 10 ml PBS + 0.1 % Tween 20, and incubated for one hour with HRP-conjugated secondary antibody. The blots were washed again as described above, and ECL Western blotting detection reagents (Amersham Pharmacia AB, Uppsala, Sweden) were used to detect the antibodies.

2.6. ATP measurement.

Each 400 µl aliquot of hepatocytes incubating at 37° was mixed with 100 µl 10 % perchloric acid while still shaking. After centrifugation to remove the protein precipitate, 100 µl of perchloric acid extract was neutralized with 200 µl 0.1 M NaOH and diluted with 2 ml H_2O. The ATP concentration in this neutralized extract was measured by the luciferin/luciferase method, using a kit from Sigma.

3. Results

3.1. Inhibition of autophagy by homocysteine, potentiated by adenosine.

Freshly prepared L-homocysteine was an effective suppressant of hepatocytic autophagy, producing an 80% inhibition at 4 mM (Figure 1). At higher concentrations, significant toxicity became evident, as indicated by a precipitous drop in cellular viability. Although the homocysteine concentrations needed to inhibit autophagy were some ten times higher than the normal intrahepatic levels of the amino acid [42,43], the ability of a single amino acid to achieve this

extent of autophagy suppression is unique [16], suggesting that a further characterization of the effect might be worthwile. Only the L-stereoisomer was effective, no suppression being observed with a DL-homocysteine preparation (results not shown).

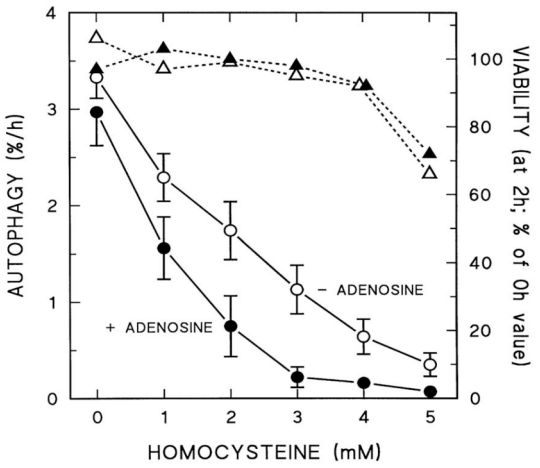

Fig. 1. **Suppression of autophagy in rat hepatocytes by homocysteine and adenosine.** Hepatocytes ($3.5 \cdot 10^6$ cells/ml) were incubated for two hours at 37°C with 0–5 mM homocysteine in the absence (○) or presence (●) of 0.5 mM adenosine (along with 10 μM 5-iodotubercidin and 50 μM 2'-deoxycoformycin as inhibitors of adenosine metabolism). The net autophagic sequestration of endogenous LDH (○,●) was measured and expressed as %/h, each value being the mean ± S.E. of four experiments. Dotted lines: viability (% intact cells, as measured by LDH retention in triplicate cell samples) of hepatocytes after 2 h of incubation in the absence (△) or presence (▲) of 0.5 mM adenosine (+ iodotubercidin and deoxycoformycin); single experiment.

The intrahepatic metabolism of homocysteine may proceed along three pathways (Figure 2): (1) it can condense with adenosine to form *S*-adenosylhomocysteine (AdoHcy; SAH), an important regulator of intracellular methylation; (2) it can be methylated to methionine, or (3) it can be converted to cysteine, to be used in protein synthesis or for the formation of glutathione [44]. To assess the AdoHcy pathway, adenosine (1 mM) was added along with homocysteine, in the presence of 2'-deoxycoformycin to prevent the metabolism of adenosine to inosine, and in the presence of 5-iodotubercidin to prevent its phosphorylation to the autophagy-suppressive metabolite AMP [18,28]. As shown in Figure 1, adenosine (0.5 mM) had little effect of its own under these conditions, but it markedly potentiated the effect of homocysteine, causing a 93% inhibition of autophagy at 3 mM of the latter. The possibility of the autophagy suppression being mediated by intracellularly formed AdoHcy therefore had to be considered. Both homocysteine and adenosine are taken up effectively by hepatocytes, and cause the accumulation of large amounts of AdoHcy, catalyzed by the enzyme *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1) [42,45,46]. In contrast, neither AdoHcy nor its precursor *S*-adenosylmethionine (AdoMet; SAM) are significantly taken up by hepatocytes [42,47]. They were both tested in our system at concentrations up to 5 mM, but had little or no effect on hepatocytic autophagy (results not shown).

3.2. Effects of homocysteine, adenosine and methionine on intracellular levels of S-adenosylhomocysteine and S-adenosylmethionine.

To check to what extent intracellular AdoHcy levels might correlate with inhibition of autophagy, AdoHcy (as well as AdoMet) was measured by HPLC in hepatocyte extracts. In

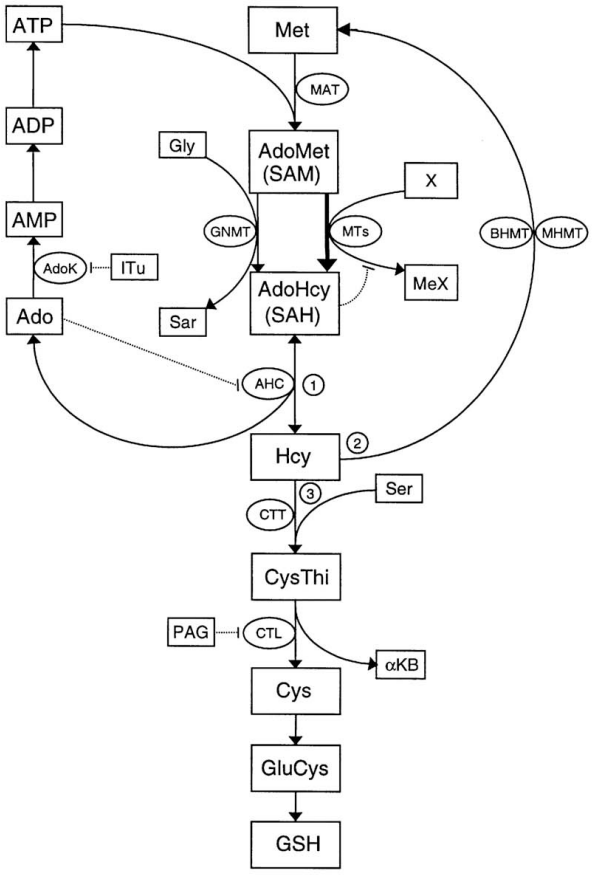

Fig. 2. **Pathways of homocysteine metabolism.** αKB, β-ketobutyrate; Ado, adenosine; AdoHcy, *S*-adenosylhomocysteine; AdoK, adenosine kinase; AdoMet, *S*-adenosylmethionine; AHC, adenosylhomocysteinase; BHMT, betaine-homocysteine methyltransferase; CTL, cystathionine γ-lyase; CTT, cystathionine β-synthase; CysThi, cystathionine; GluCys, γ-glutamylcysteine; Gly, glycine; GNMT, glycine *N*-methyltransferase; GSH, glutathione; Hcy, homocysteine; MAT, methionine adenosyltransferase; Met, methionine; MeX, methylated X; MHMT, 5-methyltetrahydrofolatehomocysteine methyltransferase; MTs, methyltransferases; PAG, propargylglycine; Sar, sarcosine; Ser, serine; X, any methyltransferase substrate.

control cells, the levels of both metabolites were extremely low (Figure 3A). As expected, homocysteine (2 mM) raised the level of AdoHcy markedly, without any detectable effect on AdoMet (Figure 3B). Methionine (1 mM), on the other hand, raised the levels of both metabolites, apparently accelerating the overall flux through the cellular AdoMet and AdoHcy pools (Figure 3C). Adenosine (1 mM), like homocysteine, elevated the AdoHcy level selectively (Figure 3D). In combination, homocysteine and adenosine acted synergistically, giving very high AdoHcy levels as well as raising the AdoMet level (Figure 3E). Somewhat surprisingly, a combination of methionine and adenosine produced exactly the same pattern (Figure 3F).

Despite the similar effects of methionine and homocysteine on intracellular AdoHcy levels, methionine did not detectably inhibit autophagy, be it in the absence or presence of adenosine (Table 1). AdoHcy is, therefore, unlikely to mediate the autophagy-suppressive effect of homocysteine. The same argument would apply to AdoMet and the AdoHcy/AdoMet ratio, which, in the presence of adenosine, were similarly affected by methionine and homocysteine. The data would, therefore, seem to suggest that homocysteine inhibits autophagy independently of AdoHcy or AdoMet formation, although Ado or AdoHcy may potentiate the effect of homocysteine indirectly, e.g., through nonspecific cytotoxicity [48]. The inability of methionine to inhibit autophagy indicates,

furthermore, that the methylation pathway(s) from homocysteine to methionine (Figure 2) play no role in mediating the autophagy-suppressive effect of homocysteine.

Table 1
Effects of homocysteine, methionine and adenosine on hepatocytic autophagy and intracellular levels of AdoHcy and AdoMet

Additions during incubation	Autophagy (%/h)	AdoMet (nmol/g cells)	AdoHcy (nmol/g cells)
None	2.88 ± 0.12 (2)	43 ± 6 (3)	5 ± 1 (3)
Homocysteine (2 mM)	1.74 ± 0.30 (4)	100 ± 22 (3)	40 ± 7 (3)
Homocysteine (2 mM) + adenosine[a]	0.75 ± 0.32 (4)	585 ± 88 (3)	364 ± 16 (3)
Methionine (1 mM)	2.81 ± 0.07 (2)	297 ± 21 (2)	55 ± 4 (2)
Adenosine (1 mM)	2.13 ± 0.29 (2)	11 ± 1 (2)	68 ± 2 (3)
Methionine (1 mM) + adenosine (1 mM)	2.16 ± 0.36 (2)	477 (1)	351 (1)

Isolated rat hepatocytes were incubated for 2 h at 37°C in the presence of the metabolites and inhibitors indicated. At the end of the incubation, the cells were washed once and extracted with 10 % trichloroacetic acid for measurement of intracellular AdoMet and AdoHcy levels by HPLC, or electrodisrupted for measurement of autophagic activity. Each value is the mean ± S.E. (or range) of the number of experiments indicated.
[a]Adenosine concentration 0.5 mM in the autophagy experiments; 1 mM in the experiments used for HPLC analysis.

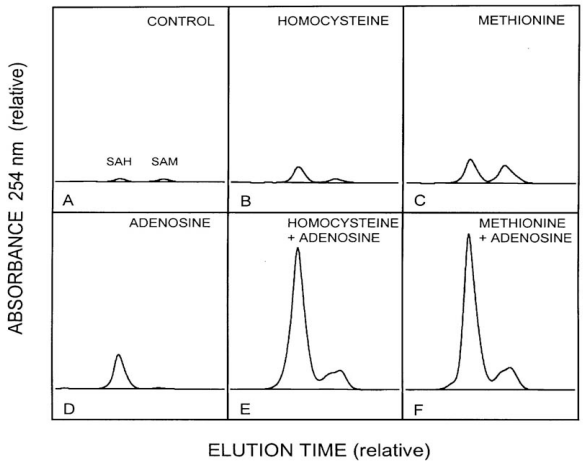

Fig. 3. **Hepatocellular levels of AdoMet and AdoHcy after incubations with homocysteine, adenosine or methionine.** Hepatocytes were incubated for 2h at 37°C with (A), no additions; (B), 2 mM homocysteine; (C), 1 mM methionine; (D), 1 mM adenosine; (E) homocysteine + adenosine; (F), methionine + adenosine. After incubation, the cells were homogenized in 10% TCA, and the acid extracts fractionated by HPLC.

3.3. Effects of homocysteine-related metabolites on autophagy

Okadaic acid, a potent inhibitor of hepatocytic autophagy [26], has been shown to induce overphosphorylation of a cytosolic 32-kDa protein [24], recently identified by mass spectrometry as glycine-*N*-methyltransferase (GNMT; our unpublished results). This enzyme is believed to regulate biological methylation by a wasteful conversion of AdoMet to AdoHcy through formation of the functionless metabolite sarcosine (*N*-methylglycine) [35]. Since homocysteine is a downstream product of this reaction, it could conceivably feedback-regulate the flux through GNMT and hence alter the pools of glycine and sarcosine. Attempts were made to measure intracellular sarcosine levels by an HPLC method, but the levels were too low to be reliably detectable. As shown in Figure 4A, neither sarcosine nor its precursor, glycine, inhibited autophagy, and are thus unlikely to be mediators of the homocysteine inhibition. At high concentrations (5 mM), a slight stimulation of autophagy was in fact observed both with these amino acids and with methionine, contrasting with the progressive dose-dependent inhibition by homocysteine (Figure 4A). None of these metabolites were, however, able to antagonize the autophagy-suppressive effect of homocysteine (results not shown).

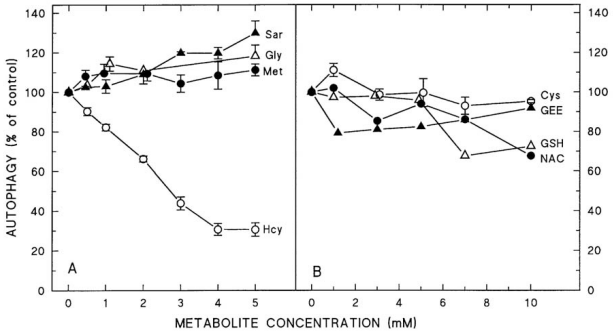

Fig. 4. **Effects on autophagy of metabolites related to homocysteine metabolism.** Hepatocytes were incubated for two hours at 37°C in the presence of (A), homocysteine (Hcy, ○), methionine (Met, ●), glycine (Gly, △) or sarcosine (Sar, ▲); or (B), cysteine (Cys, ○), *N*-acetylcysteine (NAC, ●), reduced glutathione (GSH, △) or glutathione ethyl ester (GEE, ▲) at the concentration indicated. The net autophagic sequestration of endogenous LDH was measured and expressed as % of the control rate (which averaged 2.6 %/h). Each value is the mean ± S.E (or range) of 2-4 experiments.

