Purification of Reversibly Oxidized Proteins (PROP) Reveals a Redox Switch Controlling p38 MAP Kinase Activity

Dennis J. Templeton*, Myo-Sabai Aye, Joshua Rady, Fang Xu, Janet V. Cross*

Department of Pathology, University of Virginia School of Medicine, Charlottesville, Virginia, United States of America

Abstract

Oxidation of cysteine residues of proteins is emerging as an important means of regulation of signal transduction, particularly of protein kinase function. Tools to detect and quantify cysteine oxidation of proteins have been a limiting factor in understanding the role of cysteine oxidation in signal transduction. As an example, the p38 MAP kinase is activated by several stress-related stimuli that are often accompanied by in vitro generation of hydrogen peroxide. We noted that hydrogen peroxide inhibited p38 activity despite paradoxically increasing the activating phosphorylation of p38. To address the possibility that cysteine oxidation may provide a negative regulatory effect on p38 activity, we developed a biochemical assay to detect reversible cysteine oxidation in intact cells. This procedure, PROP, demonstrated in vivo oxidation of p38 in response to hydrogen peroxide and also to the natural inflammatory lipid prostaglandin J2. Mutagenesis of the potential target cysteines showed that oxidation occurred preferentially on residues near the surface of the p38 molecule. Cysteine oxidation thus controls a functional redox switch regulating the intensity or duration of p38 activity that would not be revealed by immunodetection of phosphoprotein commonly interpreted as reflective of p38 activity.

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* E-mail: dtempleton@virginia.edu (DJT); jvc5b@virginia.edu (JVC)

Introduction

Cell signal transduction is a summation of positive stimuli and homeostatic negative feedback. Positive regulation of protein kinase cascades, such as those activated during mitogenic signaling, differentiation, and pathogenic processes such as inflammation and cancer, is particularly well studied. Most commonly, activation of a kinase cascade results from sequential phosphorylation of protein kinases by other kinases positioned upstream. In contrast, other than the action of phosphatases that remove the activating phosphorylation, inhibitory or negative feedback mechanisms for damping or silencing protein kinase cascades are less well understood. In fact, mainly due to the utility of anti-phosphopeptidope antibodies in identifying phosphate on specific proteins, it has become common in the literature for the presence of an activating phosphorylation on a signaling kinase to be interpreted as an ‘active’ kinase pathway. This unfortunate simplification ignores more subtle means of signal regulation.

Many signaling events, including UV irradiation, inflammatory cytokines, and mitogen stimulation, are accompanied by generation of reactive oxygen species [1,2]. These oxidative species include those that acquire one electron from NAD(P)H and those that acquire two electrons from NAD(P)H [3]. One-electron oxidoreduction reactions result in the release of high-energy species generated by the unused electron from NAD(P)H. These species include superoxide, hydroxyl radical, and others, that likely contribute to damaging modifications of proteins and lipids. Two-electron oxidoreduction mainly results in the in situ generation of hydrogen peroxide, that can be used by endogenous enzymes to oxidize cysteine residues on proteins. These modifications take several forms, including oxidation to sulfenic acid (R-SOH) or formation of disulfides involving intra- and intermolecular connections between proteins or S-thiolation of proteins by glutathione and cysteine. Reversible oxidation of proteins on cysteines is potentially a means for controlling signal transduction, since it adds an often-charged, bulky moiety to the protein primary structure, and is reversible, much like protein modifications resulting from phosphorylation [4,5]. For example, the activity of the MEKK1 protein kinase is regulated by reversible glutathionylation of a single cysteine residue in the ATP binding pocket [6]. Several other kinases have been shown to be activated or inhibited by reversible cysteine oxidation [7,8,9,10,11,12,13,14,15], though mainly with non-physiologic stimuli.

