COMMENTARY

Protein kinase function and glutathionylation

Anthony N. ANSELMO and Melanie H. COBB[1]

Department of Pharmacology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9041, U.S.A.

Intracellular reactive oxygen species are generated as a by-product of normal metabolic processes and can both damage cellular constituents and function as important signalling species. This signalling often involves changes in the thiol redox balance. As an antioxidant, glutathione serves in maintaining the reduced state of cellular protein thiol groups. The paper by Cross and Templeton appearing in this issue of the *Biochemical Journal* describes a mechanism by which glutathionylation plays a key role in the regulation of the kinase activity of MEKK1 [MAP (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase; MAP3K] in response to oxidative stresses. This type of post-translational-modification glutathionylation may represent a general mechanism by which protein kinase function can be regulated.

Key words: glutathione, glutathionylation, kinase, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase kinase (MEKK1), oxidative stress, reactive oxygen species (ROS).

The regulation of intracellular levels of ROS (reactive oxygen species) such as H_2O_2, $^{\bullet}O_2^-$ (superoxide anions) and $^{\bullet}OH$ (hydroxyl radicals) is a matter of life and death. ROS are the unavoidable by-products of the metabolic processes essential for life. Yet at high levels they can cause the destruction of cellular membranes (through a cascade of lipid peroxidation) and can cause damage to DNA, which will have adverse consequences for the integrity of the cell. Without adequate cellular defence mechanisms, the result would be guaranteed cell death. However, ROS are successfully countered by cellular enzymes such as the SOD (superoxide dismutases; $2^{\bullet}O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) and catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$), among others, as well as by non-enzyme antioxidants such as ubiquinone and, especially, glutathione. Unfortunately, environmental UV and γ-irradiation, and chemical oxidants, have the potential to increase intracellular ROS and shift the redox balance in favour of oxidation. The resulting oxidative stress, if left unchecked, culminates in cell death (reviewed in [1]). ROS have been extensively studied in terms of their consequences for cell viability; however, the direct role of ROS in oxidative-stress-induced signal transduction has only begun to be addressed.

Glutathione, a tripeptide (L-γ-glutamyl-L-cysteinylglycine) in which a glutamic acid residue is linked through its γ-carboxy group to cysteinylglycine, is the most abundant non-protein thiol in the cell. The intracellular glutathione concentration is in the millimolar range in cells, with its concentration highest (at 10 mM) in the liver and lens. GSTs (glutathione S-transferases) catalyse condensation reactions between glutathione and electrophilic functional groups in endogeneous and xenobiotic hydrophobic chemical substrates (e.g. products of cytochrome P450, pesticides). The result is an increased water solubility of the glutathionylated products that allows for their efficient subsequent excretion (reviewed in [2]). Thus the primary function of glutathione here is detoxification. In a similar manner the glutathione peroxidases use glutathione to catalyse the reduction of hydroperoxides, including H_2O_2. As an antioxidant, glutathione also plays a role in maintaining the reduced state of cellular protein thiol groups. Thus, through its reducing potential in the cell, it confers protection against protein thiol oxidation. However, upon oxidative stress, the intracellular concentrations of both GSSG (oxidized glutathione) and protein–glutathione mixed disulphides increase. The spontaneous production of mixed disulphides is readily reversible when the redox balance of the cell is restored to its pre-oxidation state. Thus glutathionylation may represent a general mechanism by which protein kinase function may be regulated.

In this issue of the *Biochemical Journal*, Cross and Templeton [3] describe a mechanism by which glutathionylation plays a key role in the regulation of the kinase activity of MEKK1 [MAP (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase; MAP3K] in response to oxidative stresses such as H_2O_2 and the redox-cycling reactive quinone menadione (vitamin K3). MEKK1 is a protein kinase that has been studied for the last decade for its role in such varied cellular processes as proliferation, differentiation and programmed cell death. Through MS analysis, the authors demonstrate that treatment of cells with menadione induces glutathionylation of Cys^{1238} of the kinase domain of MEKK1. Although menadione has alkylating activity in addition to oxidant activity, its ability to inhibit MEKK1 activity through alkylation can be blocked *in vitro* by GSH (reduced glutathione) at concentrations typically observed in cells. Thus the authors demonstrate that it is indeed the oxidant potential of menadione that mediates glutathionylation and concomitant inhibition of MEKK1. The modification of this cysteine residue in response to oxidation takes on functional significance, because mutation of Cys^{1238} to valine abrogates the inhibitory effects of H_2O_2 and menadione on MEKK1 kinase activity. Interestingly, Cys^{1238} is located in the glycine-rich loop of subdomain I of the kinase domain, a region that is critical for optimal binding and co-ordination of ATP. As such, it is tempting to speculate that glutathionylation of Cys^{1238} might interfere sterically with the function of the glycine-rich loop. In any case, this is the first identification of this specific mechanism for the inhibition of kinase catalytic activity by oxidative stress.