A major pathway of homocysteine metabolism is by condensation with serine to form cystathionine, which is subsequently cleaved by the enzyme cystathionine γ-lyase (CTL) to form (by transsulfuration) cysteine and α-ketobutyrate. The cysteine can then be utilized for the formation of glutathione (GSH; Figure 2). As shown in Figure 4B, neither cysteine nor its more cell-permeable analogue, *N*-acetylcysteine (NAC), had much effect on hepatocytic autophagy. The same was true for GSH and its more cell-permeable ethyl ester (GEE). Furthermore, a CTL inhibitor, propargylglycine [49], had no effect on autophagy either in the presence or absence of homocysteine (Figure 5). The autophagy-suppressive effect of homocysteine would thus not seem to be mediated by any of the metabolites along the propargylglycine-sensitive transsulfuration pathway.

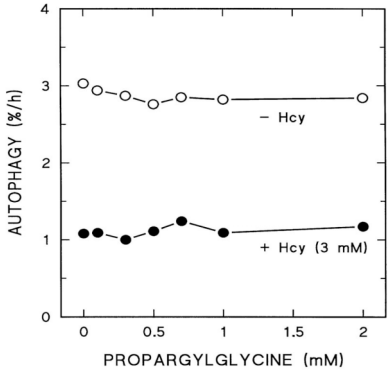

Fig. 5. **Lack of homocysteine antagonism by the cystathionine lyase inhibitor, propargylglycine.** Hepatocytes were incubated for two hours at 37°C with propargylglycine at the concentration indicated, in the absence (○) or presence (●) of 3 mM homocysteine. The net autophagic sequestration of endogenous LDH was measured and expressed as %/h. Data without Hcy are the means of two independent experiments; data with Hcy are from a single experiment.

3.4. Effects of homocysteine and other amino acids on S6 phosphorylation.

The ribosomal protein S6 becomes phosphorylated in hepatocytes in response to autophagy-suppressive amino acid mixtures, and has been implicated in the regulation of autophagy [50]. To see if homocysteine might share this biochemical amino acid effect, S6 phosphorylation was probed with phosphospecific antibodies against Ser235/236 or Ser240/244. As shown in Figure 6, treatment of hepatocytes with an autophagy-suppressive amino acid mixture [15] stimulated phosphorylation at both positions, whereas homocysteine did not. Homocysteine would thus not seem to affect hepatocellular protein metabolism by the same mechanism as the amino acid mixture. Immunoblotting of several other phosphoproteins indicated that homocysteine in fact tended to suppress protein phosphorylation in general (results not shown), suggesting a relatively nonspecific mechanism of action.

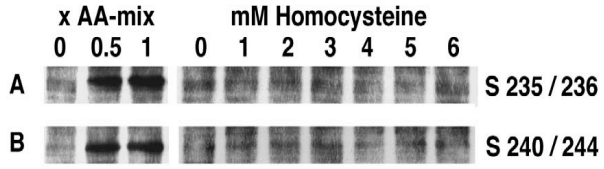

Fig. 6. **Effect of homocysteine and an autophagy-suppressive amino acid mixture on S6 phosphorylation.** Hepatocytes were incubated for two hours with an autophagy-suppressive, complete amino acid mixture (15) at a total amino acid concentration of 13 mM (1x) or at one-half this concentration (0.5x). Alternatively, the cells were incubated with homocysteine at the concentration indicated (0-6 mM). After incubation, the cells were solubilized in an SDS-containing lysis buffer, and the resulting extracts were analysed by gel electrophoretic separation and immunoblotting with two different phosphospecific antibodies against ribosomal protein S6 (A, anti-Ser235/236; B, anti-Ser240/244).

3.5. Effect of homocysteine on intracellular ATP levels

One general mechanism through which homocysteine might interfere with protein phosphorylation could be by reducing the availability of the phosphate donor, ATP. Measurements of intracellular ATP levels by the luciferin-luciferase method revealed that homocysteine indeed effected a strong, dose-dependent reduction in ATP (Figure 7). At 5 mM homocysteine, the hepatocellular ATP content was less than 10 % of the control value. This lack of ATP would account for the reduced protein phosphorylating activity in homocysteine-treated hepatocytes, and also provides a plausible explanation for the autophagy-suppressive effect of homocysteine. Hepatocytic autophagy has been shown to be strongly ATP-dependent, ATP apparently being required at all steps in the autophagic-lysosomal pathway, and particularly at the initial sequestration step [10]. Homocysteine is thus likely to inhibit autophagy by reducing the cellular ATP content.

When ATP levels decline, a reciprocal increase in AMP levels will cause activation of the AMP-activated protein kinase (AMPK), which serves to protect cells against ATP depletion by shutting down several ATP-requiring processes, including autophagy [18,28]. To see if AMPK might be involved in mediating the autophagy-suppressive effect of homocysteine, the activity state of AMPK was checked by immunoblotting with an antibody against the phosphorylated, active form of AMPK. No AMPK activation was observed (results not shown), suggesting that the ATP level, altered in this case by homocysteine, can control autophagic activity independently of AMPK.

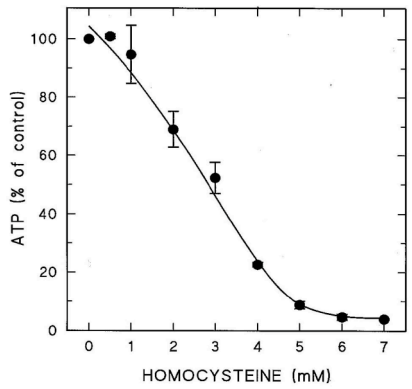

Fig. 7. **Effect of homocysteine on intracellular ATP levels.** Hepatocytes were incubated for one hour at 37°C with homocysteine at the concentration indicated. Metabolic activity was then stopped by the addition of perchloric acid (to a final conc. of 2 %) while the cells were still incubating, and ATP was measured in neutralized perchloric acid extracts by the luciferin-luciferase method. Each value is the mean ± range of two independent experiments.

4. Discussion

The complex intrahepatic metabolism of homocysteine made it necessary to examine a number of possible mechanisms for its autophagy-suppressive effect. The potentiating ability of adenosine would be highly suggestive of mediation by AdoHcy, readily formed in isolated hepatocytes by the reversible adenosylhomocysteinase reaction [42]. Adenosine alone has previously been shown to be an effective suppressant of hepatocytic autophagy, but its effect was abrogated by the adenosine kinase inhibitor, 5-iodotubercidin, and mimicked by AICAR, an activator of the AMP-activated protein kinase (AMPK), suggesting mediation by AMP and possibly by AMPK [18,28]. In the present study, adenosine was always administered along with 5-iodotubercidin, thus eliminating AMP-mediated effects while allowing the formation of AdoHcy. However, although homocysteine was found to elevate AdoHcy leves in isolated hepatocytes both in the absence and presence of adenosine, methionine induced very similar changes without affecting autophagy, thus ruling out AdoHcy as a mediator. Furthermore, since the two amino acids also affected AdoMet levels similarly (in the presence of adenosine), neither AdoMet nor the AdoHcy/AdoMet ratio would seem likely to serve as autophagy-suppressive signals. Adenosine, which raised the AdoHcy level at least as effectively as did homocysteine (probably reflecting its ability to inhibit adenosylhomocysteinase in the absence of homocysteine, cf. [42]), was much less autophagy-suppressive, further disqualifying AdoHcy as an autophagy inhibitor.

Apart from being converted to AdoHcy, homocysteine can serve as an intracellular substrate for methionine formation. The methyl group can be transferred from betaine, by betaine-homocysteine methyltransferase (BHMT), or from 5-methyltetrahydrofolate, by 5-methyltetrahydrofolate-homocysteine methyltransferase (MHMT, methionine synthase) [44]. The reaction product, methionine, does not inhibit autophagy, but the consumption of 5-methyltetrahydrofolate in the MHMT reaction could conceivably relieve an inhibitory effect of this folate on glycine *N*-methyltransferase (GNMT) [51]. GNMT has been postulated to regulate intracellular methylation by short-ciruiting the flux, consuming AdoMet for formation of the presumably functionless metabolite, sarcosine [35]. Methylation in general would be impaired both by the lack of AdoMet and by the accumulation of AdoHcy, which inhibits transmethylases in general, but not GNMT [52]. Since okadaic acid, a potent inhibitor of autophagy [26], has been found to induce GNMT phosphorylation [24,53], presumably associated with activation of the enzyme [54], autophagy

suppression might be a plausible function for one of the catalytic products of the GNMT reaction, the amino acid sarcosine. However, neither sarcosine nor its precursor, glycine, were able to inhibit hepatocytic autophagy in the present experiments. They were, furthermore, unable to antagonize the autophagy-suppressive effect of homocysteine, thus ruling out any role as positive regulators of autophagy.

A third possible metabolic route for homocysteine is the transsulfuration pathway, by which homocysteine is first condensed with serine to form cystathionine (Figure 2), catalyzed by cystathionine β-synthase. Subsequent cleavage of cystathionine, catalyzed by the propargylglycine-sensitive enzyme, cystathionine γ-lyase, leaves the sulfhydryl group with the serine moiety, thus giving rise to cysteine and eventually to glutathione [44]. However, neither propargylglycine nor the metabolic products cysteine and glutathione (including their cell-permeable analogues *N*-acetylcysteine and glutathione ethyl ester) had much effect on autophagy, suggesting that the autophagy-suppressive effect of homocysteine was not mediated through the transsulfuration pathway.

Collectively, the data obtained in the present study indicate that homocysteine does not need to be metabolized in order to exert its autophagy-suppressive effect. This is a characteristic shared with the other autophagy-suppresive amino acids, compatible with a common mechanism of action. Amino acids are the major regulators of hepatic autophagy, shutting down the process when amino acid concentrations in the portal blood are high [2,55,56]. Autophagy is controlled at the initial sequestration step by a subset of amino acids, among which leucine assumes a prominent role, apparently by acting synergistically with other amino acids like tryptophan, phenylalanine, tyrosine and histidine [16,57]. The mechanism of amino acid inhibition is not clear, although some evidence for a leucine surface receptor has been presented [58-61]. Leucine and other amino acids can stimulate phosphorylation of the ribosomal protein S6, of S6 kinase, and of protein synthesis regulators like 4E-BP1, apparently through an mTOR-dependent pathway [62-64]. S6 phosphorylation has been shown to correlate well with the suppression of autophagy by amino acids, and has been suggested to play a causative role [50]. However, since homocysteine failed to stimulate S6 phosphorylation, this mechanism can be ruled out as an explanation of its autophagy-suppressive effect.

Our observation that homocysteine tended to inhibit protein phosphorylation in general, provided a clue to its mechanism of action. Homocysteine was eventually found to strongly reduce the hepatocellular levels of ATP, a phosphate donor that is absolutely required for protein phosphorylation. ATP is also required for autophagy: all steps in the autophagic-lysosomal pathway are ATP-dependent, in particular the initial sequestration step, resulting in strongly reduced autophagy when ATP levels are low [10]. In addition, the excess AMP generated during ATP depletion may contribute to inhibition of autophagy through the autophagy-suppressive AMP-activated protein kinase, AMPK [18,28]. These two mechanisms of autophagy suppression - ATP depletion and AMPK activation - can be difficult to tell apart. It is, therefore, interesting that homocysteine generated a situation where ATP was depleted in the absence of AMPK activation, thus demonstrating the ability of ATP to control autophagy independently of AMPK

The ability of homocysteine to reduce intracellular ATP levels is likely to be responsible for other of its inhibitory effects, e.g., on hepatocytic protein synthesis [46], and for the general cytotoxicity observed at high homocysteine concentrations in the present study. The unresponsiveness of AMPK in this situation may contribute to the cytotoxicity, since its protective closure of ATP-consuming cellular processes [65] does not occur. Whether ATP depletion is relevant to the role of homocysteine as a recognized risk factor for cardiovascular disease [66,67] is an open question. The concentrations of homocysteine required to reduce ATP in isolated hepatocytes are an order of magnitude above physiological intrahepatic homocysteine levels [42,43], and two orders of magnitude above normal plasma concentrations [68]. Nevertheless, mitochondrial abnormalities have been observed in the hepatocytes of human patients with hyperhomocysteinemia as well as in aortic endothelial cells from hyperhomocysteinemic rats [69]. The role of homocysteine as a perturbant of mitochondrial function and cellular energy metabolism would, therefore, seem worthy of further study.

Acknowledgments

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PAPER III

PAPER IV

Toxin-induced Tail Phosphorylation of Hepatocellular S6 Kinase: Evidence for a Dual Involvement of the AMP-activated Protein Kinase in S6 Kinase Regulation

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SUMMARY

Several protein phosphatase-inhibitory toxins (okadaic acid, microcystin, calyculin A, cantharidin, tautomycin) administered to isolated rat hepatocytes were found to induce phosphorylation in the tail region of S6 kinase (S6K; p70S6K1) as detected with a phosphospecific antibody against doubly phosphorylated Thr-421/Ser424. AICAR, an adenosine analogue that elicits activation of the hepatocellular AMP-activated protein kinase (AMPK), similarly stimulated S6K tail phosphorylation. The flavonoid naringin prevented the effects of AICAR, okadaic acid and microcystin on AMPK activation as well as on S6K tail phosphorylation, suggesting AMPK as a mediator of the latter. The effects of AICAR and the toxins were rapamycin-resistant; in contrast, amino acids induced an S6K tail phosphorylation that was rapamycin-sensitive, suggesting mediation by the protein kinase mTOR. Amino acids activated S6K by phosphorylation at Thr-389, but the toxins did not, and AICAR in fact suppressed the activating phosphorylation induced by the amino acids. The possibility thus must be considered that the phosphorylated S6K tail may transmit a toxic signal independently of S6K enzymatic activity. Despite their inability to activate S6K, the toxins (but not AICAR) stimulated phosphorylation of the ribosomal protein S6, presumably by activating some other S6-phosphorylating protein kinase.