Compared to the study of protein phosphorylation, study of specific oxidation on proteins is difficult. Antibodies against S-glutathionylation have been described, and protein sulphhydrils have been labeled with fluorescent maleimides or iodoacetamides. We have developed an unbiased method of quantifying reversible cysteine oxidation in proteins using an affinity capture technique we call Purification of Reversibly Oxidized Proteins (PROP). We have applied this technique to study the regulatory oxidation of the p38 MAPK signaling kinase. We have found that p38 is oxidized following exposure of cells to exogenous hydrogen peroxide or prostaglandin J2, and that oxidation results in kinase inactivation despite continued phosphorylation on the activating residues detected by immunoblot. Prostaglandin J2 (PGJ2) is an inflammatory...
mediator that has been characterized for its role in inducing oxidative signals [16,17]. As such, the oxidation of p38 following exposure of cells to PGJ2 reflects a relevant physiologic signaling event that might occur during inflammation. The PROP procedure provides a facile means to quantify oxidation of known protein targets and to identify new targets of potential regulatory oxidation events.

Results

Inhibition of p38 activity by protein oxidation

To test the effect of oxidation on p38 kinase activity, we treated cells with hyperosmotic sorbitol to activate p38, with 10 mM H$_2$O$_2$, or both. As expected, we found that both osmotic shock and hydrogen peroxide, or both, induced phosphorylation of p38 detected with anti-phospho-p38 antibodies (Figure 1A, top). We then tested the in vitro kinase activity of p38 by using immune complexes containing p38 from aliquots from these same cells, measuring phosphorylation of the p38 substrate ATF2 (Figure 1A, bottom). Osmotic shock induced p38 kinase activity as expected, but H$_2$O$_2$ treatment did not induce kinase activity despite strongly inducing p38 phosphorylation (lane 2). Additionally, H$_2$O$_2$ treatment of osmotically-shocked cells completely extinguished the p38 kinase activity, despite having no effect on p38 phosphorylation (lane 4). These results suggest that H$_2$O$_2$ blocks p38 kinase activity through a mechanism that does not decrease activating phosphorylation, probably cysteine oxidation.

To test this possibility, we devised a method to quantify oxidation of specific proteins. We developed and optimized a procedure for Purification of Reversibly Oxidized Proteins (PROP) described in detail in Methods. This procedure is outlined in Figure 1B. Briefly, cell metabolism is stopped in strong acid, then cell proteins are dissolved in guanidine denaturing buffer. Reduced protein thiols are blocked by exhaustive treatment with N-ethylmaleimide, then the NEM is inactivated and protein reduction effected by treatment with an excess of dithiothreitol (DTT) at elevated temperatures. DTT is known to reverse sulfenic acid and S-nitrosylation, as well as most disulfide bonds. Proteins are recovered through precipitation, and the newly-reduced thiols resulting from the originally oxidized sites are recovered by a commercial preparation of activated thiolpropyl agarose beads, that contain a dipyridyl disulfide group that forms spontaneous disulfides with free thiols. The originally-oxidized proteins are bound to the activated beads and recovered, washed and eluted in DTT-containing sample buffer for immunoblot analysis. Summarizing briefly, the procedure efficiently blocks unoxidized cysteines, and efficiently recovers proteins that were initially oxidized. Validation of the PROP procedure is described below in conjunction with Figure 2.
We suspected that H$_2$O$_2$ induced oxidation of one or more cysteine residues on p38. We used the PROP procedure to measure reversible oxidation on p38. HeLa cells treated as above with hyperosmotic sorbitol and/or H$_2$O$_2$ were subjected to the PROP procedure, and the recovered proteins analyzed for p38 (Figure 1C, top). Immunoblotting for p38 revealed recovery of p38 in the PROP pull-down of the H$_2$O$_2$ treated samples, reflecting reversible p38 oxidation in cells treated with H$_2$O$_2$, whether or not the cells were osmotically shocked. Minimal oxidized p38 was detected in untreated cells or osmotically shocked cells without H$_2$O$_2$. Equal expression of p38 in all samples was confirmed by immunoblotting of an aliquot of the total cell lysates (Figure 1C, bottom).