A number of protein kinases have been reported previously to be regulated by cysteine glutathionylation within their catalytic domains in response to oxidation. The sites of modification, however, differ from that of MEKK1. Perhaps, the more interesting of these include PKC (protein kinase C) and cAPK (cAMP-dependent protein kinase)/PKA (protein kinase A). The kinase activity of PKC prepared from rat brain can be directly inhibited by the

[1] To whom correspondence should be sent (email melanie.cobb@utsouthwestern.edu).

thiol-specific oxidant diamide [1,1′-azobis-(*N*,*N*′-dimethylformamide)] *in vitro* in a manner that is potentiated in the presence of glutathione [4]. This inhibition of kinase activity could be reversed by the subsequent addition of a potent reducing agent such as DTT (dithiothreitol). Although Cross and Templeton [3] did not determine the site of glutathionylation, the PKCs do not contain a cysteine residue in the glycine-rich loop of subdomain I of its kinase domain that corresponds to the Cys^{1238} of MEKK1. Since these findings were made, it has been demonstrated that diamide can negatively regulate PKA activity through the glutathionylation of Cys^{199} in the activation loop of the catalytic C-subunit [5]. This site is protected from glutathionylation in the PKA tetramer (R_2C_2), only becoming accessible after activation.

Glutathionylation of cysteine anywhere within the interior of the catalytic site of a protein kinase may be a general regulatory mechanism. Cysteine modifications within the glycine-rich loop of subdomain I or within subdomain II could interfere with ATP binding. Cdk1/2 (cyclin-dependent kinases 1/2) are inhibited during the cell cycle by phosphorylation of T (threonine) and Y (tyrosine) in their glycine-rich GXGTYG motif and re-activated in part by dephosphorylation by Cdc25 phosphatases [6,7]. Thus the mechanism of inhibition of MEKK1 by glutathionylation bears structural similarity to the normal control of the cell-cycle kinases. As Cross and Templeton [3] have noted, a handful of kinases, including polo family kinases and the src-family tyrosine kinase Lck (lymphocyte kinase), exist with the corresponding Cys^{1238} of MEKK1. They, too, may be regulated through their glycine-rich loops. A survey of protein kinases reveals other similar modes of potential regulation. The MAPK JNK (c-Jun N-terminal kinase) contains a cysteine residue just one amino acid C-terminal to the relatively conserved valine residue (Val^{57} in PKA) in the glycine-rich loop. Consistent with the possibility of the regulation of JNK activity by glutathionylation, it has previously been demonstrated that GSTπ interacts with, and inhibits, JNK activity in response to H_2O_2 [8]. The kinase WNK ['with no lysine (K)'] contains a cysteine residue in place of a conserved ATP-binding lysine residue within subdomain II of its functional catalytic domain. While a lysine residue in subdomain I compensates for a lack of the conserved lysine residue in subdomain II, it is fair to speculate that modification of this cysteine residue might be inhibitory to catalytic activity.

Modification of cysteine residues in protein kinase activation loops might block kinase activation by preventing activation-loop phosphorylation or by changing protein–substrate interactions. Regulation of PKA by glutathionylation is likely to be the result of the latter of these possibilities. Interestingly, Cys^{199}, which lies in the activation loop of PKA, is conserved in kinases such as AKT1/Raca, S6K (S6 kinase) and even PKCα. In addition to the glycine-rich loop and the activation loop, the catalytic loop may also be a site for cysteine glutathionylation. Here, the result would be a block in the phospho-transfer reaction.

While Cross and Templeton [3] have undoubtedly uncovered a novel mode for redox regulation of a kinase, they also speculate on the biological significance of their discovery. MEKK1 was originally discovered as a consequence of its similarity to the protein Ste11p, a MAPK kinase kinase involved in the mating-pheromone response pathway in yeast. Since then, overexpression studies by various groups have implicated MEKK1 in the ERK1/2, JNK and p38 MAPK pathways. In addition, MEKK1 expression has been reported to activate nuclear factor (NF-κB) and to inhibit apoptosis (reviewed in [9]).