INTRODUCTION

A variety of protein phosphatase-inhibitory toxins of biological origin have been shown to exert toxic effects on mammalian organisms and cells. Microcystins from blue-green algae are acutely hepatotoxic when administered to experimental animals or inadvertently ingested by livestock (Runnegar *et al.,* 1986), and their presence in drinking water is suspected to contribute to liver cancer in certain parts of China (Ueno *et al.,* 1996; Carmichael, 1994). Okadaic acid from marine dinoflagellates is well documented to be a major contributant to diarrhetic shellfish poisoning (Tester, 1994). However, when administered intravenously, okadaic acid evokes hepatotoxic rather than enterotoxic symptoms (Berven *et al.,* 2001). Isolated rat hepatocytes (Seglen, 1976) would, therefore, seem to be a suitable experimental system in which to study the mechanisms of action of protein phosphatase-inhibitory environmental toxins in general (Fladmark *et al.,* 1998).

Okadaic acid and other protein phosphatase-inhibitory toxins have been shown to induce hyperphosphorylation of cytoskeletal proteins like keratin (Blankson *et al.,* 2000) and plectin (Ruud Larsen *et al.,* 2002) in isolated rat hepatocytes, and to cause disruption of the intracellular network of keratin intermediate filaments (Blankson *et al.,* 1995). The toxins also inhibit hepatocellular processes like autophagy, endocytosis and protein synthesis (Holen *et al.,* 1993; Gordon *et al.,* 1995), and elicit apoptotic cell death when administered to hepatocytes in culture (Bøe *et al.,* 1991; Blankson *et al*., 2000). Two toxic mechanisms can be distinguished on the basis of toxin dose-response characteristics and antagonistic effects of the grapefruit flavonoid, naringin: (1) a naringin-sensitive mechanism that seems to involve protein phosphatase PP2A, used by microcystin and low concentrations of okadaic acid, and (2) a naringin-resistant mechanism used by calyculin A, cantharidin and tautomycin that probably involves PP1 (Holen *et al*., 1993; Gordon *et al*., 1995; Ruud Larsen *et al*., 2002). Such differential naringin sensitivity is also seen in the activating effects of toxins on the AMP-activated protein kinase (AMPK), suggesting AMPK as a mediator of the toxicity (Ruud Larsen *et al*., 2002). This hypothesis is strengthened by the ability of AICAR, an adenosine analogue that activates AMPK after being converted intracellularly to an AMP analogue, to mimic many of the hepatocellular effects of the toxins (Samari and Seglen, 1998; Ruud Larsen *et al*., 2002). The major function of AMPK is to save energy when cellular ATP levels are low (and AMP levels correspondingly high), a goal achieved by phosphorylation of key metabolic enzymes (Hardie *et al.,* 1998). AMPK may conceivably exert a similar function in the preservation of cellular resources under conditions of toxic stress.

In a search for mediators of toxic signalling downstream of AMPK, we have used proteomic methods and phospho-specific antibodies to identify toxin- and naringin-sensitive hepatocellular phosphoproteins (Ruud Larsen *et al*., 2002). One protein thus identified is S6 kinase, which plays an essential role in cellular mass growth by phosphorylating the ribosomal protein S6, thereby directing the ribosomes to mRNAs specifically associated with protein synthetic capacity (ribosomal proteins and translation factors) (Dufner and Thomas, 1999). S6 and S6K have also been implicated in the regulation of autophagy, which is suppressed by S6K-activating amino acids (Blommaart *et al.,* 1995). An AMPK-dependent shutdown of these energy-requiring processes may well be a functional adaptive response to toxic stress.

In the present study we have investigated the effects of toxins on S6K phosphorylation, using a phosphospecific antibody directed against the tail region of the enzyme (where an activation-priming phosphorylation at T421/S424 takes place) as well as an antibody detecting the enzyme-activating phosphorylation at T389. The results suggest that the protein phosphatase-inhibitory toxins induce an AMPK-dependent phosphorylation of the S6K tail in the absence of any concomitant enzyme activation. The possibility thus must be considered that S6K tail phosphorylation may convey a toxic signal independently of S6K enzymatic activity, causing, e.g., autophagy suppression.

[1]To whom correspondence should be addressed: Proteomics Section, Department of Cell Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Tel.: 47-22-93-59-47; Fax: 47-22-93-45-80; E-mail: per.seglen@labmed.uio.no. | ABBREVIATIONS AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PP, protein phosphatase; S6K, S6 kinase; SDS, sodium dodecyl sulfate | KEYWORDS rat, liver, hepatocyte, okadaic acid, microcystin, calyculin A, cantharidin, tautomycin, naringin, AICAR, S6 kinase, AMP-activated protein kinase.

MATERIALS AND METHODS

Reagents. Phosphospecific polyclonal rabbit antibodies against Thr389-phosphorylated or Thr421/Ser424-phosphorylated S6K, and horseradish peroxidase-linked anti-rabbit IgG antibody, were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Okadaic acid and microcystin-LR were from Alexis Biochemicals (Läufelfingen, Switzerland); calyculin A, cantharidin and tautomycin from Calbiochem (San Diego, CA, USA), and 5-aminoimidazole-4-carboxamide riboside (AICAR) from Toronto Research Chemicals (North York, On, Canada). Rainbow molecular weight markers (RPN 756) and the ECL western blotting detection kit were from Amersham Biosciences (Little Chalfont, Bucks., UK). Sodium dodecyl sulfate (SDS), acrylamide and bisacrylamide were obtained from BioRad (Hercules, CA, USA). Dry milk powder was from Nestlé (Vevey, Switzerland), and nitrocellulose membranes from Osmonics (Westborough, MA, USA). Methanol and acetic acid were from Merck (Whitehouse Station, NJ, USA), and alkaline phosphatase (MB grade) from Roche Appl. Sci., Penzberg, Germany. Other biochemicals were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Animals and cells. Isolated hepatocytes were prepared from 18-h starved male Wistar rats (250-300 g; Harlan UK Ltd., Shaws Farm, Oxon, UK) by two-step collagenase perfusion (Seglen 1976), purified by differential centrifugation, and resuspended in suspension buffer fortified with 2 mM Mg^{2+} and 15 mM pyruvate (Seglen 1976). Aliquots of cell suspension, each containing about 30 mg cells (wet mass) in a volume of 0.4 ml, were incubated for 60 min at 37°C in shaking centrifuge tubes.

Gel electrophoresis and immunoblotting. Cell incubations were terminated by adding 2 ml of ice-cold Tris-buffered saline (TBS; 20 mM Tris-base, 0.1 % Tween-20, pH 7.6) to each tube, followed by centrifugation of the cells at 1600 rev/min for 4 min in the cold (4°C); this washing was repeated once. The cells were lysed for 30 min on ice in one ml of lysis buffer containing 0.4% SDS, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, and 20 mM Tris-base, pH 7.2. The resulting whole-cell extracts were diluted 1:2 in double-strength SDS gel-loading buffer (single strength, 2% SDS, 1 M mercaptoethanol, 0.1% bromophenol blue, 10% glycerol, 50 mM Tris-HCl, pH 6.8) and boiled for 5 min at 95°C. After measuring the protein contents of the extracts by the method of Bradford (Bradford, 1976), using the BCA protein assay kit from Pierce (Rockford, USA), samples containing 20 μg were separated by SDS gel electrophoresis for approximately 40 min at 200 V in 10% polyacrylamide gels containing 0.1 % SDS. Molecular weight markers were included in all gels.

Gel-separated proteins were transferred to nitrocellulose blotting membranes using a semi-dry transfer unit (Bio-Rad Laboratories, Hercules, CA, USA) with Towbin's blotting buffer (192 mM glycine, 20% methanol, 25 mM Tris-base, pH 8.3). The membranes were blocked by overnight incubation at 4°C with 5% dry milk in TBS containing 0.2% Tween-20 (TBS-T), and washed three times for 10 min in TBS-T. For detection of total or phosphorylated AMPK⋄, the membranes were first incubated overnight at 4°C with the respective antibodies (diluted 1:1000 in TBS-T). After washing three times with TBS-T the membranes were incubated for one hour at room temperature with anti-rabbit-horseradish peroxidase (diluted 1:2000 in TBS-T), washed three times and visualized by chemiluminescence using the ECL Western Detection Kit (Amersham Biosciences).

RESULTS

Toxin-induced phosphorylation of the S6K tail region.

The availability of a phosphospecific antibody that recognizes simultaneous phosphorylation of T421 and S424 in the C-terminal tail region of S6K, where multiple phosphorylations are involved in conformational changes required for activation of this enzyme (Dufner and Thomas 1999), has allowed us to investigate the effects of protein phosphatase-inhibitory toxins on S6K tail phosphorylation. Okadaic acid, microcystin-LR, calyculin A, cantharidin or tautomycin all induced a dose-dependent immunoreactivity in the upper of two adjacent bands in the 70 kDa region of the gel when administered to isolated rat hepatocytes incubated at 37°C (Fig. 1), okadaic acid being the most potent of the toxins. The present study has concentrated on this 70 kDa band, taken to represent the cytosolic p70S6K (henceforth referred to as S6K), although some toxin-induced immunostaining could be observed at higher molecular mass values as well.

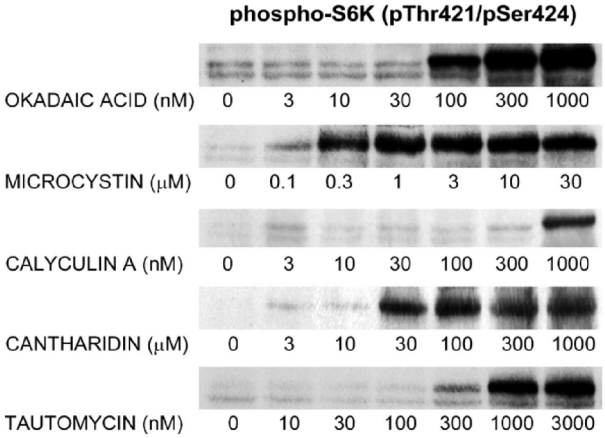

FIG. 1. **Toxin-induced S6K tail phosphorylation.** Freshly isolated rat hepatocytes were incubated for 1 h at 37°C with okadaic acid, microcystin-LR, calyculin A, cantharidin or tautomycin at the concentrations indicated. Gel-separated cell extracts were immunoblotted with a phosphospecific antibody against Thr421/Ser424-phosphorylated S6K.

Immunoblotting with a general S6K antibody (Fig. 2A) showed that only the upper of the two 70 kDa bands was immunoreactive. A reduction in the mobility of this band, sometimes evident as an upward-directed band broadening, was observed at increasing concentrations of the toxins tested (okadaic acid, microcystin, calyculin A and tautomycin). To check if the mobility shift might reflect toxin-induced S6K phosphorylation, cell extracts were treated with alkaline phosphatase prior to gel electrophoresis and immunoblotting. As shown in Fig. 2B (upper lane), the phosphatase treatment abolished the mobility shifts induced by selected toxins (okadaic acid, microcystin and tautomycin), and strongly reduced the signal obtained with the phosphospecific pThr421/pSer424 S6K antibody (lower lanes), thus demonstrating that the mobility shift was indeed due to S6K phosphorylation.

Differential naringin sensitivity of toxin-induced S6K tail phosphorylation.

The toxins used have previously been shown to induce activating phosphorylations of the AMP-activated protein kinase (AMPK) by two different mechanisms: a naringin-sensitive mechanism associated with PP2A inhibition (okadaic acid, microcystin) and a naringin-resistant mechanism associated with PP1 inhibition (calyculin A, cantharidin, tautomycin) (Ruud Larsen *et al.*, 2002). To see if

S6K tail phosphorylation might similarly exhibit differential naringin sensitivity, the effect of this flavonoid was examined. As shown in Fig. 3, naringin, while having no effect on its own, antagonized the phosphorylation-stimulatory effects of okadaic acid and microcystin, but not the effects of calyculin A, cantharidin or tautomycin. The two toxin mechanisms operating on AMPK can thus also be observed at the level of S6K tail phosphorylation, consistent with the possibility of the latter phosphorylation being mediated by AMPK.

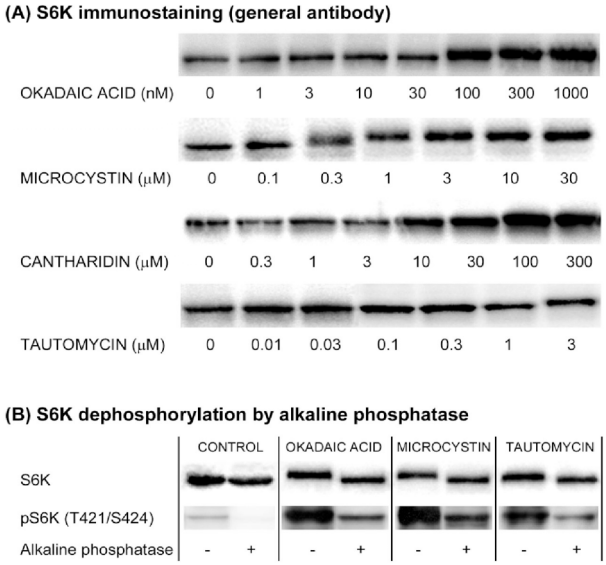

FIG. 2. **Phosphorylation-induced S6K mobility shift.** (A) Hepatocytes were incubated for 1 h at 37°C with okadaic acid, microcystin, cantharidin or tautomycin at the concentrations indicated. Cell extracts were immunoblotted with a general antibody against S6K. (B) Extracts of cells incubated with okadaic acid (300 nM), microcystin (3 μM) or tautomycin (1 μM) were treated with alkaline phosphatase (150 units/ml) for 3h at 37°C as indicated. The treated extracts were gel-separated and immunoblotted with a general S6K antibody (upper lane) or a T421/S424-phosphospecific antibody (lower lanes).