Validation of the PROP procedure

Extracts containing p38 oxidized by H$_2$O$_2$ were used to validate the PROP procedure. We first tested the efficiency of recovery of oxidized proteins using the thiol bead pull down (Figure 2A). Using either detergent extracts of unoxidized cells (four left lanes) or extracts of cells oxidized with 10 mM H$_2$O$_2$ to oxidize p38 (four right lanes), we tested the efficiency of the thiol bead pull-down. Little p38 was recovered on the beads from the untreated, unoxidized sample. In contrast, the 1st precipitation of the oxidized p38 recovered much of the input p38, while little remained in solution that could be precipitated in subsequent repeat precipitations.

Figure 2. Validation of efficiency and specificity of PROP procedure. A. Specificity and efficiency of activated thiol bead purification. Extracts containing unoxidized p38 (left) or p38 from cells oxidized with 10 mM H$_2$O$_2$ (right) were sequentially incubated with activated thiol beads after PROP (1st, 2nd, or 3rd PD respectively). Compared to the starting material (“input”) little p38 was recovered on the beads from the untreated, unoxidized sample. In contrast, the 1st precipitation of the oxidized p38 recovered much of the input p38, while little remained in solution that could be precipitated in subsequent repeat precipitations. B. Efficiency of sulfhydryl blockade using NEM. HeLa cells treated with 10 mM H$_2$O$_2$ were processed through PROP, with equal aliquots taken at several points along the procedure. Proteins were treated with Biotin-maleimide to label free sulfhydryls that had escaped NEM blockade, and biotin was detected with anti-biotin immunoblot.

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right lanes), an aliquot of the input sample was analyzed adjacent to equivalent fractions of serial precipitations using the activated thiol sepharose beads. Essentially no p38 was precipitated from the unoxidized cell extract (lanes 2, 3, and 4). Oxidized p38 was recovered in the first pull down from the oxidize cell (lane 6) but not from the repeatedly precipitated extracts (lanes 7 and 8). Thus, the precipitation of oxidized proteins does not recover unoxidized proteins, and a single precipitation is essentially complete.

Next, the efficiency of blocking of unoxidized proteins using NEM in the PROP procedure was tested. In the absence of blocking NEM (Figure 2b, lanes 1–4), unblocked thiols were readily detected using biotin-maleimide and detection of biotin label using anti-biotin immunoblotting (lane 2). No anti-biotin signal was evident without the biotin-maleimide treatment (lane 1). Biotin-maleimide labeling increased when extracts were treated with DTT to reduce oxidized thiols (lane 3). The majority of proteins that could be labeled with biotin-maleimide were removed using activated thiol beads (lane 4). Importantly, the number of free protein thiols in this reaction is far higher than in the precipitation step used in the actual PROP procedure because of the lack of NEM blockade; thus this inefficiency does not reflect the efficiency of precipitation documented in Panel A.

Compared to these unblocked samples, NEM blockade of thiols in oxidized or unoxidized samples was greatly diminished (lanes 5 and 6); thus NEM blockade of free thiols is efficient. As predicted labeling with biotin-maleimide could be increased in the oxidized cell sample using DTT (lane 8), and less so in unoxidized cells (lane 7). Following precipitation of these cell extracts using activated thiol beads (lanes 9 and 10) essentially no proteins were labeled with biotin-maleimide. Thus, again thiol bead precipitation using activated thiol beads efficiently removed the initially-oxidized proteins from cell extracts.

Hydrogen peroxide blocks p38 activity in intact cells

While oxidation of cells reduced the activity of p38 measured in 

in vitro assays, we sought to confirm that this reflected the activity of p38 in intact cells. We repeated the experiment shown in Figure 1, activating cells with hyperosmotic sorbitol to activate p38, and subsequently treating cells with hydrogen peroxide to inhibit p38 (Figure 3). Again, p38 phosphorylation (second panel) was strongly activated by both hyperosmotic sorbitol or hydrogen peroxide, or both. However, immunoblotting of the phosphorylated p38 substrate ATF2 (top panel) showed the intact p38-ATF2 axis was blocked in cells treated with H2O2. Again PROP revealed oxidation of p38 by H2O2 treatment (bottom two panels).

