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JB Review

Regulation of intracellular signalling through cysteine oxidation by reactive oxygen species

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Reactive oxygen species (ROS) have been regarded as harmful molecules that damage various molecules inside cells by oxidation and are responsible for ageing and various human diseases. However, recent studies have revealed an opposite aspect of ROS that these are actively generated in cells and mediate physiological intracellular signalling as second messengers. Several proteins have been shown to function as effectors for ROS, which are sensitively and reversibly oxidized by ROS. Such ROS-effector proteins commonly possess a highly reactive cysteine (Cys) residue, of which oxidation changes the protein function, thus enabling signal transmission to downstream targets. Among the ROS effectors, protein tyrosine phosphatase (PTP), thioredoxin (TRX) and peroxiredoxin (PRX) family proteins possess special domains/motifs to maintain the reactivity of Cys and utilize them to respond to ROS. Progressively advancing identification of ROS-effector proteins reveals the pleiotropic functions of ROS in physiological and pathological cell biology.

Keywords: intracellular signalling/peroxiredoxin/protein tyrosine phosphatase/reactive oxygen species/thioredoxin.

Abbreviations: Ask1, apoptosis signal-regulating kinase 1; ATM, ataxia-telangiectasia mutated; CRMP, collapsin response mediator protein; Cys, cysteine; Duox, Dual oxidase; Dvl, dishevelled; FAD, flavin adenine dinucleotide; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MKP, MAP kinase phosphatase; MST1, mammalian Ste20-like kinase 1; NADPH, nicotinamide adenine dinucleotide phosphate; Nox, NADPH oxidase; NRX, nucleoredoxin; O_2^-, superoxide; PDGF, platelet-derived growth factor; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PKM2, pyruvate kinase M2; PRL, phosphatase of regenerating liver; PRX, peroxiredoxin; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; PTEN, phosphatase and tensin homologue deleted from chromosome 10; ROS, reactive oxygen species; SFK, Src family tyrosine kinase; TNFα, tumour-necrosis factor α; TRX, thioredoxin.

Reactive oxygen species (ROS) have long been regarded as harmful molecules generated as by-products of respiration causing oxidative damages to various cellular components. The accumulation of such damages is thought to be responsible for ageing and multiple disorders, such as cancers, neurodegenerative diseases and diabetes mellitus (*1*). However, accumulating evidences from recent studies have shed light on a novel aspect of ROS; i.e. ROS mediate physiological intracellular signalling. There are multiple types of ROS-generating enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH), oxidase (Nox) and Dual oxidase (Duox), which are activated by physiological stimuli and produce ROS to cause appropriate cell responses (*2*). Contrary to the great advances in understanding the ROS-generation mechanism, the molecular signalling events downstream of ROS are poorly understood. This review article describes the importance of ROS-effector proteins, which are sensitively and reversibly oxidized by ROS, in ROS-mediated signalling.

ROS as Second Messengers

Stimulation of culture cells with very high level of hydrogen peroxide (H_2O_2) results in cell death. In contrast, it has been known that moderate amount of H_2O_2 can augment cell proliferation and that the level of intracellular H_2O_2 is consistently elevated in various cancer cell lines (*3*). Therefore, at appropriate level, ROS have been suggested to function as signalling molecules that positively affect cell proliferation. In 1990, Shibanuma *et al*. reported the importance of H_2O_2 in the proliferation response to platelet-derived growth factor (PDGF) in culture cells (*4*). They clearly demonstrated that the level of H_2O_2 increased in response to PDGF stimulation. Furthermore, the addition of H_2O_2 to the culture medium by itself stimulated phosphorylation of proteins, which was inhibited by catalase, an H_2O_2-degrading enzyme. A similar finding using endothelial cells was also reported by Sundaresan *et al*. (*5*). Another important discovery was reported in 1999 with the identification of the ROS-generating enzyme Mox1 (now known as Nox1) (*6*). Suh *et al*. isolated the novel cDNA encoding Mox1, which was similar to NADPH oxidase, the superoxide (O_2^--generating enzyme involved in killing of pathogenic bacteria by neutrophils. In a striking contrast, Mox1 was expressed predominantly in colon but rarely in leukocytes. Unexpectedly, suppression of Mox1 expression by antisense oligonucleotides resulted in the reduction not only of the ROS level, but also of the proliferation rate, thus suggesting the growth-promoting role of Mox1 and O_2^-. On the basis

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of these seminal findings, it became evident that ROS not only damage cells, but also play a second messenger role and regulate cell proliferation.