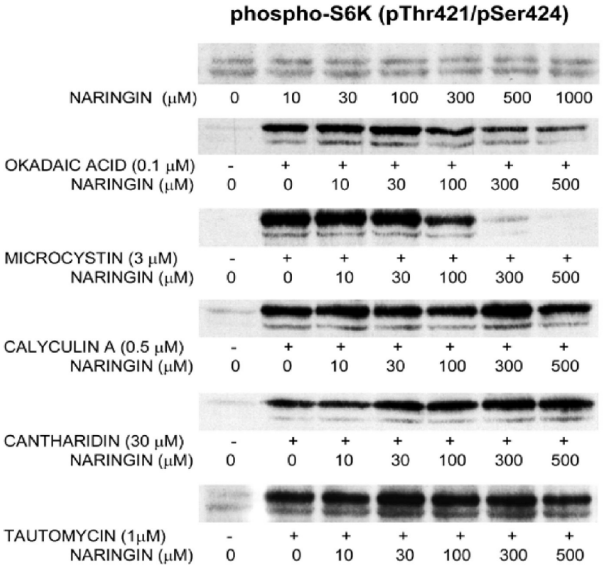

FIG. 3. **Effect of naringin on toxin-induced S6K tail phosphorylation.** Hepatocytes were incubated for 1 h at 37°C with toxins and/or naringin at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr421/Ser424-phosphorylated S6K.

Effects of AICAR and amino acids on S6K tail phosphorylation.

To further examine the possibility of an AMPK involvement, we tested the effect of AICAR, an adenosine analogue converted by hepatocytes to an AMP analogue that activates AMPK (Samari and Seglen 1998) in a naringin-sensitive manner (Ruud Larsen *et al*., 2002). AICAR had no effect on the absolute S6K levels as indicated by immunoblotting with the general S6K antibody (results not shown), but induced a dose-dependent phosphorylation of S6K at T421/S424 that was antagonized by increasing concentrations of naringin (Fig. 4A). This observation would be consistent with an involvement of AMPK in S6K tail phosphorylation. In contrast, a mixture of amino acids, which activate S6K through an mTOR-dependent pathway (Shigemitsu *et al.,* 1999; Blommaart *et al*., 1995), elicited a dose-dependent S6K tail phosphorylation that was resistant to naringin (Fig. 4B). Amino acids induced a more pronounced mobility shift than did the other phosphorylation stimulants, effectively causing a split of the upper 70 kDa band, probably indicating phosphorylation at more sites than with AICAR or toxins.

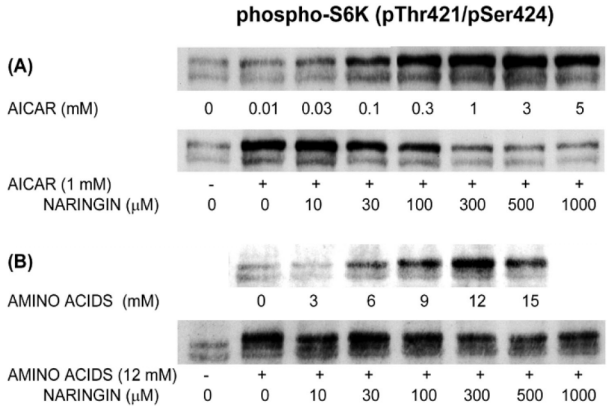

FIG. 4. **AICAR- and amino acid-induced S6K tail phosphorylation: effect of naringin.** Hepatocytes were incubated for 1 h at 37°C with a physiological amino acid mixture, AICAR and/or naringin at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr421/Ser424-phosphorylated S6K.

The ability of AICAR to induce phosphorylation of S6K in a position generally associated with activation of the enzyme is surprising, since several previous studies have shown that AICAR inhibits S6K activity, preventing an activating phosphorylation at T389 (Kimura *et al.,* 2003; Dubbelhuis and Meijer, 2002; Krause *et al.,* 2002). We examined, therefore, if AICAR might antagonize the effects of amino acids and toxins on S6K tail phosphorylation. However, as shown in Fig. 5, AICAR did not have any antagonistic effects on tail phosphorylation induced by the other effectors.

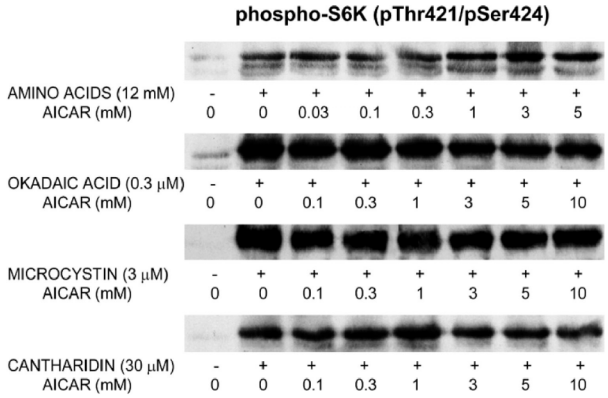

FIG. 5. **Lack of effect of AICAR on S6K tail phosphorylation induced by amino acids or toxins.** Hepatocytes were incubated for 1 h at 37°C with amino acids or toxins in the absence or presence of AICAR at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr421/Ser424-phosphorylated S6K.

The toxin-induced S6K tail phosphorylations are rapamycin- and wortmannin-resistant.

The above results suggest that the naringin-sensitive S6K tail phosphorylations induced by okadaic acid, microcystin and AICAR proceed by a mechanism different from the naringin-resistant phosphorylation induced by amino acids. However, since amino acids could conceivably share the mechanism used by the naringin-resistant toxins, the effect of rapamycin, an inhibitor of mTOR-mediated amino acid effects on S6K (Shigemitsu *et al*., 1999; Blommaart *et al*., 1995), was examined. As shown in Fig. 6, rapamycin effectively antagonized the amino acid-induced S6K tail phosphorylation, but had no detectable effect on the phosphorylations induced by AICAR or any of the toxins. Thus, whereas amino acids seem to induce S6K tail phosphorylation through a rapamycin-sensitive, mTOR-mediated mechanism, AICAR and the toxins would appear to use a different, probably AMPK-mediated mechanism.

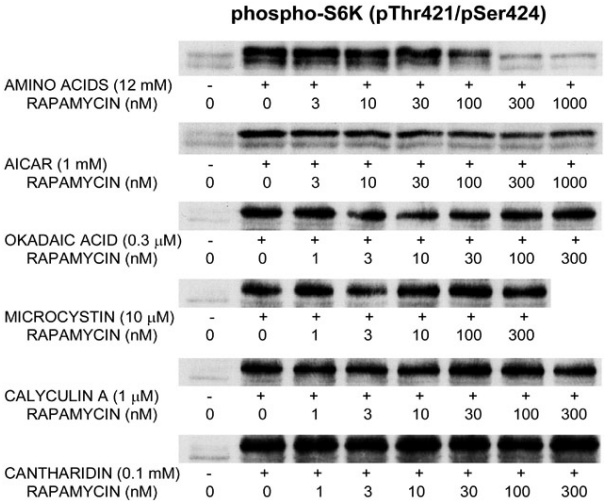

FIG. 6. **Effects of rapamycin on S6K tail phosphorylation induced by amino acids, AICAR or toxins.** Hepatocytes were incubated for 1 h at 37°C with amino acids or toxins in the absence or presence of AICAR at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr421/Ser424-phosphorylated S6K.

In addition to the two mechanisms dicussed above, S6K is known to be regulated by hormones and growth factors (e.g., by insulin) through a wortmannin-sensitive pathway that involves PI 3-kinase(Shigemitsu *et al*., 1999). However, wortmannin did not antagonize any of the hepatocellular S6K tail phosphorylations, be they induced by amino acids, AICAR, okadaic acid, microcystin or cantharidin, nor did wortmannin have any effect on its own (Fig. 7). Furthermore, we have not been able to observe any effects of toxins on the phosphorylative activation of protein kinase B, a common effector in the PI 3-kinase pathway (results not shown). The PI 3-kinase pathway would thus seem unlikely to be involved in toxin-induced S6K phosphorylation.

Effects of toxins, amino acids and AICAR on S6 phosphorylation.

To see if the toxin-induced S6K tail phosphorylation was accompanied by S6K activation, S6K activity in the hepatocytes was examined as *in situ* phosphorylation of the enzyme's substrate, ribosomal protein S6. Using a phospho-specific antibody directed against double phosphorylation of the adjacent S6 positions S235 and S236, it could be shown that all of the toxins tested (okadaic acid, microcystin, calyculin A, cantharidin and tautomycin) as well as amino acids induced a dose-dependent phosphorylation of a double

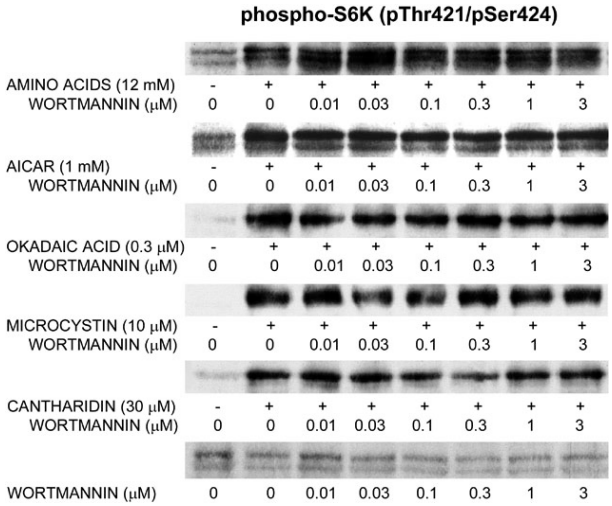

FIG. 8. **Effects of toxins, amino acids and AICAR on S6 phosphorylation.** Hepatocytes were incubated for 1 h at 37°C with toxins, amino acids or AICAR at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Ser235/236-phosphorylated ribosomal protein S6.

band around 32 kDa, corresponding to the position of S6 (Fig. 8), consistent with activation of S6K (or some other S6-phosphorylating enzyme). In contrast, AICAR failed to induce S6 phosphorylation (some inhibition of the basal S6 phosphorylation was in fact indicated, but the absolute levels were too low for reliable interpretation), indicating that the drug did not stimulate hepatocellular S6K activity.

These and the following experiments also included immunoblotting with a phosphospecific antibody directed against S6 doubly phosphorylated at Ser240 and Ser244, with results more or less identical to those obtained with the pSer235/236 antibody.

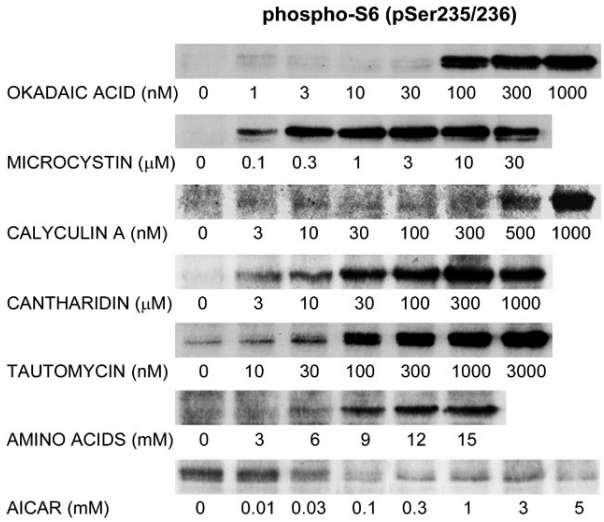

FIG. 8. **Effects of toxins, amino acids and AICAR on S6 phosphorylation.** Hepatocytes were incubated for 1 h at 37°C with toxins, amino acids or AICAR at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Ser235/236-phosphorylated ribosomal protein S6.

Differential naringin sensitivity of S6 phosphorylation.

S6 phosphorylation induced by okadaic acid or microcystin was antagonized by naringin, whereas phosphorylation induced by calyculin A, cantharidin or tautomycin was not (Fig. 9), parallelling the differential naringin sensitivity of the AMPK activation and S6K tail phosphorylation elicited by these two groups of toxins. The amino acid-induced S6 phosphorylation was, as expected, unaffected by naringin (Fig. 9).

No antagonism between AICAR and naringin could be reliably detected at the low, basal levels of S6 phosphorylation.

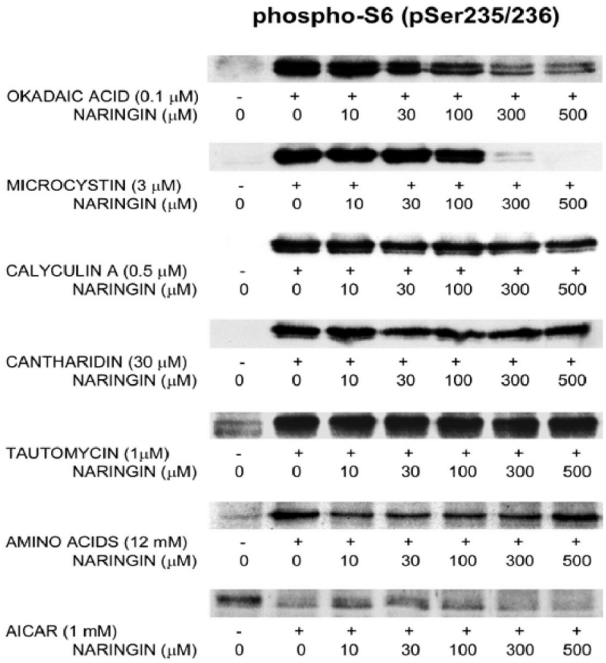

FIG. 9. **Effect of naringin on S6 phosphorylation induced by toxins, amino acids or AICAR.** Hepatocytes were incubated for 1 h at 37°C with toxins, amino acids or AICAR in the absence or presence of naringin at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Ser235/236-phosphorylated ribosomal protein S6.

Effects of AICAR, rapamycin and wortmannin on S6 phosphorylation.

In contrast to the inability of AICAR to antagonize the S6K tail phosphorylation elicited by amino acids (Fig. 5), this AMPK activator completely suppressed the amino acid-induced S6 phosphorylation at S235/236 (Fig. 10A). The inhibitory effect of AICAR was markedly antagonized by naringin, consistent with mediation by AMPK. These results thus corroborate previous studies which have indicated that AICAR induces an AMPK-dependent inhibition of hepatocellular S6K activity (Kimura *et al*., 2003; Dubbelhuis and Meijer 2002; Krause *et al*., 2002).