The natural cell oxidant prostaglandin J2 also regulates p38 oxidation and activity

Since 10 mM H2O2 is a super-physiological oxidative stimulus, we tested the natural prostaglandin, PGJ2, for oxidation on p38. PGJ2 is an arachidonic acid metabolite associated with inflammation that has been implicated in regulatory oxidation of other proteins, for example p53 [16,17].

Hyperosmotic sorbitol, H2O2, and PGJ2, all induced p38 phosphorylation (Figure 4, Panel A, top). As before, H2O2 completely blocked p38 kinase activity induced by hyperosmotic shock, as measured by in vitro phosphorylation of ATF2 substrate (Figure 4A, middle, compare lanes 2 and 3); while 100 μM PGJ2 reduced p38 activity by 38% (compare lanes 2 and 5). These results were reproducible over five independent experiments; sorbitol activation of p38 was inhibited by both H2O2 (71.5+/− 21.6% inhibition, p = .0004) and PGJ2 (57.3+/−11.5% inhibition, p = .004).

Both H2O2 and PGJ2 induced p38 oxidation, as revealed by PROP (Figure 4B). PGJ2 induced 25% of the amount of oxidation induced by H2O2 (Figure 4B, top, lanes 3 and 5), consistent with the fractional reduction in p38 kinase activity. Sorbitol treatment alone did not cause p38 oxidation. Thus, quantifying and comparing p38 phosphorylation and kinase activity shows a discordance between anti-phospho-p38 signal and actual kinase activity. For both H2O2 and PGJ2, inhibition of p38 kinase activity paralleled the degree of oxidation observed by PROP.

The induction of p38 oxidation by PGJ2 was dose responsive (Figure 5A), with distinct oxidation detected with PROP at the lowest dose tested, 25 μM. Oxidation was maximal at the 100–200 μM range. P38 oxidation induced by 100 μM PGJ2 was also time dependent, with reversible oxidation detected at 1 hour after treatment, and increasing at the 3 hour time point (Figure 5B). Reversible oxidation of p38 was no longer detected at 6 hours or subsequent time points. Time dependence was not observed using 10 mM H2O2, which rapidly oxidized and inhibited p38 (data not shown).

Figure 3. Oxidation by H2O2 inhibits p38 kinase activity in intact cells. HeLa cells were seeded in 6-well plates. At 30 min prior to harvesting, the indicated wells were treated with 10 mM H2O2 and 10 min later, the indicated wells were treated with hyperosmotic 0.4 M sorbitol to activate p38. PROP pulled down oxidized p38 only after H2O2 treatment (lanes 3 and 4). Both sorbitol and H2O2 induced phosphorylation of endogenous p38 (lanes 2,3,4). However, while endogenous phosphorylated ATF-2, an in-vivo substrate of p38, was seen after activation of p38 with hyperosmotic sorbitol (lane 2) phosphorylation of ATF-2 was absent in cells treated with both hyperosmotic sorbitol and H2O2. Thus, in vivo activity of p38 (indicated by phosphorylation of the p38 substrate, ATF-2) did not correlate with the conventional measure of p38 activity, i.e. phosphorylation of p38. The amount of endogenous p38 following any treatment (bottom panel) was unchanged. The figure is representative of two independent experiments.