Target Proteins for ROS

ROS induce oxidation. Considering the importance of switchability between ON and OFF states in intracellular signalling, proteins that can be sensitively and reversibly oxidized by ROS are candidates for mediating the signalling function of ROS. Among the 20 amino acids that comprise proteins, cysteine (Cys) is of particular interest, because the thiol moiety (–SH) in the side chain of Cys is very sensitive to oxidation and can form disulfide bonds with another thiol moiety. It is well known that disulfide bonds can be reduced back to the free thiol moiety under physiological intracellular conditions. Therefore, the Cys residues that exist on the protein surface are considered to be the physiological targets for ROS. This oxidative reaction has been thought to occur non-specifically, but recent studies revealed the presence of highly reactive Cys selectively oxidized by ROS.

Protein Tyrosine Phosphatase

Protein tyrosine phosphatases (PTPs) are enzymes that catalyse the chemical reaction to dephosphorylate phosphotyrosine in proteins. Among all PTPs, one Cys residue is conserved in the catalytic centre and functions as a transient acceptor for the phosphate during reaction. The thiol moiety (–SH) of this Cys is kept chemically active, which makes it prone to oxidation by ROS. Therefore, PTPs are generally sensitive to oxidation and indeed, several PTPs have been shown to be negatively regulated by oxidation of their catalytic Cys (*7*, *8*). This is consistent with the abovementioned concomitant increase of H_2O_2 and tyrosine phosphorylation in endothelial cells stimulated with PDGF (*5*). Thus, the inactivation of PTPs by ROS seems to cooperate with the activation of tyrosine kinases to efficiently induce tyrosine phosphorylation by growth factors.

As shown in Fig. 1, the thiol moiety (–SH) is oxidized to become the sulfenyl moiety (–SOH), which is known to be reduced to the thiol moiety by various antioxidants that exist in cells. However, the sulfenyl moiety (–SOH) can be further oxidized to the sulfinyl moiety ($–SO_2H$) and sulfonyl moiety ($–SO_3H$). These highly peroxidized moieties cannot be reduced under the normal intracellular condition. There are several molecular mechanisms to avoid such peroxidation, which accounts for the reversible regulation of the function of PTPs by the redox reaction.

Disulfide (–SS–) bond formation

Phosphatase and tensin homologue deleted from chromosome 10 (PTEN), which is known to suppress human cancers, is a PTP domain-containing protein that catalyses the dephosphorylation of phosphotyrosine and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (*9*–*11*). When PTEN is subjected to oxidation, the catalytic Cys forms an intramolecular disulfide bond with another Cys (*12*, *13*) and protects itself from further irreversible oxidation. The disulfide bond can be reduced under normal intracellular conditions with the help of reducing enzymes, such as thioredoxin (TRX). The oxidation of PTEN inactivates its phosphatase activity and presumably contributes to augment the signal intensity relayed by phosphorylation. Therefore, such disulfide bond formation in PTEN is an intricate mechanism to allow the reversible redox regulation of the phosphatase activity.

Sulfenylamide (–SN–) bond formation

Protein-tyrosine phosphatase 1B (PTP1B) is a PTP domain-containing protein involved in the regulation of insulin signalling and has been shown to be