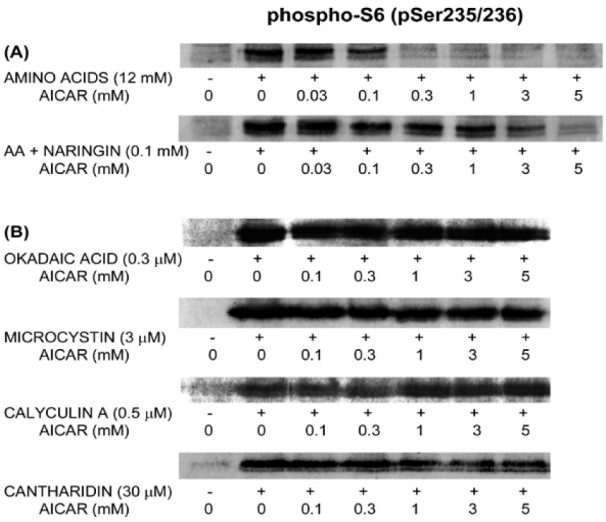

FIG. 10. **Effect of AICAR on S6 phosphorylation induced by toxins or amino acids.** Hepatocytes were incubated for 1 h at 37°C with (A) amino acids (with or without naringin) or (B) toxins in the absence or presence of AICAR at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Ser235/236-phosphorylated ribosomal protein S6.

The apparent ability of AMPK both to inhibit S6 phosphorylation (elicited by amino acids) and to stimulate S6 phosphorylation (elicited by toxins) raises a paradox. As shown in Fig. 10B, toxin-induced S6 phosphorylation was not much affected by S6K-inhibitory concentrations of AICAR, possibly indicating that the toxins stimulated S6 phosphorylation by an S6K-independent mechanism.

Rapamycin, at 300 nM, completely abolished the amino acid-induced S6 phosphorylation, while having no effect on toxin-induced phosphorylation (Fig. 11). These observations are consistent with an mTOR-mediated amino acid effect, whereas the toxins apparently cause S6 phosphorylation by a different mechanism. Wortmannin had no effect on S6 phosphorylation elicited by either amino acids or toxins (results not shown).

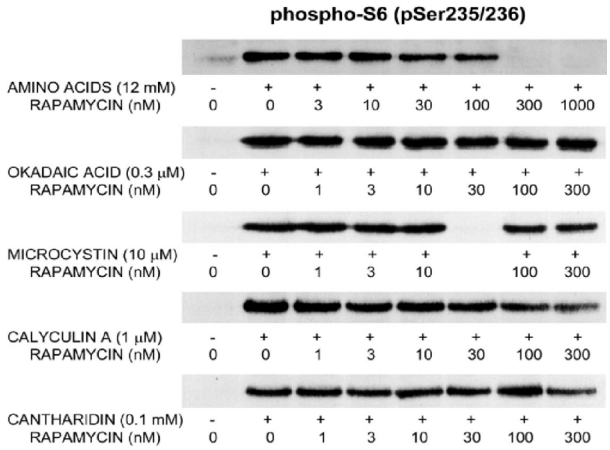

FIG. 11. **Effect of rapamycin on S6 phosphorylation induced by amino acids or toxins.** Hepatocytes were incubated for 1 h at 37°C with amino acids or toxins in the absence or presence of rapamycin at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Ser235/236-phosphorylated ribosomal protein S6.

Effects of toxins, amino acids and AICAR on S6K-activating phosphorylation at Thr-389.

Phosphorylation at T389 is absolutely required for S6K activity, and can be used as a convenient indicator of S6K activation. As shown in Fig. 12A, an amino acid mixture significantly stimulated T389 phosphorylation in isolated hepatocytes, as detected with a phosphospecific antibody directed against this site. The phosphorylation was rapamycin-sensitive, indicating mediation through mTOR, and eliminated by AICAR as previously observed (Kimura *et al*., 2003; Dubbelhuis and Meijer 2002; Krause *et al*., 2002), whereas naringin had no effect. The amino acid-antagonistic effect of AICAR was overcome by naringin (Fig. 12B), supporting the contention that AICAR inhibits S6K activity by signaling through the AMPK pathway.

Okadaic acid and other toxins did not stimulate T389 phosphorylation (Fig. 12C), despite their ability to promote S6 phosphorylation as demonstrated above. This surprising observation suggests that the toxins do not activate S6K, implicating that some other kinase must be responsible for the toxin-induced S6 phosphorylation.

DISCUSSION

The present study shows that protein phosphatase-inhibitory toxins stimulate S6K tail phosphorylation at T421/S424 in isolated rat hepatocytes. Tail phosphorylation is also elicited by an amino acid mixture, but by a different mechanism: whereas the amino acid-induced tail phosphorylation is blocked by rapamycin, suggesting mediation by the protein kinase mTOR, the toxin-induced phosphorylation is not.

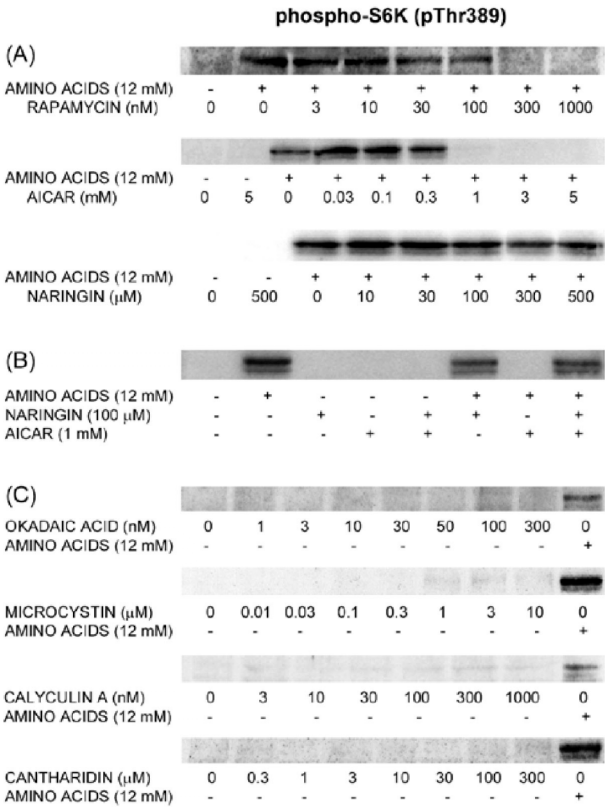

FIG. 12. **Effects of amino acids, AICAR, toxins and naringin on the phosphorylative activation of S6K.** Hepatocytes were incubated for 1 h at 37°C with (A, B), amino acids, rapamycin, AICAR, naringin or (C), toxins at the various combinations and concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr389-phosphorylated S6K.

Two types of evidence indicate that the toxin-induced S6K tail phosphorylation may be mediated by the AMP-activated protein kinase, AMPK: (1) AICAR, an adenosine analogue that activates AMPK after being converted intracellularly to the AMP analogue, ZMP (Sabina *et al.,* 1985), causes S6K tail phosphorylation. The phosphorylation is prevented by naringin, a flavonoid previously shown in our laboratory to antagonize AICAR-induced AMPK activation (Ruud Larsen *et al*., 2002). (2) S6K tail phosphorylation induced by the toxins okadaic acid or microcystin is similarly prevented by naringin, which also inhibits okadaic acid-induced AMPK activation (Ruud Larsen *et al*., 2002). Although other toxins (calyculin A, cantharidin and tautomycin) stimulate S6K tail phosphorylation through a naringin-insensitive mechanism, this naringin-insensitivity is parallelled by the effects of these toxins on AMPK activation (our unpublished results). Thus both groups of toxins may stimulate S6K tail phosphorylation through the activation of AMPK. The naringin-sensitive and naringin-resistant mechanisms are thought to reflect the differential effects of the toxins on the inhibition of protein phosphatases PP2A and PP1, respectively (Ruud Larsen *et al*., 2002).

In vascular smooth muscle cells, the MAP kinase pathway and the PI 3-kinase-mTOR pathway have been shown to cooperate in phosphorylating S6K at S411 (Eguchi *et al.,* 1999), but MAP kinase does not seem to be capable of phosphorylating rat liver S6K at T421/S424 (Mukhopadhyay *et al.,* 1992), and as shown in the present study, inhibitors of PI 3-kinase (wortmannin) or mTOR (rapamycin) do not affect toxin-induced S6K tail phosphorylation. The protein kinase Cdc2 may also play a role in S6K tail phosphorylation at S411(Papst *et al.,* 1998; Mukhopadhyay *et al*., 1992), but this site seems to be phosphorylated independently of T421/S424 (Le *et al.,* 2003). A more likely candidate would be the stress-activated protein kinase, JNK, which has been strongly implicated in T421/S424 phosphorylation in several epithelial cancer cell lines (Le *et al*., 2003), and which is activated in rat hepatocytes by AICAR and okadaic acid (Ruud Larsen *et al*., 2002) as well as by the other toxins studied here (our unpublished results).

The mechanism by which toxin-induced protein phosphatase inhibition causes activation of AMPK, a protein kinase, is not clear. AMPK is reportedly dephosphorylated at pT172 *in vivo* by PP2C rather than by the toxin-sensitive phosphatases, PP1 and PP2A (Moore *et al.,* 1991; Davies *et al.,* 1995), but there is some evidence that the latter may regulate dephosphorylation of the PP2C α subunit (Kobayashi *et al.,* 1998), and hence control AMPK dephosphorylation indirectly. An alternative possibility is that the toxins prevent dephosphorylation of other, as yet uncharacterized sites involved in the regulation of AMPK activity (Woods *et al.,* 2003b), or that they somehow promote T172 phosphorylation of AMPK by its upstream protein kinase, LKB1 (Hawley *et al.,* 2003; Hong *et al.,* 2003; Woods *et al.,* 2003a).

The ability of AICAR to induce S6K tail phosphorylation is somewhat surprising, since this AMPK activator has previously been shown to inhibit S6K activity by preventing the activating phosphorylation of the enzyme at T389, elicited, e.g., by amino acids (Kimura *et al*., 2003; Dubbelhuis and Meijer 2002; Krause *et al*., 2002). This effect of AMPK could be secondary to an inhibitory phosphorylation of mTOR, an S6K kinase (Cheng *et al.,* 2004). The present study confirms that AICAR does, in fact, inhibit both the amino acid-induced T389 phosphorylation and the activity of S6K as indicated by reduced S6 phosphorylation in intact cells. Both of these inhibitory effects of AICAR are antagonized by naringin, suggesting that they are mediated by AMPK. AMPK would thus appear to have a dual effect on S6K: it promotes tail phosphorylation, but suppresses T389 phosphorylation and S6K activity.

Could the S6K tail phosphorylation serve an independent physiological function, unrelated to the S6-phosphorylating role of S6K? It is noteworthy that agents that either stimulate (amino acids), inhibit (AICAR) or have no effect (toxins) on S6K activity can all induce S6K tail phosphorylation. These diverse effectors have one other property in common: they all inhibit hepatocytic autophagy. Previous investigators have pointed to a correlation between the abilities of amino acids to induce S6 phosphorylation and to suppress autophagy, suggesting that phosphorylated S6 might somehow act as an autophagy inhibitor (Blommaart *et al*., 1995). Although the ability of AICAR to inhibit autophagy while suppressing S6 phosphorylation does not support an autophagy-regulatory role for S6, the effect of AICAR on S6K tail phosphorylation would be compatible with a non-enzymatic, autophagy-regulatory role for S6K. The possibility is not entirely unprecedented: in yeast, the protein kinase Atg1 has ben found to regulate autophagy (albeit in a stimulatory capacity) independently of its kinase activity (Abeliovich *et al.,* 2003).

Since both algal toxins and AICAR have been shown to elicit hepatocellular apoptosis (Blankson *et al*., 2000; Meisse *et al.,* 2002), the possibility should also be considered that AMPK-induced S6K modifications might play a role in apoptotic signalling. Inactivation of S6K has been shown to favour apoptosis under several conditions (Harada *et al.,* 2001; Tee and Proud, 2001; Bonatti *et al.,* 2000); in addition, phosphorylation of the S6K tail could conceivably act as a separate pro-apoptotic signal. Whether tail-phosphorylated S6K can promote apoptosis or suppress autophagy independently of the enzyme's enzymatic (S6-phosphorylating)

activity will need to be investigated by means of S6K mutations. Unfortunately, such mutations cannot presently be generated in hepatocytes, due to the extremely limited proliferation capacity of these cells in culture.

Future studies are also required to resolve another paradox arising from the present results: how can the toxins stimulate S6 phosphorylation without activating S6K? The most likely answer is that another kinase, activated by the toxins, is responsible for the S6 phosphorylation. One candidate would be the closely related enzyme, S6K2 (p70βS6K), which differs sufficiently from S6K (S6K1) to preclude immunological cross-reactivity (Gout *et al.,* 1998; Shima *et al.,* 1998), and which might, therefore, have escaped detection by the pT389 antibody. Furthermore, both the S6K and the S6K2 transcripts generate larger (p85) translation products with a nuclear localization, probably engaged in the phosphorylation of S6 at nucleolar sites (Dufner and Thomas 1999). Thirdly, although protein kinases in the related RSK family (p90RSK 1-3) generally serve other cellular functions (Frödin and Gammeltoft, 1999), they may be capable of phosphorylating S6, as observed, e.g., in human peripheral blood mononuclear cells after leptin treatment (van den Brink *et al.,* 2000), in okadaic acid-treated maturing oocytes (Gavin and Schorderet-Slatkine, 1997) or in mutant mice which lack both S6K and S6K2 (Pende *et al.,* 2004).

Given the numerous signalling pathways and cellular processes regulated by toxin-sensitive protein phosphatases, it is perhaps not surprising that the toxins may have complex effects even on a single biochemical event such as S6 phosphorylation. Whether the toxin effects on S6 and S6K are a cause or a manifestation of cellular toxicity remains to be shown.