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Oxidized, inactive p38 can be reactivated by reduction in vitro

We hypothesized that the lack of p38 activity recovered from cells treated with H$_2$O$_2$ or PGJ$_2$ was the result of reversible oxidation of p38, as detected using the PROP procedure. To test this hypothesis, cells were treated with combinations of sorbitol, H$_2$O$_2$, or PGJ$_2$ as before. Aliquots of these samples were immunoblotted to assess p38 expression and phosphorylation (Figure 6A), and the remaining lysate used for in vitro p38 kinase assay. We divided the bead-bound immune complexes into two aliquots before assay; one aliquot was treated with 10 mM DTT at room temperature for 30 minutes, while the other aliquot did not receive DTT. In vitro kinase assay was performed using phosphorylation of the ATF2 substrate as the readout (Figure 6B).

DTT treatment of p38 from untreated cells did not increase activity (sample 1), and had only minor effects on the mildly activated p38 from cells treated with PGJ$_2$ alone (sample 2).

However, DTT treatment of p38 from cells treated with both sorbitol and PGJ$_2$ (sample 3) strongly (2.5 fold) activated p38 compared to the p38 that was not treated with DTT. In fact, DTT restored 104% of the p38 activity from the PGJ$_2$/sorbitol sample (sample 3) compared to DTT-treated p38 from cells treated with sorbitol but no oxidants (sample 6). Thus, in vitro exposure to DTT completely restores the activity of p38 inhibited by PGJ$_2$.

DTT treatment also increased the activity of p38 recovered from cells treated with H$_2$O$_2$ alone (sample 4) or the combination of H$_2$O$_2$ and sorbitol (sample 5), the amount of activity recovered was reduced (66%) compared to the activity recovered from cells treated with sorbitol alone or sorbitol with PGJ$_2$. Thus, in vitro exposure to DTT completely restores the activity of p38 inhibited by PGJ$_2$.

Mapping of p38 oxidation sites

The p38 protein kinase has 4 cysteine residues, none of which are predicted to be disulfide bonded. We constructed mutants of p38 containing cysteine-to-serine substitution of all 4 cysteine residues (C4S), four mutants in which each individual cysteine is
mutated to serine, as well as four mutants in which single cysteines were added back to the 4CS mutant individually. We tested proteins encoded by each of these mutants for reversible oxidation induced by PGJ2, using PROP. Each of the mutant p38 proteins with individual cysteine to serine substitutions was still oxidized after exposure of cells to PGJ2 as measured by PROP, suggesting that oxidation occurred on more than one cysteine residue (data not shown). Analysis of the mutant p38 proteins in which individual cysteines were added back to the 4CS mutant allowed us to assess the oxidation at each cysteine residue after PGJ2 treatment. Summary results averaging three experiments are shown in Figure 7a. Oxidation of the 4CS mutant protein was not detected, as expected. The mutants with only individual cysteine residues retained weak or strong oxidation, depending on the...
cysteine residues, with C119 and C162 oxidized to an extent greater than residues C211 and C39. Thus, to the extent that these variant proteins reflect the activities of the natural p38, we conclude that the major sites of reversible oxidation are C119 and C162. The mutant proteins with only a single cysteine residue did not retain catalytic activity, so it was not possible to confirm the anticipated effects on activity using these mutants.

The structure of p38 has been determined [18], and is available in the Protein Data Bank (http://www.pdb.org/pdb/explore/explore.do?structureId = 1WFC). This structure confirms that p38 does not have disulfide bonds. It also shows (Figure 7B) that cysteines C119 and C162 lie topologically adjacent to each other near the surface of the protein, while the other two cysteines are buried within the protein matrix. This suggests that these two sites would be more accessible for oxidation following exposure of the cells to PGJ2. Importantly, the migration of p38 from oxidant treated cells on non-reducing SDS-PAGE was similar to that of p38 from untreated cells, and to p38 following reduction with DTT (data not shown), suggesting that intramolecular disulfide bonding or disulfide bridges to another protein is likely not involved in p38 inhibition.