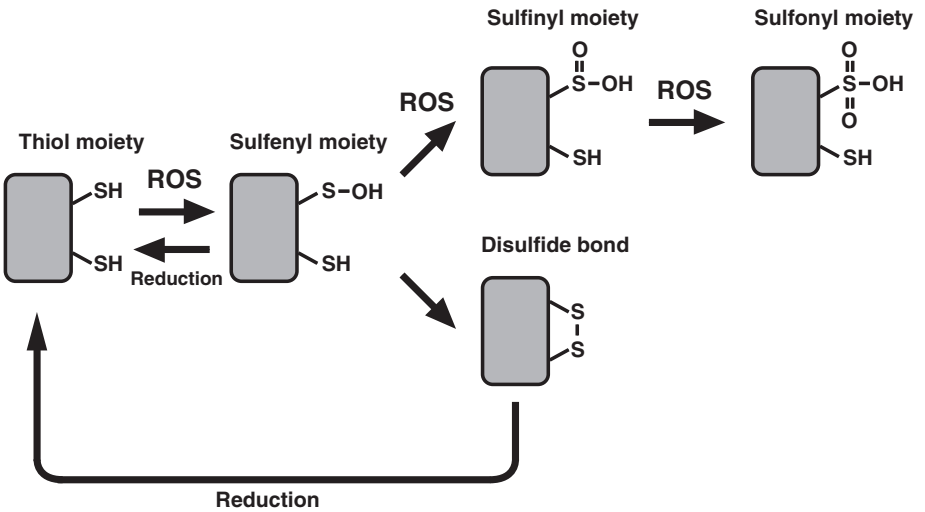

Fig. 1 Cys oxidation by ROS. The thiol moiety is oxidized to become the sulfenyl moiety (–SOH). The sulfenyl moiety can form disulfide bond with another thiol moiety. The sulfenyl moiety and disulfide bond can be reduced by various antioxidants in cells, such as TRX. The sulfenyl moiety can also become the sulfinyl ($–SO_2H$) and sulfonyl moiety ($–SO_3H$) upon further oxidation, which cannot be reduced under the normal intracellular environment.

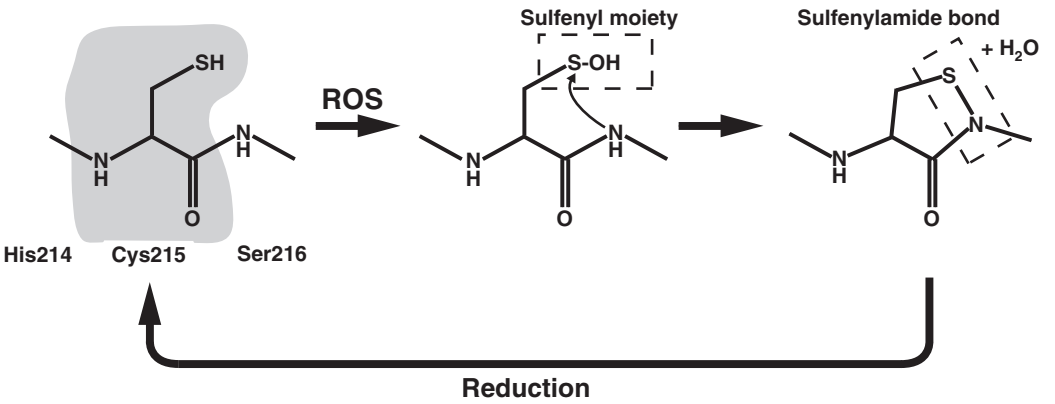

Fig. 2 Sulfenylamide bond formation in PTP1B. The thiol moiety of Cys215 (shaded) becomes the sulfenyl moiety upon oxidation. Then, the sulfenyl moiety reacts with the nitrogen atom in the peptide bond of Ser216 and forms sulfenylamide bond. The sulfenylamide bond can be reduced to the thiol moiety in cells.

regulated by oxidation (*14*). In 2003, two back-to-back papers were published in *Nature*, reporting on a novel structure of PTP1B that is completely different from any known oxidized forms of proteins (*15*, *16*). X-ray crystallographic analyses revealed that the oxidized Cys in the catalytic centre forms the sulfenylamide (–SN–) bond with nitrogen atom in the main chain in the polypeptide (Fig. 2). This sulfenylamide bond formation protects it from further oxidation, as does the disulfide bond. In addition, the sulfenylamide bond can also be reduced under normal intracellular condition and thus, enables reversible redox regulation.

The aforementioned mechanisms via the formation of disulfide bonds and sulfenylamide bonds can clearly explain the reversible redox regulation. Especially, the disulfide bond formation is observed in other several PTPs, such as phosphatase of regenerating liver (PRL) 3 (*17*). However, there are reports indicating the different mechanisms of redox regulation of PTPs. MAP kinase phosphatase (MKP) is a PTP domain-containing protein involved in stress signalling pathway by negatively regulating the activity of c-Jun N-terminal kinase (JNK). When cells are stimulated with tumour necrosis factor α (TNFα), ROS are actively generated and oxidizes MKP, which results in the inhibition of its phosphatase activity and contributes to sustained activation of JNK (*18*). The catalytic centre Cys in MKP is shown to be important for this redox regulation. However, in this case, oxidized MKP forms high molecular weight protein complexes including a sulfenyl moiety and then targeted to degradation by proteasomes. So far as we know, the detailed molecular nature of this high-weight complex of MKP remains uncharacterized. Also, a report shows an unexpected mechanism of redox regulation in the case of another PTP domain-containing protein Sdp1; oxidized Sdp1 shows a stronger catalytic activity than its reduced form (*19*). In this case, disulfide bond formation occurs in a pair of Cys, which exist near the catalytic centre Cys. This disulfide bond formation induces a structural change in Sdp1, which augments the recognition of its substrate. This appears to be an unusual case and there has been no report indicating a similar mechanism working in other PTPs, but it should be noted that oxidation of PTPs does not always lead to inactivation of the phosphatase activity.

