Cell, Vol. 121, 667–670, June 3, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.05.016

Redox Redux: Revisiting PTPs and the Control of Cell Signaling

Minireview

Nicholas K. Tonks*
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

The architecture of the active site of members of the protein tyrosine phosphatase (PTP) superfamily renders these enzymes sensitive to reversible oxidation and inactivation. The importance of reversible oxidation of PTP superfamily members in controlling the signal output following an extracellular stimulus is discussed.

Great progress has been made in defining phosphorylation-dependent signaling events that underlie physiological responses to extracellular stimuli. Although initially considered in terms of linear cascades of sequential phosphorylation reactions that culminate in a particular signaling outcome, it is now appreciated that the responses to stimuli comprise integrated networks of signaling pathways that function in a precisely coordinated manner. Control over these signaling networks is exerted at the level of both phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases, respectively. Disturbance of this delicate balance between kinase and phosphatase activity has been shown to be a cause of human diseases including cancer, diabetes, and inflammation. Therefore, an understanding of the regulation and function of both the kinases and phosphatases in the human proteome is a prerequisite to defining the molecular mechanisms that underlie signal transduction in health and disease and ultimately to revealing opportunities for therapeutic intervention.

Phagocytic cells, such as macrophages, contain a multiprotein NADPH oxidase complex that produces a burst of reactive oxygen species (ROS) as part of the innate immune response to infection. NADPH oxidase catalyzes the single electron reduction of molecular oxygen to produce superoxide, which is rapidly converted to H_2O_2. A family of NADPH oxidases has now been identified in nonphagocytic cells, which produce ROS in a regulated manner at lower levels than in phagocytes, to control the signaling response to a wide variety of physiological stimuli (Lambeth, 2004). H_2O_2 is a small, neutral molecule that is readily produced and removed following a physiological stimulus and can freely diffuse through cellular membranes; thus, it exhibits properties that are ideal for a regulator of signaling (Finkel, 2003). A substantial body of literature now points to the importance of regulated ROS production as a new tier of control over tyrosine phosphorylation-dependent signal transduction.

Reversible Oxidation and Inactivation of PTPs

As protein tyrosine phosphatases (PTPs) have become established as critical regulators of signal transduction in their own right, attention has been drawn to defining how the activity of these enzymes can be regulated in vivo. There are ~100 human genes in the PTP superfamily, which rivals the diversity and complexity of the family of PTKs. The PTP superfamily encompasses two broad categories of enzymes: the classical pTyr-specific phosphatases and dual specificity phosphatases (DSPs), which may also dephosphorylate Ser/Thr residues, and nonprotein substrates, such as inositol phospholipids. The signature motif [I/V]HCXXGXXR[S/T], which defines the PTP superfamily, contains an invariant Cys residue, which functions as a nucleophile in catalysis. Due to the unique chemical environment of the PTP active site, this Cys residue displays an unusually low pKα, which enhances its nucleophilic properties but renders it susceptible to oxidation. Work from several labs has demonstrated that PTPs are an important target of ROS in the induction of an optimal tyrosine phosphorylation response to a variety of physiological stimuli (Rhee et al., 2003). Oxidation of the active site Cys abrogates its nucleophilic properties, thereby inhibiting PTP activity. Thus, one function of ROS produced following agonist stimulation is to inactivate transiently the critical PTP(s) that provide an inhibitory constraint upon the system, thus facilitating the initiation of the signaling response to that stimulus. Importantly, this mode of regulation has the potential to represent a general feature of the PTP superfamily as a whole.

A key element of a signaling response is reversibility. For oxidation to represent a mechanism for reversible regulation of PTP function, it is essential that the active site Cys residue is not oxidized further than sulphenic acid (S-OH); higher oxidation to sulphinic S-O_2H or sulphonic S-O_3H acid is usually irreversible. A crystallographic analysis of PTP1B showed that following oxidation of the active site Cys to sulphenic acid, oxygen is rapidly eliminated to produce a cyclic sulphenamide, a 5-atom ring structure in which a covalent bond is formed between the Cys sulfur atom and the main chain nitrogen of the adjacent Ser residue (Salmeen et al., 2003; van Montfort et al., 2003). This induces profound changes in the architecture of the active site, in which residues that are normally buried now adopt solvent-exposed positions. These structural changes, which are readily reversible in physiological reducing agents, may serve a dual purpose; to protect the enzyme from higher-order, irreversible oxidation of the active site Cys and, by exposing the oxidized Cys to cellular reducing agents, to facilitate reduction back to the active form of the enzyme. DSPs, such as cdc25 and PTEN, as well as the low Mr PTP, are also sensitive to oxidation. Unlike the classical PTPs, these enzymes contain a second Cys residue within the active site. Following oxidation of the nucleophilic cysteine within the signature motif, a disulfide bond is formed with the vicinal Cys, which protects the enzymes from higher oxidation and irreversible inactivation (Buhrman et al., 2005; Lee et al., 2002). The S-S bond can be readily reduced,

*Correspondence: tonks@cshl.edu

ensuring the transient nature of the modification and returning the enzymes to their active form.