Discussion

We have developed a new procedure, PROP, to detect and quantify oxidation of cell proteins, and have used it to detect oxidation and inhibition of the stress-activated p38 kinase resulting from both H2O2 and from the physiologic mediator of inflammation, prostaglandin J2. The PROP assay is similar in concept to the Jaffrey assay for protein nitrosothiols [19], in that protein thiols are first blocked with thiol-reactive reagents, and then modified thiols revealed with DTT (or ascorbate, for nitrosothiol) before recovery using thiol affinity methods. PROP may have some benefit over this prior assay because it involves fewer steps (no chemical modification of the target cysteines are required for recovery, instead direct thiol affinity is achieved using a one-step commercial reagent). While the Jaffrey procedure blocks thiols in SDS solutions, we found in pilot studies that the use of guanidine instead of SDS as a denaturant resulted in superior blocking of non-oxidized cysteines. Precipitation of guanidine-denatured protein by methanol is an efficient way of recovering protein while removing small cysteine-modifying chemicals including NEM and DTT.

In addition to the Jaffrey method, there are several other approaches for labeling and detecting reversible oxidation of protein targets. Most of these rely on a similar strategy of blocking unmodified cysteines, then reversing the modification and labeling with a thiol reactive reagent that provides a means of detection. Among the available options are fluorescent labels, biotinylated agents, mobility shift, and radioactive compounds [20,21,22,23,24,25,26]. These approaches each have limitations that the PROP procedure obviates. For example, while fluorescent or radioisotopic labeling can enable detection of labeled proteins, these approaches do not purify the labeled (oxidized) proteins away from non-oxidized proteins, that is necessary for proteomics analysis to characterize unidentified proteins. Biotin labeling (as in the Jaffrey or biotin switch method) enables a somewhat efficient purification approach. However, the biotin label on the purified protein can be problematic for mass spectroscopy identification of the modified residue on the oxidized proteins. In the PROP procedure, proteins are released from the thiol beads with reducing agent (e.g. DTT, TCEP etc) in an unmodified form, leaving them completely compatible with downstream applications such as mass spectrometry, or for isotopic labeling such as with maleimides containing heavy isotopes. Alternate methods of protein release with ascorbic acid or arsenite, can be used to elute proteins with specific types of cysteine oxidations. Some biotin-switch methods rely on labeling of cell lysates, which introduces the variability of post-lysis oxidation, despite precau-

![Figure 7. Oxidation of individual cysteine residues in p38.](image-url)
tions of cell lystate processing in an anaerobic chamber [27]. Our approach uses rapid fixation of cells using 10% trichloroacetic acid, which immediately prevents post-lytic oxidation. As the acid is removed, proteins are continuously in the presence of thiol-blocking maleimide.

A seemingly simple aspect of the procedure that provides a major advantage is the use of guanidine as a denaturing agent. Guanidine denaturation provided superior blocking of free sulhydryls when compared to any other approach we attempted, allowing sensitive detection of very small amounts of oxidized protein due to the absence of background labeling that results from inefficient blocking. Removing guanidine can be troublesome, but our use of methanol precipitation of proteins efficiently removes the denaturant, the reductant, and the blocking reagent simultaneously. The resulting protein precipitate is solubil only in strong denaturants (in our case the same guanidine buffer as used for lysis) but this reagent is suitable for use with the activated thiol agarose beads.

Protein thiols are subject to several levels of oxidation. Thiols can form disulfides through interaction with other protein thiols or with small thiols such as cysteine or glutathione. Alternately, thiol oxidation can lead to sulfenic acid (R-SO\(_2\)H) or sulfonic (R-SO\(_3\)H) acids. Additionally, nitroso-thiols can result from endogenous or exogenous nitric oxide. Disulfides, nitroso-thiols, and sulfenic acid are non-enzymatically converted to thiols in the presence of reducing agents such as glutathione or DTT, and are certainly detected using the PROP procedure in which modified proteins are subjected DTT. These modifications are likely also reversible in vivo and therefore represent potential regulatory events. Higher order oxidations (sulfinic and sulfonic) are unlikely to be reversed by DTT treatment during PROP. Based on our observations, it is possible that \(H_2O_2\) induces some higher-order modifications of p38, given the lack of recovery of full activity by DTT treatment in vitro (Figure 4). In contrast, since essentially all of the activity of the PGJ2 inhibited p38 is recovered by DTT treatment, PGJ2-induced oxidation of p38 appears to be completely reversible, suggesting lower order events at the dose range tested.