ACKNOWLEDGMENTS

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PAPER V

Stimulation of Hepatocytic AMP-Activated Protein Kinase by Okadaic Acid and other Autophagy-suppressive Toxins

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Autophagic activity in isolated rat hepatocytes has previously been shown to be strongly suppressed by okadaic acid and other protein phosphatase-inhibitory toxins (I. Holen, P.B. Gordon & P.O. Seglen [1993], *Eur. J. Biochem.* 215, 113-122) as well as by AICAR (5-amino-imidazole-4-carboxamide riboside), a direct activator of the AMP-activated protein kinase (AMPK; H. Samari & P.O. Seglen [1998], *J. Biol. Chem.* 273, 23758-23763). To investigate whether AMPK might be a mediator of the toxin effects, a phosphospecific antibody directed against the AMPK-activating phosphorylation at Thr172 was used to assess the activation status of this enzyme. AICAR as well as all the toxins tested (okadaic acid, microcystin-LR, calyculin A, cantharidin and tautomycin) induced strong, dose-dependent AMPK phosphorylation, correlating with AMPK activity *in situ* (in intact hepatocytes) as measured by the AMPK-dependent phosphorylation of acetyl-CoA carboxylase (ACC) at Ser79. All treatments induced the appearance of multiple, phosphatase-sensitive, low-mobility forms of the AMPKα-subunit, consistent with phosphorylation at several sites besides Thr172. The flavonoid naringin, an effective antagonist of okadaic acid-induced autophagy suppression (P.B. Gordon, I. Holen & P.O. Seglen [1995], *J. Biol. Chem.* 270, 5830-5838), inhibited the AMPK phosphorylation and mobility shifting induced by AICAR, okadaic acid or microcystin, but not the changes induced by calyculin A or cantharidin. Furthermore, naringin antagonized the autophagy-suppressive effects of AICAR and okadaic acid, but not the autophagy suppression caused by cantharidin. These results suggest that AMPK mediates the autophagy-suppressive effects of toxins as well of AICAR. The activation of AMPK by Thr172 phosphorylation can apparently be influenced by both a naringin-sensitive and a naringin-resistant mechanism, probably involving protein phosphatases PP2A and PP1, respectively.

INTRODUCTION

Autophagy is a process used by eukaryotic cells to degrade their own cytoplasm under conditions of nitrogen starvation or stress, in order to obtain amino acids and other small molecules needed for the maintenance of essential cell functions. Autophagy is executed by specialized intracellular membrane cisternae called *phagophores*, which excise and sequester pieces of cytoplasm, forming closed vacuoles called *autophagosomes*. The autophagosomes may fuse with endosomes to form intermediary vacuoles called *amphisomes*, and eventually the vacuolar contents of sequestered cytoplasm is delivered to *lysosomes* for degradation [1].

Studies of autophagy (Atg) mutations [2] in yeast have indicated that a functional phagophore requires the protein Atg8 (LC3-II in mammalian cells), covalently bound to phosphatidylethanolamine (PE) in the phagophore membrane by the help of a ubiquitylation-like conjugation system [3]. An Atg12-Atg5 conjugate, formed by a second conjugation system [4], seems to be associated with the phagophore during the autophagic sequestration process, but upon vacuole closure (autophagosome formation) Atg12-Atg5 detaches, while Atg8 remains [5]. Other proteins, mostly variants of cyto-solic enzymes, have been found to be selectively associated with autophagosomal membranes in rat liver cells, but it is not clear whether these have an autophagic function or just represent degradation intermediates [6].

In addition to the conjugation reactions, phagophore formation is dependent on a lipid kinase complex that includes the PI 3-kinase Atg6 (beclin-1 in mammalian cells) and a "switching complex" that includes the protein kinase Atg1 [7]. Atg1, which appears to regulate yeast autophagy independently of its protein kinase activity [8], has no mammalian homologue, but several other kinases have been suggested to be involved in the regulation of autophagy both in yeast and mammalian cells [9]. For example, the AMP-activated protein kinase (AMPK), a Ser/Thr-kinase, has been implicated in the negative control of hepatocellular autophagy [10]; surprisingly, its yeast homologue, Snf1, has the opposite effect [11]. Pho85, a cyclin-activated Ser/Thr-kinase, antagonizes Snf1 and suppresses autophagy in yeast [11]. Furthermore, the rapamycin-sensitive protein/lipid kinase, TOR, inhibits autophagy both in yeast and mammalian cells [12;13], and may partly mediate the autophagy-suppressive effect of amino acids [14-16]. In yeast, TOR has been shown to promote the phosphorylation and inactivation of Atg13, an Atg1-stimulatory protein factor [17;18]. Other protein kinases that have been implicated in the regulation of autophagic activity include the cAMP-dependent protein kinase (PKA) [19], the mitogen-activated protein kinase (MAPK) [20], stress-activated protein kinases [21;22], protein kinase B (PKB/Akt) [23], p70 S6 kinase [22;24;25], Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) [26;27] and protein-tyrosine kinases [28]. eIF2α kinases appear to be required for the long-term maintenance of autophagic capacity in both yeast and mammalian cells, probably through phosphorylation of a transcriptional activator (Gcn4) of autophagy genes [29].

In rat hepatocytes, overphosphorylation induced by various protein phosphatase-inhibitory toxins has been shown to inhibit autophagic activity completely [30;31]. Dose-response considerations indicated the involvement of a type 2A protein phosphatase (PP2A), whereas the autophagy-inhibitory protein kinase(s) have remained elusive. Although the auto-

[1] **H.R. Samari and M.T.N. Møller contributed equally to the present study**. [2]To whom correspondence should be addressed: Proteomics Section, Department of Cell Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Tel.: 47-22-93-59-47; Fax: 47-22-93-45-80; E-mail: per.seglen@labmed.uio.no. | ABBREVIATIONS ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; CA, calyculin A; CaMK-II, Ca^{2+}/calmodulin-dependent protein kinase II; CT, cantharidin; CTR, control; eIF2α, eukaryotic initiation factor 2α; GAIP, Gα interacting protein; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MC, microcystin-LR; mTOR, mammalian target of rapamycin; NA, naringin; OA, okadaic acid; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PP, protein phosphatase; SDS, sodium dodecyl sulfate. | KEYWORDS acetyl-CoA carboxylase, AICAR, AMPK, calyculin, cantharidin, microcystin, naringin, tautomycin.

phagy-suppressive effect of okadaic acid, an algal toxin, is effectively antagonized by CaMK-II inhibitors [30;31], the flavonoid naringin, which does not inhibit CaMK-II [22], is an equally effective antagonist, indicating an enzyme other than CaMK-II as the mediator of the toxin effect. In a search for this naringin-sensitive protein kinase, we examined the effects of various toxins and of naringin on AMPK [10]. The results, parts of which have been reported in a preliminary form [22], show that AMPK can be phosphorylation-activated by naringin-sensitive as well as by naringin-resistant mechanisms, depending on the toxin administered. This differential naringin sensitivity is also observed at the level of autophagic sequestration. The suppression of hepatocellular autophagy during toxic stress may thus be mediated by complex signalling events upstream of AMPK.

EXPERIMENTAL PROCEDURES

Reagents

Okadaic acid and microcystin-LR were from Alexis Biochemicals (Läufelfingen, Switzerland); calyculin A, cantharidin and tautomycin from Calbiochem (San Diego, CA, USA), and 5-aminoimidazole-4-carboxamide riboside (AICAR) from Toronto Research Chemicals (North York, On, Canada). Polyclonal rabbit antibodies against the AMPK α subunit, Thr172-phosphorylated AMPKα, and Ser79-phosphorylated acetyl-CoA carboxylase, as well as horseradish peroxidase-linked anti-rabbit IgG antibody, were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Rainbow molecular weight markers (RPN 756) and the ECL western blotting detection kit were from Amersham Biosciences (Little Chalfont, Bucks., UK). Sodium dodecyl sulfate (SDS), acrylamide and bisacrylamide were obtained from BioRad (Hercules, CA, USA). Dry milk powder was from Nestlé (Vevey, Switzerland), and nitrocellulose membranes from Osmonics (Westborough, MA, USA). Methanol and acetic acid were from Merck (Whitehouse Station, NJ, USA), metrizamide from Nycomed Pharma AS (Oslo, Norway), and alkaline phosphatase (MB grade) from Roche Appl. Sci., Penzberg, Germany. Other biochemicals were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Animals and cells

Hepatocytes were isolated from 18-h starved male Wistar rats (250-300 g; Harlan UK Ltd., Shaws Farm, Oxon, UK) by two-step collagenase perfusion [32], purified by differential centrifugation, and resuspended in suspension buffer fortified with 2 mM Mg^{2+} and 15 mM pyruvate [32]. For immunoblotting studies, 0.4-ml aliquots of cell suspension, each containing about 30 mg cells (wet mass), were incubated for 60 min at 37°C in shaking centrifuge tubes. In autophagy experiments, 2-ml aliquots of cell suspension, containing about 20 mg cells, were incubated for 120-140 min at 37°C in 5-cm Nunclon petri dishes precoated with albumin to prevent attatchment of the cells to the substratum [33].

Gel electrophoresis and immunoblotting

Incubations were stopped by adding 2 ml of ice-cold Tris-buffered saline (TBS; 20 mM Tris-base, 0.1 % Tween-20, pH 7.6) to each tube, followed by centrifugation of the cells at 1600 rev/min for 4 min in the cold (4°C); this washing was repeated once. The cells were lysed for 30 min on ice in one ml of lysis buffer containing 0.4% SDS, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, and 20 mM Tris-base, pH 7.2. The resulting whole-cell extracts were diluted 1:2 in double-strength SDS gel-loading buffer (single strength, 2% SDS, 1 M mercaptoethanol, 0.1% bromophenol blue, 10% glycerol, 50 mM Tris-HCl, pH 6.8) and boiled for 5 min at 95°C. After measuring the protein contents of the extracts by the method of Bradford [34], using the BCA protein assay kit from Pierce (Rockford, USA), samples containing 20 µg were separated by SDS gel electrophoresis for approximately 40 min at 200 V in 10% polyacrylamide gels containing 0.1 % SDS. Molecular weight markers were included in all gels.

The separated proteins were transferred to nitrocellulose blotting membranes using a semi-dry transfer unit (Bio-Rad Laboratories, Hercules, CA, USA) with Towbin's blotting buffer (192 mM glycine, 20% methanol, 25 mM Tris-base, pH 8.3). The membranes were blocked by overnight incubation at 4°C with 5% dry milk in TBS containing 0.2% Tween-20 (TBS-T), and washed three times for 10 min in TBS-T. For detection of total or phosphorylated AMPKα, the membranes were first incubated overnight at 4°C with the respective antibodies (diluted 1:1000 in TBS-T). After washing three times with TBS-T the membranes were incubated for one hour at room temperature with anti-rabbit-horseradish peroxidase (diluted 1:2000 in TBS-T), washed three times and visualized by chemiluminescence using the ECL Western Detection Kit (Amersham Biosciences).

Measurement of autophagy

Autophagy was measured as the net sequestration of an endogenous cytosolic enzyme, lactate dehydrogenase (LDH), into sedimentable autophagic vacuoles (mostly autolysosomes) during 2h of incubation at 37°C, with the proteinase inhibitor leupeptin (0.3 mM) added to prevent intralysosomal LDH degradation [35]. After incubation, the cells were sedimented at 1600 rev/min for 4 min in the cold (4°C), resuspended in 4 ml ice-cold unbuffered (electrolyte-free) 10 % sucrose, and centrifuged again. The resuspension and centrifugation was repeated once more, and finally the cell pellet was resuspended in 0.5 ml 10% sucrose, briefly warmed to 37°C and electrodisrupted by a single high-voltage (2 kV/cm) pulse. The disrupted cells were centrifuged through a metrizamide/sucrose cushion, and the amount of LDH in the resulting cytosol-free sediment was measured and expressed as per cent (autophagically sequestered per hour) of the total cellular LDH in the sample [35].

RESULTS

Stimulation of AMPK phosphorylation by AICAR but not by amino acids

Autophagic activity in isolated rat hepatocytes has been shown to be strongly suppressed by adenosine, AICAR and various adenosine analogues [10;36]. The autophagy suppression can be eliminated by the adenosine kinase inhibitor, 5-iodotubercidin, suggesting mediation by AMP and AMP analogues and a possible involvement of the AMP-activated protein kinase, AMPK [10;36]. Hepatocytic autophagy can also be inhibited by amino acid mixtures [14;16] as well as by okadaic acid and other algal toxins [30], but the mechanisms of action of these agents are not known.

AMPK is activated allosterically by AMP, but in addition its activity is absolutely dependent on phosphorylation at Thr172 by an upstream protein kinase [37], recently identified as LKB1 [38;39]. The availability of a commercial, phospho-specific antibody that detects phosphorylation of AMPK at Thr172 has allowed a closer examination of the effects of autophagy suppressants on AMPK activation. As shown in Fig. 1A, the treatment of freshly isolated rat hepatocytes with AICAR induced a dose-dependent phosphorylation of AMPK at Thr172, indicating activation of the enzyme. Presumably, the direct binding of AMP to AMPK alters its susceptibility towards phosphorylation by LKB1, although it has also been suggested that the AMPK-phosphorylating enzyme itself

could be allosterically activated by AMP [37]. Some basic AMPK phosphorylation could usually be detected, but it was very variable, probably reflecting various degrees of hypoxia sustained during hepatocyte preparation. This variable background probably accounted for the variability in the AICAR concentrations needed to obtain a detectable effect, ranging from 0.1 to 1 mM.

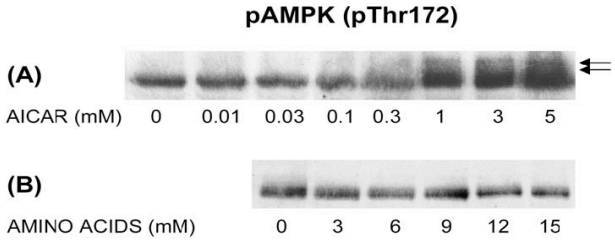

Figure 1 **Stimulation of AMPK phosphorylation by AICAR**. Freshly isolated rat hepatocytes were incubated for 1 h at 37°C with (A) AICAR, or (B) a physiological amino acid mixture, at the concentrations indicated. Cell extracts were immunoblotted with an antibody against the Thr172-phosphorylated AMPK α subunit.