TRX Family Proteins

TRX

TRX is highly conserved through evolution even from prokaryotes and catalyses the chemical reaction to reduce disulfide bonds in target proteins (*20*). TRX possesses a pair of redox-active Cys in its catalytic centre (TRX motif). TRX reduces its target proteins, such as peroxiredoxin (PRX), by transferring disulfide bonds to the Cys pair and becomes oxidized to form a disulfide bond in itself (Fig. 3). Oxidized TRX is then reduced and recycled by the reducing enzyme TRX-reductase. Therefore, TRX can also be reversibly oxidized and reduced as PTPs.

TRX contributes to maintain the global redox environment in cells and protects cells from oxidative stress by reducing a number of intracellular oxidized proteins. In contrast, it has been reported that TRX also participates in the regulation of a specific signalling pathway. Apoptosis signal-regulating kinase 1 (Ask1) is a protein kinase that can induce apoptosis and is involved in ROS-induced cell death (*21*). As stated above, stimulation of cells with TNFα results in ROS generation and cell death, and Ask1 plays a critical role in this ROS-mediated cell death. TRX normally binds to Ask1 and suppresses its kinase activity (*22*). However, when cells are stimulated with TNFα, TRX becomes oxidized and forms intramolecular disulfide bonds (Fig. 4). Oxidized TRX loses its ability to associate with Ask1. Then, Ask1 free from TRX can be activated and induce apoptosis. Collectively, it has become clear that TRX is involved in the regulation of Ask1 signalling by using its redox-sensitive nature.

Nucleoredoxin

Nucleoredoxin (NRX) was discovered as an oxidoreductase that contains a pair of redox-active Cys in its catalytic centre, as does TRX. The original report indicated that NRX mainly localizes in the nucleus (*23*), but it was later shown that NRX also exists in the cytoplasm (*24*). NRX was also identified as a major binding protein for dishevelled (Dvl), an essential

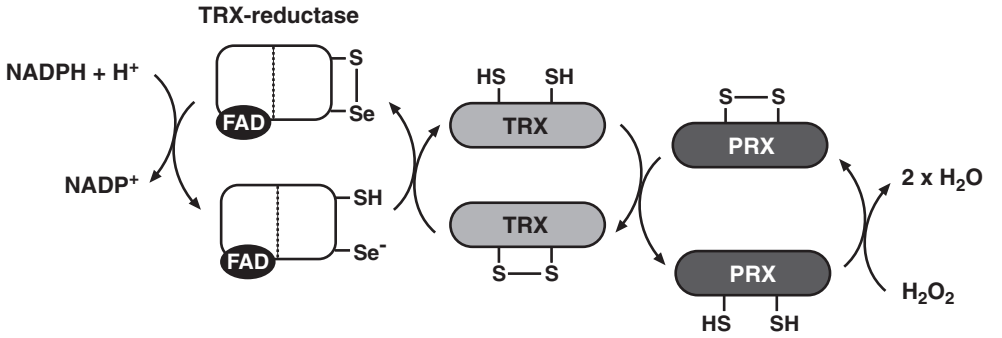

Fig. 3 The TRX system and PRX. PRX possesses a reactive Cys, which can reduce H_2O_2 and, in turn, is oxidized to form a disulfide bond (intra- or intermolecular, which depends on PRX isoforms). TRX reduces its target oxidized proteins, such as PRX, and becomes oxidized to form a disulfide bond in itself. Oxidized TRX is reduced and reactivated by TRX-reductase. TRX-reductase is a dimeric protein that contains selenocysteine and flavin adenine dinucleotide (FAD) and catalyses the electron transfer from NADPH to TRX.