The use of PROP or similar approaches will allow protein oxidation reactions to be identified and quantified. Known targets of cysteine oxidation can be analyzed by immunoblot detection, similar to the approach used here for p38. In addition, since proteins are liberated with the target cysteines in their native state, the PROP procedure offers opportunities for proteomic studies to identify new protein targets of oxidation. We have performed pilot studies in which proteins from oxidized cells are recovered using PROP, released in DTT, labeled with iodoacetamide, and subjected to mass spectrometry analysis, with promising results.

While protein kinase activation usually involves phosphorylation of highly conserved serine, threonine or tyrosine residues within the “activation loop” of the protein kinase, the inhibitory redox switches that we and others have identified impact several divergent protein kinase regions. For example, MEKK1 is targeted by oxidation of a cysteine residue within the ATP binding domain [6]. PKA is oxidized within the activation loop in a manner that may regulate dephosphorylation of the activating threonine [28]. PKG1 is activated through oxidation to form a cysteine-paired dimer [7]. In other instances, several cysteine residues are oxidized, accompanying inactivation of the kinase, including JAK2, [9], and SAPK/JNK (Cross and Templeton, unpublished). In only a few instances have sites of oxidation been mapped to specific cysteines.

On the other hand, kinases that are related to one another likely share functional regulatory mechanisms, including the inhibitory redox switch. We identified the surface cysteines C119 and C162 of p38 as major sites of oxidation using mutant studies. On the linear subdomain map of p38 aligned with other protein kinases [29], C119 of p38 lies within subdomain V. Notably, p38 alpha and beta share a cysteine codon analogous to C119, while JNK1, 2, and 3 share a nearby cysteine in the same subdomain, and ERK isoforms lack cysteines in domain V. Cysteine C162, which is found in subdomain VIb, is shared among all p38 isoforms, and all JNK and ERK isoforms excepting ERK 3 and 4, but is otherwise lacking in other members of the CMGC kinase family (http://kinase.com/human/kinase/groups/cmgc.ahn). This observation leads to the prediction that these conserved cysteine residues may serve to control other MAP kinase family members much as they do p38.

The case by which specific cysteines and not others are oxidized may reflect the relative degree ionization of the target cysteine. Some oxidizable cysteines are also targets of electrophilic attack by drugs or signal-regulatory compounds originating outside the cell. For example the oxidized cysteine found in MEKK1 [6] is also the target of covalent alkylation by phenylethylisothiocyanate and sulforaphane, cancer chemopreventive compounds containing an electrophilic isothiocyanate group [30]. Other cysteines identified as oxidation sites thus represent potential sites of therapeutic regulation by drug candidates that may also covalently modify these target cysteines. Covalent targeting of regulatory cysteine residues is potentially the mechanism through which the protein kinase C (PKC) inhibitor bisindolyl maleimide (BIM) functions, since the maleimide moiety should make covalent bonds with target cysteines. While BIM is considered an ATP mimetic, we note that conventional and novel PKCs, that are inhibited by BIM, all have a cysteine residue within the ATP binding domain, while the atypical isoforms of PKC ( iota and zeta), which are resistant to BIM, lack this cysteine. This appears not to have been explored in the literature.