In addition to increasing the pThr172 immunoreactivity of the single AMPK band seen in unstimulated cells, AICAR treatment induced immunoreactivity at two adjacent bands of lower mobility (Fig. 1A, arrows). Since Thr172 phosphorylation was detectable in all three bands, additional structural or conformational changes associated with AMPK activation must be responsible for the mobility shift. Phosphorylation at additional sites is one likely possibility, particularly since both Thr258 and Ser485 in the AMPK α subunit have been shown to be subject to phosphorylation, apparently by the same enzyme [40]. Presumably, binding of the AICAR metabolite, ZMP, increases the susceptibility of AMPK towards phosphorylation at several sites.

Unlike AICAR, a physiological mixture of amino acids had no effect on AMPK phosphorylation (Fig. 1B). The mixture has previously been shown to inhibit autophagy maximally at about 12 mM, the "single-strength" (1x) concentration corresponding to the amino acid levels in portal blood after a protein-rich meal [16]. The autophagy-suppressive effect of amino acids would thus not appear to be mediated by AMPK.

Activation of AMPK by okadaic acid, microcystin and other toxins

To see if other autophagy suppressants might stimulate AMPK phosphorylation, the effects of various protein phosphatase-inhibitory toxins were examined. Okadaic acid and other algal toxins have previously been shown to suppress hepatocytic autophagy virtually completely, with dose characteristics suggesting the involvement of a type 2A protein phosphatase (PP2A) [30]. As shown in Fig. 2, the toxins okadaic acid, microcystin-LR, calyculin A, cantharidin and tautomycin all stimulated AMPK phosphorylation in a dose-dependent manner. Okadaic acid and calyculin A were the most potent stimulants, with detectable effects at 30 nM and maximal effects at 100-300 nM. The effect of microcystin was detectable at 100 nM, whereas tautomycin and cantharidin required micromolar concentrations to produce detectable effects. The toxins induced a more pronounced mobility shift than did AICAR; at the highest toxin concentrations the AMPK form of lowest mobility was predominant.

To check that phosphorylation of AMPK at Thr172 really correlated with AMPK activity, the phosphorylation of cellular acetyl-CoA carboxylase (ACC) in toxin-treated hepatocytes was examined. ACC is specifically phosphorylated by AMPK at Ser79 [41], making this phosphosite a convenient indicator for the activity of AMPK in intact cells. As shown in Fig. 3A, ACC phosphorylation was strongly stimulated by all the toxins tested (okadaic acid, microcystin, calyculin A and cantharidin), confirming the assumption that AMPK phosphorylation at Thr172 is accompanied by AMPK activation. ACC phosphorylation was also clearly stimulated by the established AMPK activator, AICAR (Fig. 3B). The ability of the toxins to stimulate AMPK phosphorylation and AMPK activity would be compatible with a role for AMPK in mediating their autophagy-suppressive effects.

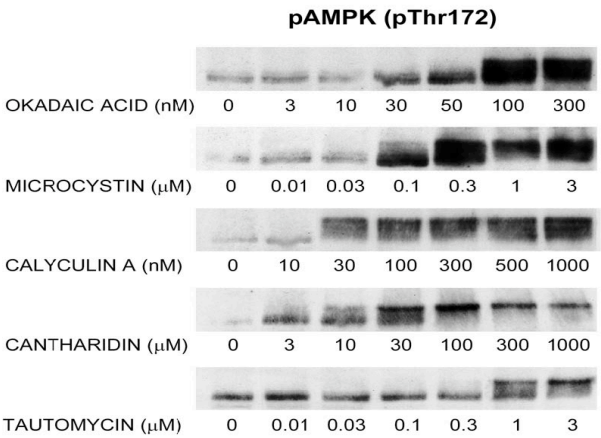

Figure 2 **Stimulation of AMPK phosphorylation by various toxins**. Hepatocytes were incubated for 1 h at 37°C with okadaic acid, microcystin-LR, calyculin A, cantharidin or tautomycin at the concentrations indicated. Cell extracts were immunoblotted with an antibody against Thr172-phosphorylated AMPKα.

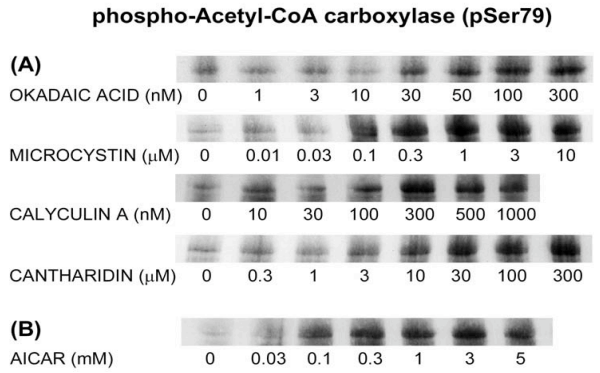

Figure 3 **Toxin- and AICAR-induced phosphorylation of acetyl-CoA carboxylase (ACC), an AMPK substrate.** Hepatocytes were incubated for 1 h at 37°C with (A), okadaic acid, microcystin-LR, calyculin A or cantharidin, or (B), AICAR, at the concentrations indicated. Cell extracts were immunoblotted with an antibody against Ser79-phosphorylated ACC.

Differential naringin sensitivity of toxin-induced AMPK phosphorylation

The autophagy-suppressive effect of okadaic acid has been shown to be effectively antagonized by naringin, a grapefruit flavonoid believed to inhibit a protein kinase involved in the negative regulation of autophagy [42]. Several other okadaic acid effects on rat hepatocytes are also naringin-sensitive, such as the inhibition of endocytosis [42], the disruption of keratin and plectin cytoskeletal networks [42-44], and the induction of apoptotic cell death [43].

As shown in Fig. 4A, naringin strongly antagonized the effect of okadaic acid on AMPK (Thr172) phosphorylation, largely preventing the formation of phosphorylated low-mobility forms. Similarly, the AMPK phosphorylation induced by microcystin-LR was virtually abolished by naringin. In contrast, naringin did not antagonize AMPK phosphorylation induced by calyculin A or cantharidin. These results parallel our previous observations on hepatocytic plectin

phosphorylation, which suggested the involvement of two phosphorylation mechanisms: one naringin-sensitive mechanism probably involving inhibition of PP2A by microcystin or low concentrations of okadaic acid, and one naringin-resistant mechanism probably involving PP1 inhibition by calyculin A, cantharidin or tautomycin [22]. The hypothesis is based in large measure on the known relative specificities of the toxins towards PP1 and PP2A in intact cells and cell-free systems.

In accordance with its effects on AMPK phosphorylation, naringin antagonized the stimulatory effects of okadaic acid and microcystin on phosphorylation of the AMPK substrate, ACC, whereas the stimulatory effects of calyculin A and cantharidin were unaffected (results not shown). Both the naringin-sensitive and the naringin-resistant toxin mechanism thus clearly regulate AMPK activity.

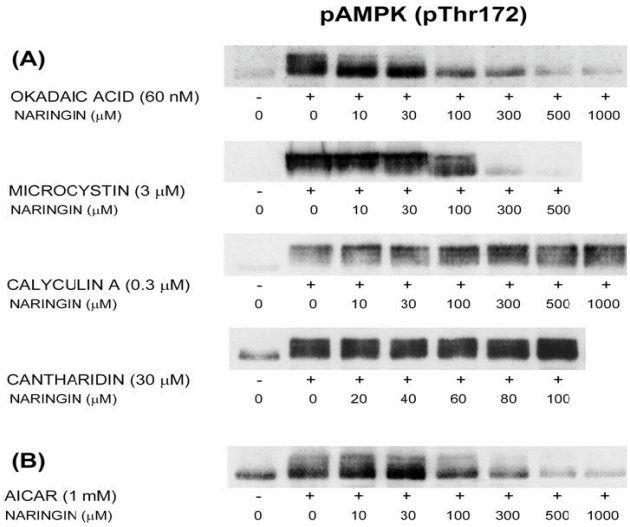

Figure 4 **Differential naringin sensitivity of AMPK phosphorylation induced by algal toxins or AICAR.** Hepatocytes were incubated for 1 h at 37°C with (A), okadaic acid (60 nM), microcystin-LR (3 μM), calyculin A (0.3 μM) or cantharidin (30 μM), or (B), AICAR (1 mM), at various concentrations of naringin. Cell extracts were immunoblotted with an antibody against Thr172-phosphorylated AMPK.

AICAR-induced AMPK phosphorylation is also naringin-sensitive

Interestingly, the AMPK phosphorylation induced by AICAR was also strongly antagonized by naringin (Fig. 4, bottom). This effect of naringin could reflect a direct binding to AMPK, e.g., in competition with AMP, resulting in reduced susceptibility of the enzyme towards phosphorylation. Alternatively, naringin could inhibit LKB1 or some other upstream kinase involved in AMPK phosphorylation, or it could otherwise interfere with signalling through LKB1. Flavonoids are structurally similar to adenine nucleotides, and inhibit a variety of protein kinases by competing with ATP for binding to the catalytic site [45-47]. The ability of naringin to suppress the direct AMPK activation by AICAR would seem to suggest that the naringin-sensitive, putatively PP2A-mediated, toxin mechanism disussed above is more proximal to AMPK than the naringin-resistant, putatively PP1-mediated mechanism.

Low-mobility forms of AMPK are not detectable prior to toxin treatment

The low-mobility AMPK forms detected by the anti-pAMPK(pThr172) antibody in toxin- or AICAR-treated cells could be the result of a treatment-induced mobility shift, or they could represent pre-existing molecular forms that become detectable as the result of treatment-induced Thr172 phosphorylation. To distinguish between these two possibilities, immunoblotting was performed with a general anti-AMPK antibody capable of detecting unphosphorylated as well as phosphorylated AMPK α subunits. As shown in Fig. 5A, a mobility shift was detected with this general antibody (upper lane) just as well as with the phosphospecific antibody (lower lane) following treatment with either microcystin or calyculin A. Similar shifts (not shown) were observed with okadaic acid, cantharidin or AICAR. The mobility shifts induced by microcystin (Fig. 5A), okadaic acid or AICAR were naringin-sensitive, whereas the shifts induced by calyculin A (Fig. 5A) or cantharidin were not. The results obtained with the general anti-AMPK antibody thus mimic those obtained with the phosphospecific antibody, showing that the low-mobility forms of AMPK do not pre-exist in detectable amounts in control cells, but are formed as a result of the treatment with AICAR or phosphorylation-inducing toxins.

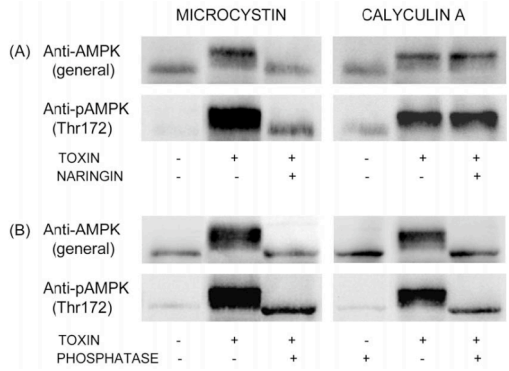

Figure 5 **Toxin-induced, naringin-sensitive AMPK mobility shifts as detected by general or phosphospecific anti-AMPK antibodies.** (A) Hepatocytes were incubated for 1 h at 37°C with microcystin-LR (3 μM) or calyculin A (0.5 μM) in the absence or presence of naringin (100 μM). Cell extracts were immunoblotted with a general antibody against the AMPK α subunit (upper lane) or with a phosphospecific antibody against Thr172-phosphorylated AMPK (lower lane). (B) Extracts (containing ~6 mg protein/ml) from hepatocytes incubated with or without microcystin or calyculin as in (A) were treated for 3h at 37°C with alkaline phosphatase (150 units/ml) as indicated.

To check whether the toxin-induced low-mobility AMPK forms might represent phosphorylated enzyme species, the cell extracts were pretreated with alkaline phosphatase prior to gel electrophoresis. The phosphatase treatment completely eliminated the low-mobility bands induced by microcystin or calyculin A (Fig. 5B), indicating that they indeed represented AMPK α subunits modified by toxin-induced phosphorylation at sites additional to Thr-172. Some immunoreactivity remained in the high-mobility band after phosphatase treatment of extracts from toxin-treated cells, perhaps indicating that the pThr172 site was more resistant to dephosphorylation than the other sites.

Autophagy-suppressive toxin effects are differentially naringin-sensitive

To see if the differential naringin sensitivity of the AMPK-activating toxins might be reflected at the level of autophagy, the autophagic activity of hepatocytes treated with either okadaic acid (naringin-sensitive) or cantharidin (naringin-resistant) was examined. As shown in Fig. 6A, both okadaic acid and cantharidin suppressed autophagy effectively, okadaic acid being approximately two orders of magnitude more potent than cantharidin. The autophagy-inhibitory effect of okadaic acid was effectively antagonized by naringin, but

the inhibition by cantharidin was not (Fig. 6B). Although naringin itself, for reasons not known, reduced the autophagic activity rather more than in previous experiments [42], there was clearly very little additional effect of okadaic acid at the highest concentrations of the flavonoid, whereas cantharidin was strongly autophagy-suppressive at all naringin concentrations. Thus, like AMPK phosphorylation, autophagy can apparently be modulated by either naringin-sensitive or naringin-resistant mechanisms, depending on the nature of the toxin applied. This observation is consistent with an involvement of AMPK in the down-regulation of hepatocellular autophagy under toxic stress.