There have been several reports of transient oxidation and inactivation of specific PTPs in various signaling contexts. Two recent papers have further emphasized the importance of this regulatory modification.

Regulation of BCR Signaling Output by Reversible PTP Oxidation

Stimulation of a particular receptor can initiate multiple signaling pathways. The challenge is to understand how such pathways are coordinated and integrated, including the establishment of feedback loops that ultimately determine how the signal is propagated and the nature of the physiological outcome. Rao's lab have addressed this issue in B lymphocytes (Singh et al., 2005). The B cell antigen receptor (BCR) is a membrane bound, multiprotein complex, comprising a ligand binding immunoglobulin, which is associated with a heterodimer of Igα-Igβ (CD79a-CD79b) transmembrane proteins that serve a signaling function. Antigen stimulation induces BCR oligomerization and phosphorylation of ITAM sequences (*i*mmune receptor, *t*yrosine-based *a*ctivation *m*otif) in CD79a and b by the Src family PTK Lyn, which recruits and activates Syk. This triggers a burst of phosphorylation and the activation of downstream signaling components, such as PLCγ, which generates IP3 and releases Ca^{2+} from intracellular stores. In their analysis, Singh et al. have shown that the timing and extent of the BCR-generated signal is determined by a feedback loop between BCR-induced Ca^{2+} release and the production of ROS. They noted two, largely distinct, waves of BCR-induced phosphorylation, the first of which peaked at 1 min before declining to baseline, followed by the second, which reached a maximum by 10 min. They also showed that ligation of the BCR induced two phases of Ca^{2+} mobilization, first from the ER and then via channels that promote influx of extracellular Ca^{2+}. Interestingly, inhibition of Ca^{2+} release from the ER attentuated both waves of phosphorylation, whereas the effects of inhibition of the second capacitative Ca^{2+} influx were less pronounced. The authors concluded that the intensity of the first phase of the phosphorylation signal had a crucial impact on the extent and rate of signal progression, which focused their attention on proteins that act early in BCR signaling. They observed that the phosphorylation and activation of Lyn, the Src family PTK that initiates signaling, was dependent upon Ca^{2+}, particularly the ER pool. Thus BCR signaling triggers Ca^{2+} release, which determines the proportion of the Lyn population that becomes activated, which in turn determines the extent and rate of protein phosphorylation. But what provides the link between Ca^{2+} and tyrosine phosphorylation? This is where ROS-induced regulation of PTP function plays a critical role.

Stimulation of the BCR led to a rapid production of ROS, in particular H_2O_2, which was dependent upon PTK activity, PI 3 kinase function, and Ca^{2+}. Interestingly, suppression of BCR-induced ROS also attenuated the mobilization of intracellular Ca^{2+}, revealing a positive feedback loop between ROS and Ca^{2+}. The authors demonstrated that H_2O_2 scavengers inhibited BCR-induced Lyn phosphorylation and this was not rescued by ionomycin-induced increases in Ca^{2+}. Furthermore, exogenous ROS enhanced the phosphorylation of Lyn, but this was not attenuated by inhibition of Ca^{2+} mobilization. This suggests that Lyn phosphorylation is regulated primarily by ROS and that Ca^{2+} influences the levels of ROS that are available for this effect. BCR-induced ROS production was sensitive to DPI (diphenylene iodonium), an inhibitor of flavin-dependent oxygenases, implicating the Nox family. Indeed, using RNAi, they demonstrated that DUOX1 is critical for BCR-induced Lyn phosphorylation. The DUOX members of the Nox family are characterized by the presence of Ca^{2+} binding, EF hand motifs that regulate oxidase function. This is important because it indicates that BCR-induced Ca^{2+} mobilization directly regulates oxidase activity, providing the mechanistic link between Ca^{2+} and ROS production.