The biological relevance of cysteine modification by electrophilic compounds such as oxidized lipids[31] and dietary isothiocyanates [30] raises the possibility that reversing the resulting Michael addition and thioethercarbomates products (respectively) with DTT might result in identification of the targets of these reactions using PROP. Some evidence suggests that this is not so. For example, modification of the MEKK1 protein kinase by isothiocyanate is stable to boiling in SDS and DTT [30], though other cysteine adducts might be less stable. Similarly, numerous cell proteins labeled with biotin-prostaglandin J2, and detected with anti-biotin immunohlottting are not measurably reversed by boiling in SDS and DTT (Cross and Templeton, unpublished), though again, specific proteins might be. Owing to these observations, we predict that these reactions will not be detectable using PROP.

We [6] and others have previously detected regulatory oxidation of other protein kinases [7,8,9,10,11,12,13,14,15]. Importantly, in most of these experimental systems, oxidation is induced by hydrogen peroxide at high concentrations, diamide, menadione, or other non-physiologic agents. Our finding that micromolar concentrations of PGJ2 can induce oxidation and inhibition of p38 extends these results to agents involved in the physiologic response to disease or inflammation.

Our results suggest that oxidation may serve as a redox switch that provides a feedback mechanism for p38 and other signaling kinases. Specifically, initial activation of p38 results from phosphorylation within the “activation loop”, that is detectable using anti-phospho p38 antibodies. Subsequently, activated p38 may become oxidized at one or more cysteine residues, resulting in the loss of kinase activity. Importantly, this inactive kinase retains its
Phosphorylation and may incorrectly be interpreted as an active kinase when using anti-phospho antibody immunoblot as a surrogate measure of “activity”. Signal transduction investigators are cautioned that the simplicity of an anti-phospho-immunoblot may obscure more subtle means by which signal transduction is regulated. We recommend that for a specific signaling pathway to be deemed “activated”, a direct measurement of the catalytic activity in vitro is indicated, together with an evaluation of natural substrates of the kinase within cells.

Materials and Methods

Cell culture, reagents and transfections. HeLa cells (American Type Culture Collection, ATCC.org) were grown in Dulbecco’s Minimal Eagle’s Medium supplemented with 10% calf serum and penicillin/streptomycin, in 5% CO₂ environment. For experiments, cells were seeded in 6-well plates. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per manufacturer’s instructions. Prostaglandin J2 (Cayman Chemicals, Ann Arbor, MI) was prepared in DMEM and stored in aliquots at −20°C. Antibodies used for immunoblotting were as follows: anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-p38 and anti-phospho-ATF2 (Cell Signaling, Danvers, MA), HRP conjugated goat anti-rabbit secondary antibody (Promega, Madison, WI). Blots were developed using enhanced chemiluminescence with images captured on a Fluor-Chem HD2 (Alpha-Innotech, San Leandro, CA) in 16 bit mode without image saturation. TIFF files were quantified using ImageJ (http://rsbweb.nih.gov/ij/).

p38 expression vectors. p38 was expressed from pcDNA3.1 containing a synthetic gene (GenScript, Piscataway, NJ) encoding murine p38alpha, isoform 1 (GE:10092590), tagged at the C terminus with a chitin binding domain. For the cysteine mutants, a second synthetic gene was designed that was identical to the first except that all of the cysteine codons were mutated to serine (4CS). To prepare the single cysteine add back mutants, a second synthetic gene was designed that was identical to the first terminus with a chitin binding domain. For the cysteine mutants, a

PROCEDURE. Cells were rinsed quickly with HEPES-buffered saline and fixed in 10% TCA for 10 minutes. Beads were washed 3 times with MLB/G and once with 50 mM Tris (pH 7.4). p38 activity was assayed using 1 μg ATF2 substrate (Cell Signaling) in a 20 μL reaction containing 25 mM Tris (pH 7.4), 10 mM magnesium chloride, and 7.5 μM ATP. Samples were incubated for 30 minutes at room temperature, and then stopped by addition of 2X Laemmli sample buffer. Samples were separated by SDS-PAGE and phosphorylated ATF2 detected by immunoblotting.

Author Contributions

Conceived and designed the experiments: DJT JVC MSA. Analyzed the data: DJT JVC MSA. Wrote the paper: DJT JVC.

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