In reference to the role MEKK1 plays in oxidative stress pathways, MEKK1 expression in embryonic stem cell-derived cardiac myocytes (ESCM) inhibits apoptosis in response to H_2O_2 [10]. Further, oxidative stress-induced apoptosis was enhanced while JNK activation was attenuated in MEKK1$^{-/-}$ ECSM. This is all consistent with the hypothesis put forth by Cross and Templeton that oxidative-stress-induced apoptosis is transmitted, in part, through ASK (apoptosis signal-regulated kinase) activation coupled with MEKK1 inhibition. Thus MEKK1 may regulate the JNK pathway in response to a variety of extracellular stimuli [including LPA (lysophosphatidic acid) and growth factors] in a manner than can be surpassed by a shift in the redox balance of the cell in favour of oxidation, because glutathionylation of Cys^{1238} inhibits otherwise-active MEKK1. ASK, on the other hand, is capable of significantly contributing to JNK activation and apoptosis on release of its redox-sensitive inhibitor thioredoxin in response to oxidation [11]. In this way, the redox balance of the cell governs, to some extent, the specificity of function at the MAP3K level of MAPK pathways. Thus MAP3Ks, although having overlapping downstream signalling outputs, are not functionally redundant, as may have at first been speculated.

Indeed, the simple substitution in Nature of the generally conserved Val^{57} within the glycine-rich loop of the first subdomain of a kinase has functional consequences. Cysteine glutathionylation within the glycine-rich loop, the activation loop and catalytic loop, as well as other regions within the catalytic site of kinases, is likely to be an important general mechanism of kinase regulation. Future work in this area will almost surely entail the extrapolation of this general regulatory mechanism to other protein kinases. Structural studies should reveal important details as to these various glutathione-mediated modes of kinase inhibition. The regulation of protein function by post-translational modifications is a general theme in biology. Perhaps glutathione will be the next 'ubiquitin' to break out on the scientific scene.

REFERENCES

1 Jacobson, M. D. (1996) Reactive oxygen species and programmed cell death. Trends Biochem. Sci. **21**, 83–86

2 Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. and Turner, N. D. (2004) Glutathione metabolism and its implications for health. J. Nutr. **134**, 489–492

3 Cross, J. V. and Templeton, D. J. (2004) Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP-binding domain. Biochem. J. **381**, 675–683

4 Ward, N. E., Stewart, J. R., Ioannides, C. G. and O'Brian, C. A. (2000) Oxidant-induced S-glutathiolation inactivates protein kinase C-α (PKC-α): a potential mechanism of PKC isozyme regulation. Biochemistry **39**, 10319–10329

5 Humphries, K. M., Juliano, C. and Taylor, S. S. (2002) Regulation of cAMP-dependent protein kinase activity by glutathionylation. J. Biol. Chem. **277**, 43505–43511

6 Atherton-Fessler, S., Parker, L. L., Geahlen, R. L. and Piwnica-Worms, H. (1993) Reversible tyrosine phosphorylation and cell cycle control. Mol. Cell. Biol. **13**, 1675–1685

7 Gould, K. L. and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast $cdc2^+$ protein kinase regulates entry into mitosis. Nature (London) **342**, 39–45

8 Adler, V., Yin, Z., Fuchs, S. Y., Benezra, M., Rosario, L., Tew, K. D., Pincus, M. R., Sardana, M., Henderson, C. J., Wolf, C. R. et al. (1999) Regulation of JNK signaling by GSTp. EMBO J. **18**, 1321–1334

9 Schlesinger, T. K., Fanger, G. R., Yujiri, T. and Johnson, G. L. (1998) The TAO of MEKK. Front. Biosci. **3**, D1181–D1186

10 Minamino, T., Yujiri, T., Papst, P. J., Chan, E. D., Johnson, G. L. and Terada, N. (1999) MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes. Proc. Natl. Acad. Sci. U.S.A. **96**, 15127–15132

11 Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K. and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. **17**, 2596–2606

Received 25 May 2004/26 May 2004; accepted 26 May 2004
Published on the Internet 27 July 2004, DOI 10.1042/BJ20040873