The authors noted that BCR activation led to a rapid and transient inhibition of PTP activity that was associated with the receptor, which was blocked either by inhibition of Ca^{2+} mobilization or by antioxidants, and reversed by treatment of the BCR immunoprecipitates with reducing agents. Most interestingly, stimulation of the BCR in cells pretreated with a ROS scavenger resulted in a dose-dependent decrease in ROS, which coincided with a dose-dependent decrease in the extent of PTP inactivation and BCR-dependent Lyn phosphorylation. Thus, the authors suggest that the extent of PTP oxidation determines the activation status of Lyn and, therefore, the signal output (Figure 1). As SHP-1 is known to interact directly with the BCR and attenuate signaling, it was considered as a target for BCR-induced ROS. Having confirmed the presence of SHP-1 in the BCR complex, the authors assumed, but did not formally demonstrate, that this activity was due to SHP-1. Interestingly, when they assayed the activity of the cytosolic pool of SHP-1 directly following BCR ligation, it was not inhibited, which led them to suggest that the action of ROS was restricted to the pool of SHP-1 that was associated with the BCR. In the absence of interactions with pTyr sequences in signaling receptors, the SH2 domain-containing PTPs, such as SHP-1, adopt an inactive conformation in which their active site is occluded by the N-terminal SH2 domain, blocking access to substrate, and presumably also to ROS, and rendering them inactive. Thus, it is not clear what activity the authors are measuring in these cytosolic samples. BCR stimulation also resulted in a rapid and transient inhibition of CD45, the prototypic receptor PTP, an effect that was ablated by Ca^{2+} antagonists and antioxidants. It will be important to clarify exactly which PTPs are susceptible to oxidation in the BCR complex. In addition, the authors show that ROS continues to accumulate over a 5 min time course of BCR ligation, whereas maximal PTP inhibition was observed within 30 s and had returned to baseline within 1 min. It will also be of interest to define why oxidation is transient despite the continued production of ROS. Nevertheless, these data suggest that a Ca^{2+}-ROS feedback loop functions like a rheostat to tune BCR signal output via reversible PTP oxidation.

Oxidation of MAP Kinase Phosphatases in TNFα-Induced Regulation of JNK

A further example of this theme of coordinating signals comes from a study in Michael Karin's lab. TNFα, which is a proinflammatory cytokine involved in the innate im-

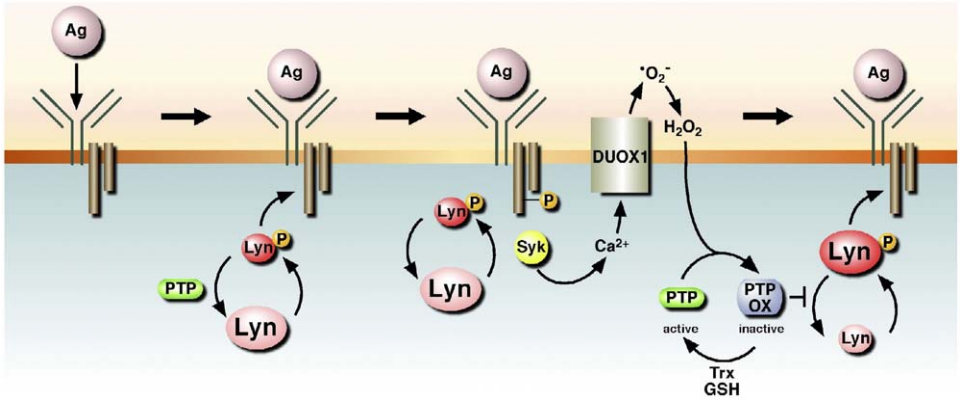

Figure 1. Redox Regulation of PTPs Determines the Activation Status of the PTK Lyn, which in Turn Controls Signal Output from the B Cell Receptor

mune response, triggers cell proliferation or cell death, depending upon the signaling pathways that it engages. The transcription factor NF-κB, which antagonizes the cell death response, is critical for this decision. In the presence of NF-κB, TNFα induces a rapid and transient activation of the JNK MAP kinase; in contrast, in NF-κB-deficient cells TNFα induces sustained JNK activation, which is a major determinant of cytotoxicity. ROS are critical second messengers in this TNFα signaling response and the ability of NF-κB to induce expression of a variety of target genes that antagonize cell death by suppressing ROS production provides a potential regulatory link with JNK activation. So how does ROS promote JNK activation? Karin and colleagues demonstrate that the MAP kinase phosphatase (MKP) subfamily of DSPs, which dephosphorylate and inactivate JNK, are critical targets of ROS (Kamata et al., 2005). Oxidation of MKPs leads to inactivation of the phosphatases and thereby serves to sustain JNK activation.