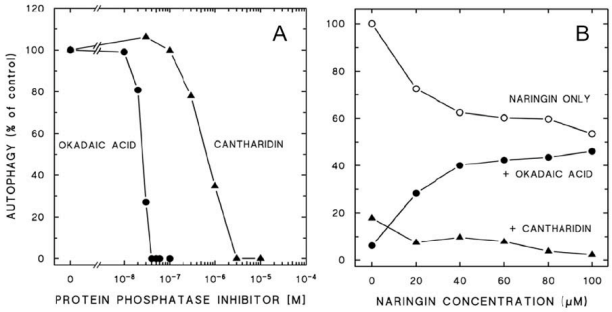

Figure 6 **Differential naringin sensitivity of autophagy-suppressive toxins**. (A) Hepatocytes were incubated for 2 h at 37°C with okadaic acid (●) or cantharidin (▲) at the concentration indicated. Autophagic activity during this period was measured and expressed as per cent of the control rate without toxins (3.3 %/h). (B) Hepatocytes were incubated for 140 min at 37°C with naringin alone at the concentration indicated (○), or together with 40 nM okadaic acid (●) or 3 μM cantharidin (▲), added 20 min after naringin. Autophagic activity during the last 2 h of incubation was measured and expressed as per cent of the control rate without additions (2.9 %/h). Each experiment was performed at least twice.

The autophagy-suppressive effect of AICAR is naringin-sensitive

Since AMPK appears to be a mediator of naringin-sensitive as well as of naringin-resistant toxic stress, it would be of interest to examine to what extent the autophagy-suppressive effect of AICAR might be naringin-sensitive. As shown in Table I, naringin eliminated the AICAR-induced suppression of autophagy as effectively as it eliminated the autophagy-suppressive effect of okadaic acid. The effects of naringin on autophagy thus parellel its effects on AMPK phosphorylation (Fig. 3), supporting the notion of AMPK as the mediator of AICAR-induced autophagy suppression [10].

Table 1 **Antagonistic effect of naringin on autophagy suppression induced by AICAR or okadaic acid**
Hepatocytes were incubated for 140 min at 37°C with or without naringin alone (at 100 μM), or together with okadaic acid (30 nM) or AICAR (100 μM), added 20 min after naringin. Autophagic activity during the last 2 h of incubation was measured and expressed as per cent per hour.

	Control	AICAR (100 μM)	Okadaic acid (30 nM)
None	2.73 ± 0.13 (4)	0.69 ± 0.20 (4)	0.63 ± 0.14 (3)
Naringin (100 μM)	1.63 ± 0.03 (4)	1.65 ± 0.03 (2)	1.62 ± 0.18 (3)

DISCUSSION

The present results show that AMPK is a target for several autophagy-suppressive toxins, and suggest that the toxins may use two different AMPK-activating mechanisms: one naringin-sensitive and one naringin-resistant. Such differential naringin sensitivity was also observed in our previous study of toxin-induced plectin phosphorylation [22], where it was pointed out that naringin sensitivity correlates quite well with the ability of the toxins to inhibit protein phosphatase 2A selectively over protein phosphatase 1. Thus, in intact cells, the naringin-antagonized toxins, okadaic acid and microcystin, seem to function as selective PP2A inhibitors, whereas the naringin-resistant toxins, calyculin A, cantharidin and tautomycin, act as selctive inhibitors of PP1 [22]. It should be noted, however, that the specificities are overlapping: given sufficiently high concentrations, all of the toxins will inhibit both PP1 and PP2A [48].

Although AMPKα can be dephosphorylated at pThr172 by mixtures of PP1 and PP2A in cell extracts [40], the dephosphorylation seems to be performed exclusively by PP2C in intact cells [49]. The effects of toxins on AMPK are, therefore, likely to be indirect, which leaves it open whether they, in fact, stimulate phosphorylation or inhibit dephosphorylation at Thr172. The ability of all the toxins to induce phosphorylation at several AMPKα sites (detectable as the appearance of low-mobility bands) indicates the possibility of indirect (permissive) effects at the level of AMPK. It has previously been demonstrated that AMPKα1 can be phosphorylated at Thr258 and Ser485 (Ser491 in α2), apparently by the same AMP-insensitive enzyme, but these sites do not appear to contribute to AMPK activation: Ser485 is in fact constitutively phosphorylated [40]. The toxin- and AICAR-induced low-mobility AMPK forms are, therefore, likely to reflect phosphorylation at sites other than Thr258 and Ser485, sites which may be permissive for the activating phosphorylation at Thr172.

AMPK is a heterotrimeric enzyme composed of a catalytic α subunit, a regulatory γ subunit and a scaffolding β subunit, all of which can be represented by at least two isoforms [50]. The γ subunit can bind two molecules of ATP, which seem to contact and inhibit the α subunit, or two molecules of AMP, which relieve the inhibition and support α subunit activity [51-53]. The γ subunit is apparently not subject to phosphorylation [40], but the ability of AICAR to stimulate Thr172 phosphorylation of the α subunit, demonstrated in the present study as well as in previous reports [22;54], indicates that relief from inhibition by ATP and the γ subunit makes the α subunit more susceptible to phosphorylation. The β subunit, on the other hand, can, like the α subunit, be phosphorylated at several sites, one of which (Ser108 in the β1 subunit) has been shown to be essential for AMPK activity [40]. It is thus conceivable that autophagy-suppressive toxins may relieve the inhibition by ATP and the γ subunit, independently of AMP, by inducing phosphorylation of either the α subunit or the β subunit. In yeast, PP2A knockouts (regulatory subunit deletions) were in fact shown to modify the interaction between the α and γ subunits of AMPK in the same way as did glucose depletion, the yeast equivalent of AMP elevation [55]. The ability of the PP2A A subunit (Tpd3 in yeast, PR65α in mammals) to anchor to a regulatory domain in the AMPK α subunit [55] would place the phosphatase in a suitable position to dephosphorylate any of the AMPK subunits. Neither pThr172, pThr258 nor pSer485/491 appeared to be substrates for AMPK-bound PP2A [55], suggesting other phosphosites to be more likely targets for primary toxin effects, as discussed above.

The AMPK kinase responsible for phosphorylation at Thr172 was recently identified as LKB1, a tumour-suppressive protein mutated in a cancer-predisposing disease, Peutz-Jeghers syndrome [38;39;56]. Although cellular LKB1 is a phosphoprotein that can be phosphorylated by a number of protein kinases [57], unphosphorylated, purified (recombinant) LKB1 was able to phosphorylate AMPKα effectively

under cell-free conditions [39], consistent with previous observations on AMPK kinase [58]. The toxins would, therefore, seem unlikely to induce AMPK activation through effects on LKB1 phosphorylation or dephosphorylation, unless additional regulatory factors operate in intact cells to render such phosphorylations relevant. Cellular LKB1 is dependent on the cooperating pseudokinase STRAD and on the scaffold protein MO25 for full activity [38;39;56], and it would be interesting to examine what effects the autophagy-suppressive toxins might have on the phosphorylation states of these three proteins.

The yeast homologue of AMPKα, Snf1, can be phosphorylated and activated by three LKB1-like protein kinases, Elm1, Pak1 and Tos3 [38;39;56]. Like mammalian AMPKα, the activity of Snf1 is regulated by interaction with an AMPK γ subunit homologue, Snf4, and requires phosphorylation at a site (Thr210) homologous to mammalian Thr172 [51;55]. This site is dephosphorylated by a yeast PP1, whereas a yeast PP2A regulates the Snf1-Snf4 interaction as discussed above [55;59]. Thus, like the hepatocellular AMPK regulation suggested by the present study, yeast cells seem to use both a PP1-dependent and a PP2A-dependent mechanism for the regulation of AMPK/Snf1 activity. The roles of the protein phosphatases in mammalian cells are less clear than in yeast, since pThr172 is thought to be dephosphorylated by PP2C rather than by PP1 [49;60]. However, since PP2Cα is a phosphoprotein subject to toxin-sensitive dephosphorylation [61], there is a possibility that a mammalian PP1 or PP2A may control Thr172 dephosphorylation indirectly through PP2C.

The measurements of toxin-induced AMPK phosphorylation (Fig. 2) suggest that toxins differ in their dose-response characteristics, best seen when comparing the most PP2A-selective toxin, okadaic acid, and the most PP1-selective toxin (in intact cells), calyculin A [62;63]. Okadaic acid induces Thr172 phosphorylation at low doses and phosphorylation of other sites (low-mobility bands) only at higher doses (where PP1 would be inhibited), whereas with calyculin A those other sites already seem to be phosphorylated when Thr172 phosphorylation becomes detectable. The characteristics of okadaic acid are shared by the other naringin-sensitive AMPK stimulants, microcystin and AICAR, suggesting that they exert their effects on Thr172 phosphorylation through related mechanisms. The ability of naringin to suppress the AICAR-induced phosphorylation may suggest that the flavonoid either competes directly with AMP/AICAR, or otherwise interferes with the AMP-sensitive interaction between the α and γ subunits of AMPK. This interaction could be influenced by phosphosites, e.g., on the β subunit that serves as a common scaffold for the α and γ subunits, subject to dephosphorylation by PP2A and perhaps to phosphorylation by a naringin-sensitive protein kinase. PP1, on the other hand, would seem to dephosphorylate sites on the α subunit that, when phosphorylated, facilitate or stabilize Thr172 phosphorylation.

The existence of both a PP2A-associated, naringin-sensitive mechanism and a PP1-associated, naringin-resistant mechanism with permissive effects on Thr172 phosphorylation implicates the involvement of several protein kinases, besides LKB1, in the regulation of AMPK activity. One candidate enzyme would be the Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II), which has been suggested as a mediator of both apoptotic and autophagy-suppressive toxin effects in hepatocytes [31;64]. This enzyme is not naringin-sensitive [22], but could be involved in the naringin-resistant, PP1-associated phosphorylation of permissive phosphosites on the α subunit. Another candidate, the cAMP-dependent protein kinase A (PKA), has been implicated in a partially naringin-sensitive suppression of hepatocytic autophagy [19], and could participate in the naringin-sensitive, PP2A-associated mechanism of AMPK regulation. As for the mechanism of action of naringin, further studies will be needed to establish whether the primary target of the flavonoid is AMPK itself or one of the AMPK kinases involved in phosphorylation of its subunits.

The good correlation between the effects of toxins, AICAR and naringin on AMPK activation and autophagy suppression is compatible with a role of this kinase in the regulation of hepatocellular autophagy under metabolic and toxic stress. Surprisingly, in yeast cells AMPK/Snf1 stimulates autophagy rather than suppressing it [11], perhaps reflecting a difference between unicellular and multicellular organisms in their choice of survival strategy when facing metabolic stress. Whereas yeast cells utilize all available sources of energy, including autophagic degradation of their own cytoplasm, mammalian cells may seek to conserve cell mass as well as saving energy by turning off processes with a short-term energy requirement, including autophagy [37]. It is, however, not known whether autophagy suppression is a general role of AMPK in mammalian cells, or a function restricted to hepatocytes

Autophagy-suppressive signaling mechanisms downstream of AMPK have not yet been clarified, but some putative elements are beginning to emerge. For example, both AICAR and the PP2A-associated toxins (okadaic acid; microcystin) induce naringin-sensitive phosphorylations of the stress-activated protein kinases SEK1 and JNK [22]. Another member of this kinase family, p38, has previously been implicated in the suppression of autophagy induced by osmotic (hypotonic) stress [21;65]. AICAR and the PP2A-associated toxins also induce naringin-sensitive phosphorylations in the regulatory tail region of S6 kinase [22], an enzyme that has been suggested to mediate amino acid-induced autophagy suppression through phosphorylation of the ribosomal protein S6 [66]. However, while stimulating the tail phosphorylation of S6 kinase (at Thr421/Ser424), AICAR paradoxically antagonizes the amino acid-induced activating phosphorylation of S6 kinase at Thr389, and blocks the ability of S6 kinase to phosphorylate S6 [22;54;67]. The autophagy suppression that accompanies AMPK activation is thus unlikely to be mediated by S6 kinase activity, but a role for tail-phosphorylated S6 kinase, independently of the catalytic function of the enzyme, cannot be excluded. It is noteworthy that amino acids, which suppress autophagy without activating AMPK (cf. the present study), are able to stimulate phosphorylation of the S6 kinase tail [25], suggesting the latter as a possible common element of several autophagy-suppressive pathways.

We previously observed that hepatocytic autophagy was strongly suppressed when the cells were depleted of ATP [68], and that the sequestration step as well as later steps in the autophagic pathway were inhibited [69]. Since AMP levels would be elevated under these conditions, the possibility should be considered that the suppression of autophagy induced by metabolic stress might reflect inhibition by AMPK rather than (or in addition to) a requirement for ATP. A role for AMPK as a general mediator of cellular stress, toxic as well as metabolic, is supported by the naringin-sensitive activation of stress-activated protein kinases like SEK1 and JNK by toxins and AICAR [22], consistent with a positioning of the latter in a stress signalling pathway downstream of AMPK. Suppression of autophagy has been shown to improve the survival of cultured hepatocytes during amino acid deprivation [70], and could well provide some survival advantage during other stressful conditions as well. However, it should be noted that prolonged activation of AMPK and its downsream effector, JNK, may elicit programmed cell death in liver cells [71], emphasizing the fundamental difference

between transient/ moderate and sustained/massive stress.

Okadaic acid and other toxins have been shown to induce naringin-sensitive phosphorylations of cytoskeletal proteins like keratin and plectin, and to cause naringin-sensitive disruption of the intracellular networks of plectin and keratin intermediate filaments [22;43;44]. These cytoskeletal alterations are probably mediated by AMPK and its downstream effectors, and could well play an instrumental role in the suppression of autophagy. The enclosure of cytoplasmic regions by the membranous phagophores [1] is likely to require some structural support, e.g., attachment to keratin intermediate filaments crosslinked by plectin. Whether the autophagy-suppressive signaling pathways terminate directly on the phagophores, or affect phagophores indirectly through effects e.g. on the cytoskeleton, will have to be examined by future experimental studies.

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Legend to first figure in the second column on page 108 (paper IV) should read:

FIG. 7. **Lack of wortmannin on S6K tail phosphorylation induced by amino acids, AICAR or toxins.** Hepatocytes were incubated for 1 h at 37°C with toxins, amino acids or AICAR in absence or presence of wortmannin at the concentrations indicated. Cell extracts were gel-separated and immunoblotted with an antibody against Thr 421/Ser424-phosphorylated S6K.

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