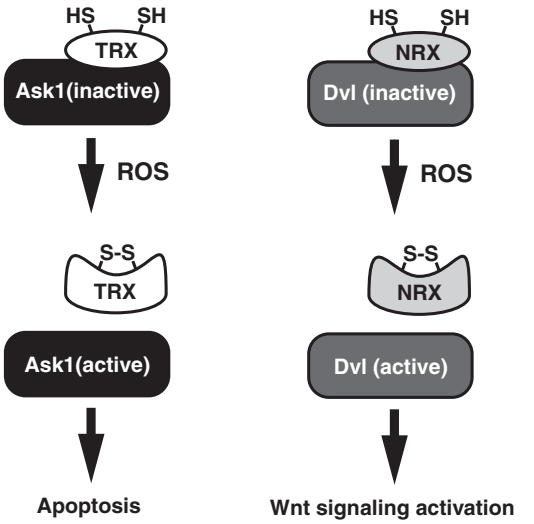

Fig. 4 Redox-dependent activation of Ask1 and Dvl. Stimulation of cells with ROS induces the oxidation of TRX, which dissociates from Ask1. Then, Ask1 free from TRX becomes activated and stimulates the downstream signalling, which results in apoptosis (left). Similar to the case of the TRX/Ask1 complex, ROS also induces the oxidization of NRX and dissociation from Dvl, which leads to the activation of Wnt signalling (right).

mediator of Wnt signalling. NRX directly binds to Dvl and inhibits its function, and thus, NRX can suppress the activity of Wnt signalling. Moreover, it was reported that NRX stabilizes Dvl by inhibiting ubiquitination and degradation of Dvl (*25*). Now, NRX is regarded as a bifunctional molecule to retain a pool of inactive Dvl for robust activation of Wnt signalling upon stimulation.

As TRX, the NRX function is regulated by oxidation. Indeed, when the purified protein complex of NRX and Dvl was incubated with H_2O_2, it readily cancelled the interaction. In addition, stimulation of cells with H_2O_2 abolished the endogenous protein complex and resulted in the activation of Wnt signalling in a manner independent of extracellular Wnt ligands. These experimental results clearly indicate the redox-responsive function of NRX on the regulation of Dvl activity (*26*), which is very similar to the case of the TRX/Ask1 complex (Fig. 4). Therefore, it might be a general feature that TRX-family proteins regulate various intracellular signalling pathways in a redox-dependent manner by forming intramolecular disulfide bonds.

PRX Family Proteins

PRX is an H_2O_2-scavenging enzyme catalysing the reaction to reduce H_2O_2 to H_2O (*27*) (Fig. 3). PRX possesses a highly reactive Cys, which is oxidized to form a disulfide bond coupled with the reduction of H_2O_2. Oxidized PRX is then reduced and recycled by the TRX system. Therefore, PRX is also considered to be a target for ROS. Indeed, it has been shown that Tpx1, the yeast homologue for PRX, mediates H_2O_2-induced activation of the p38/JNK homologue Sty1 (*28*). In this case, oxidized Tpx1 by H_2O_2 forms a transient intermolecular disulfide bond with Sty1, which stimulates activation of Sty1.

It was reported that PRX forms high molecular weight homo-oligomers when subjected to high levels of oxidative stress (*29*). These homo-oligomers are shown to have a chaperone-like activity, which contributes to the protection of cells from oxidative stress. Therefore, the function of PRX appears to be dynamically regulated by the level of oxidative stress. In addition, the PRX homo-oligomers directly bind and activate mammalian Ste20-like kinase 1 (MST1) (*30*). This activation leads to cell death and mediates apoptotic response to p53-activating anticancer drugs, such as cisplatin.

Other ROS Targets

PTPs and TRX and PRX family proteins commonly possess special protein structures that maintain redox-active Cys, which sensitively responds to ROS. However, several exceptional cases are also reported. Morinaka *et al.* (*31*) performed an *in vivo* screen for proteins that contain disulfide bonds, by using the TRX mutant that specifically forms stable complex with proteins bearing disulfide bonds. They found many proteins co-precipitated with the TRX mutant,