The authors noted that TNFα attenuated phosphatase activity toward JNK, an effect that was antagonized by the antioxidant BHA (butylated hydroxyanisole). Treatment of mice with Concanavalin A (ConA), which induces TNFα and causes liver failure, resulted in attenuated MKP activity and prolonged JNK activation. When the mice were fed a diet supplemented with BHA, JNK activation was decreased and ConA-induced liver necrosis was suppressed. In light of this, the authors focused their attention on the potential for regulation of MKP activity by oxidation. As would be expected, they demonstrated a dose-dependent oxidation of MKP-3 by H_2O_2 in vitro. However, one of most striking observations was that oxidation induced the formation of high Mr oligomers of the various MKPs that were tested. Comparison of the time course of JNK activation and MKP aggregate formation reveals that JNK phosphorylation was maximal at 20 min and had returned to baseline by 2 hr, whereas the formation of MKP aggregates was barely detectable by 4 hr and maximal by 8 hr. This, and the fact that aggregates were formed in vitro at greater concentrations of H_2O_2 than necessary for inhibition, suggests that aggregation may not directly reflect the oxidation-induced inhibition of MKP activity that regulates the JNK pathway but rather a further modification of the enzyme. Interestingly, yeast cytosolic peroxiredoxins (Prx) also undergo a shift from low Mr to high Mr forms following oxidative stress of the cells, inducing a functional change in Prx, from a peroxidase to a chaperone, which helps the yeast cell adapt to the stress response (Jang et al., 2004). It will be interesting to investigate whether oligomerization also introduces a novel function for the oxidized MKPs.

This study implicates oxidation-induced inhibition of four MKPs in the mechanism by which TNFα-induced JNK activation is sustained in NF-κB-deficient cells, raising the issue of specificity. None of the MKPs tested are specific regulators of JNK; all recognize other MAPKs (Farooq and Zhou, 2004). In fact, MKP-3 is a specific regulator of Erk. It will be particularly important to identify the molecular targets of TNFα-induced ROS under physiological conditions, including specifically which MKPs are oxidized, and to explore whether NF-κB also attenuates the activity of other MAPKs by this mechanism. Another striking aspect of this study is the sensitivity of TNFα-induced ROS production to expression of MnSOD. This suggests that mitochondria are an important source of ROS in this context, consistent with other reports of regulation of PTP function by mitochondrial ROS (Connor et al., 2005). These effects of MnSOD were also observed late in the time course of TNFα stimulation, raising questions of whether the mitochondrial ROS are a cause or an effect of the commitment to cell death. It will be interesting to explore whether other ROS-generating systems contribute earlier in the time course of TNFα-induced JNK activation.

Future Directions

These two exciting papers lend further support to the physiological importance of PTP oxidation in determining the extent and duration of a signaling response. In addition, for several receptor-PTPs, the membrane-distal PTP domain (D2) displays greater sensitivity to oxidation than the catalytically active, membrane-proximal PTP domain (D1). Oxidation induces a conformational change in D2 that can be transmitted to the extracellular segment of the RPTP, resulting in stabilization of inactive dimers (van der Wijk et al., 2004). Thus, D2 may function as a redox sensor, registering ROS production

in response to a stimulus by a conformational change that induces a functional change in the RPTP and in downstream signaling.

Despite such advances, significant challenges remain to be overcome before ROS gain universal acceptance as second messengers. Chief among these is to change the perception of ROS, such as H_2O_2, from agents of indiscriminate damage to specific regulators of signaling. One element of such specificity is the tight regulation of ROS production in response to a stimulus. The activity of NADPH oxidases is controlled by phosphorylation of regulatory proteins and interaction with the small GTPase RAC (Lambeth, 2004). As described by Singh et al., another link to signaling is provided by the regulation of the Duox enzymes by Ca^{2+}. Thus, we are beginning to understand at a mechanistic level how signaling and ROS production may be integrated.

It will also be important to define precisely the source and site of ROS production and to understand how specificity of oxidation is achieved. Not all Cys residues in proteins are susceptible to H_2O_2, which is a relatively mild oxidant; it is only those that have a low pKα and are present in the thiolate form at neutral pH that become oxidized, in contrast to the bulk of Cys residues in proteins for which the pKα is $\sim$8.5. Specificity has already been demonstrated in the selective oxidation of particular PTPs in response to physiological stimuli, as in the case of PDGF-induced oxidation of SHP-2 (Meng et al., 2002) and insulin-induced oxidation of PTP1B and TCPTP (Meng et al., 2004). Recent data even suggest that there is differential sensitivity to oxidation among PTP domains (Groen et al., 2005). Nevertheless, the conventional view of the orientation of Nox enzymes presents somewhat of a challenge. For the most part, Nox family members are localized on the plasma membrane, with the H_2O_2 that is produced in response to the stimulus being released extracellularly, then diffusing locally into the cell. It will be important to investigate to what extent this arrangement leads to oxidation of reactive Cys residues in proteins other than PTPs. This suggests that an important element of specificity is likely to be localization of the PTPs close to the site of ROS production. An interesting recent development in this context is the observation that Nox4, which regulates ROS production in response to insulin and the oxidation of PTP1B (Mahadev et al., 2004), colocalizes with PTP1B on intracellular membranes (Martyn et al., 2005). There are several powerful antioxidants, such as peroxiredoxin, that rapidly degrade H_2O_2, thereby restricting its sphere of influence within the cell and controlling its signaling potential. On the other hand, studies to date have focused on tissue culture cells exposed to an oxygen-rich environment. It will be important to demonstrate that PTP oxidation occurs under the lower physiological oxygen concentrations that are encountered in tissues in the whole animal.

There has been considerable interest in PTPs as therapeutic targets, particularly PTP1B for diabetes and obesity. However, the highly charged active site in the native enzymes, coupled with their susceptibility to oxidation, has rendered the PTPs particularly challenging targets for drug development. The changes in conformation at the active site that accompany oxidation in vitro generate a form of the PTP in which these problematic properties are circumvented and new binding surfaces for small molecule inhibitors are presented. Therefore, assuming such structural changes are induced in vivo, stabilization of the oxidized, inactive conformation of PTP1B, for example, may potentiate insulin signaling in a similar manner to inhibiting the catalytically active form of the enzyme (analogous to stabilization of inactive p210 Bcr-Abl by Gleevec) without having to resort to some of the highly charged PTP inhibitors that have been reported to date. Also, if one assumes that oxidation targets the pool of the relevant PTP that is important for regulation of the signaling response, then this strategy will target that pool specifically, possibly also reducing complications of side effects that may accompany inhibition of the native enzyme as a whole. It will be interesting to see whether future developments in our understanding of PTP oxidation will ultimately yield new therapeutic strategies.

Selected Reading

Buhrman, G., Parker, B., Sohn, J., Rudolph, J., and Mattos, C. (2005). Biochemistry *44*, 5307–5316.

Connor, K.M., Subbaram, S., Regan, K.J., Nelson, K.K., Mazurkiewicz, J.E., Bartholomew, P.J., Aplin, A.E., Tai, Y.T., Aguirre-Ghiso, J., Flores, S.C., and Melendez, J.A. (2005). J. Biol. Chem. *280*, 16916–16924.

Farooq, A., and Zhou, M.M. (2004). Cell. Signal. *16*, 769–779.

Finkel, T. (2003). Curr. Opin. Cell Biol. *15*, 247–254.

Groen, A., Lemeer, S., van der Wijk, T., Overvoorde, J., Heck, A.J., Ostman, A., Barford, D., Slijper, M., and den Hertog, J. (2005). J. Biol. Chem. *280*, 10298–10304.

Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., et al. (2004). Cell *117*, 625–635.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Cell *120*, 649–661.

Lambeth, J.D. (2004). Nat. Rev. Immunol. *4*, 181–189.

Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W., and Rhee, S.G. (2002). J. Biol. Chem. *277*, 20336–20342.

Mahadev, K., Motoshima, H., Wu, X., Ruddy, J.M., Arnold, R.S., Cheng, G., Lambeth, J.D., and Goldstein, B.J. (2004). Mol. Cell. Biol. *24*, 1844–1854.

Martyn, K.D., Frederick, L.M., von Loehneysen, K., Dinauer, M.C., and Knaus, U.G. (2005). Cell. Signal, in press.

Meng, T.C., Fukada, T., and Tonks, N.K. (2002). Mol. Cell *9*, 387–399.

Meng, T.C., Buckley, D.A., Galic, S., Tiganis, T., and Tonks, N.K. (2004). J. Biol. Chem. *279*, 37716–37725.

Rhee, S.G., Chang, T.S., Bae, Y.S., Lee, S.R., and Kang, S.W. (2003). J. Am. Soc. Nephrol. *14*, S211–S215.

Salmeen, A., Andersen, J.N., Myers, M.P., Meng, T.C., Hinks, J.A., Tonks, N.K., and Barford, D. (2003). Nature *423*, 769–773.

Singh, D.K., Kumar, D., Siddiqui, Z., Basu, S.K., Kumar, V., and Rao, K.V. (2005). Cell *121*, 281–293.

van der Wijk, T., Overvoorde, J., and den Hertog, J. (2004). J. Biol. Chem. *279*, 44355–44361.

van Montfort, R.L., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. (2003). Nature *423*, 773–777.