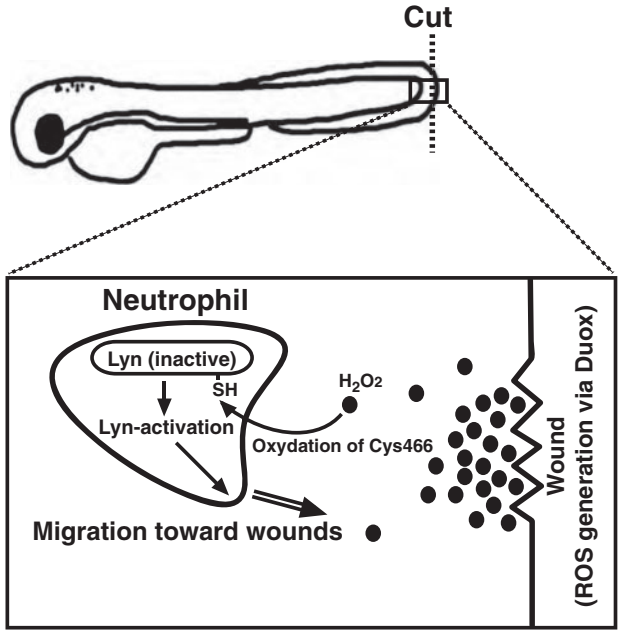

Fig. 5 H_2O_2-mediated activation of Lyn at zebrafish wounds. Upon local injury of the tail fin of zebrafish, H_2O_2 is produced at the wounded areas by ROS-generating enzyme Duox. H_2O_2 diffused from the wounds penetrates into neutrophils and oxidizes Cys466 of Lyn. Oxidized Lyn becomes activated and induces the migration of the neutrophils to the wounds.

and one of the major proteins was identified as collapsin response mediator protein (CRMP) 2, a protein that plays an essential role in repulsive axon guidance caused by Semaphorin (*32*). Stimulation of dorsal root ganglia neurons with Semaphorin resulted in the generation of H_2O_2 in the growth cones and induced oxidation of CRMP2. This oxidation occurs at Cys504 that exists in the carboxy-terminal tail region in CRMP2 and links two CRMP2 proteins. Homo-oligomerization of CRMP2 linked with the disulfide bond has been shown to be essential for Semaphorin response, but it remains unknown as to why CRMP2 is selectively oxidized by Semaphorin stimulation. CRMP2 does not possess some redox-sensitive special structural motif and Cys504 exists in the carboxy-terminally located region that is reported to be unstructured (*33*, *34*). It will be an interesting issue to clarify what determines the sensitivity of CRMP2 Cys504 to H_2O_2.

Recently, Lyn, a Src family tyrosine kinase (SFK), was shown to directly respond to H_2O_2 (*35*). In a previous study, live imaging analyses using GFP-HyPer, a GFP-based H_2O_2-specific probe (*36*), in zebrafish showed that H_2O_2 functions as a chemoattractant for recruiting leukocytes to wounded areas (*37*). Yoo *et al.* revealed that Lyn in leukocytes is activated by wound-derived H_2O_2 and triggers the chemotactic movement of leukocytes to the wounds (Fig. 5). In this process, Cys466 in Lyn seems to be the direct target of oxidation by H_2O_2, because the mutation of Cys466 to alanine specifically abolishes H_2O_2-induced activation of Lyn. Details about this activation of Lyn via Cys466 oxidation still remain unknown; i.e. why is Cys466 sensitive to H_2O_2? Does oxidized Cys466 form an intra- or intermolecular disulfide bond? Considering the cross-species conservation of Cys466 in SFKs, however, this oxidation-dependent activation mechanism might be a general feature across SFKs.

Moreover, it has been reported that oxidation of specific Cys occurs in several other proteins and plays a critical role in the regulation of their function, such as ataxia-telangiectasia mutated (ATM) (*38*) and pyruvate kinase M2 (PKM2) (*39*). As in the cases of CRMP2 and Lyn, these studies do not clarify why the specific Cys is redox-sensitive, but suggest that there still remain a number of unidentified ROS-effector proteins.

Conclusion

In this review article, we have briefly explained the roles of the target proteins for ROS in intracellular signalling, with an emphasis on highly reactive Cys that directly senses and responds to ROS. Examples of such redox-responsive proteins have been increasingly accumulating. Among them, some proteins possess special structures, such as PTP, TRX and PRX domains/motifs, which contribute to keep the redox-active Cys, but others do not. It remains an open question as to what determines the chemical reactivity of Cys in such cases, which would be important to search for novel ROS-effector proteins and ultimately understand the whole image of redox signalling.

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Conflict of interest

None declared.

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