Probing cellular protein targets of H_2O_2 with fluorescein-conjugated iodoacetamide and antibodies to fluorescein

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Abstract Recent studies suggest that H_2O_2, at subtoxic concentrations generated in response to the activation of a variety of cell surface receptors, functions as an intracellular messenger. However, the intracellular targets of H_2O_2 action have not been identified. A procedure to detect proteins with reactive cysteine residues susceptible to oxidation by intracellularly generated H_2O_2 is now described. This approach is based on the labeling of proteinaceous cysteine with 5-iodoacetamidofluorescein at pH 5.5 and immunoblot analysis of the labeled proteins with antibodies specific to fluorescein. With this procedure, many proteins in human A431 cells were shown to contain reactive cysteines and to be readily oxidized by H_2O_2 generated in response to cellular stimulation with epidermal growth factor. One of these H_2O_2-sensitive proteins was identified as protein tyrosine phosphatase 1B.

Key words: Hydrogen peroxide; Intracellular messenger; Cysteine oxidation; Growth factor; Protein tyrosine phosphatase

1. Introduction

Although H_2O_2 is generally considered a toxic by-product of respiration, recent evidence indicates that the production of this oxygen metabolite contributes to membrane receptor signaling [1–3]. In mammalian cells, various extracellular stimuli, including cytokines and growth factors, induce a transient increase in the intracellular concentration of H_2O_2 [4]. In addition to indirectly activating protein tyrosine kinases and transcription factors such as NF-κB and AP1 [5], H_2O_2 also increases the cytosolic concentration of Ca^{2+} [6], activates phospholipases (A_2, C, and D) and mitogen-activated protein kinases [7–9], enhances phosphorylation of proteins on serine (threonine) and tyrosine residues, and triggers apoptosis [10]. Thus, H_2O_2 is now recognized as a ubiquitous intracellular second messenger under subtoxic concentrations.

Because H_2O_2 is readily converted to the extremely reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction, it might seem unlikely that such a potentially dangerous molecule would serve such a crucial function. However, a precedent is provided by nitric oxide (NO), itself a reactive radical and a well-characterized intracellular messenger [11]. The observation that specific inhibition of H_2O_2 generation completely prevents signaling by platelet-derived growth factor and epidermal growth factor (EGF) strongly supports a second messenger role for this molecule [1,3]. A reactive oxygen species has also been implicated as an essential mediator of RAS-induced cell cycle progression [2].

A second messenger role for H_2O_2 requires its reversible binding to or modification of cellular components. However, systematic attempts to identify such cellular targets of H_2O_2 have not been described. Unlike other messengers such as cAMP and inositol 1,4,5-trisphosphate, the simple structure of H_2O_2 makes it improbable that it is recognized specifically by proteins. Thus, the reversible binding of H_2O_2 to proteins is likely not responsible for signal propagation. In contrast, H_2O_2 is a mild oxidant that can oxidize methionine and cysteine residues in proteins to methionine sulfoxide and cysteine sulfenic acid (or disulfide), respectively, each of which can be readily reduced back to the unmodified amino acid by various cellular reductants.

A limited number of proteins would be expected to possess a Cys-SH residue that is readily susceptible to oxidation by H_2O_2 in cells because rapid oxidation requires that the target Cys-SH have a pK_a of < 7.0, whereas the pK_a values of most protein Cys-SH residues are 8.5. Enzymes, such as protein tyrosine phosphatases and cysteine proteases (for example, papain), that contain an essential Cys-SH with a low pK_a at their active sites are potential candidates for reversible oxidation by H_2O_2 generated intracellularly.

We have now developed a procedure for identifying proteins that contain cysteine residues sensitive to intracellularly generated H_2O_2. With this procedure, we have shown that cysteine residues of several proteins become oxidized in A431 human epidermoid carcinoma cells after stimulation with EGF. One of these proteins was identified as protein tyrosine phosphatase 1B (PTP1B).

2. Materials and methods

2.1. Materials

5-Iodoacetamidofluorescein (5-IAF) and affinity-purified rabbit antibodies to fluorescein were obtained from Molecular Probes (Eugene, OR, USA). Monoclonal antibodies to PTP1B were from Transduction Laboratories (Lexington, KY, USA), and immunoprecipitating rabbit antibodies to PTP1B were from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal antibodies to fluorescein, monoclonal antibodies to phosphotyrosine, diphenyleneiodonium iodide (DPI), and purified papain were from Sigma (St. Louis, MO, USA). Rabbit antibodies to PTP1D were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Amersham (Arlington Heights, IL, USA). Glutamine synthetase was kindly provided by R. Levine. An expression vector that encodes a truncated PTP1C

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Abbreviations: EGF, epidermal growth factor; PTP1B, protein tyrosine phosphatase 1B; 5-IAF, 5-iodoacetamidofluorescein; DPI, diphenyleneiodonium iodide; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; BSA, bovine serum albumin

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(Asp[104] to Gln[532]) lacking the amino-terminal SRC homology 2 (SH2) domain was kindly provided by Dr. J. Schlessinger. The catalytically active 45-kDa recombinant PTP1C was expressed in and purified from *Escherichia coli* as described.

2.2. Cell culture and labeling with 5-IAF

A431 cells were grown to nearly confluence in 55-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were washed with minimum essential medium and deprived of serum for 16 h by incubation in Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum. Serum-deprived cells were stimulated with H_2O_2 or EGF as indicated, washed with ice-cold phosphate-buffered saline, stored by rapid freezing in liquid nitrogen, and then harvested at 4°C under anaerobic conditions by scraping into 0.5 ml of lysis buffer supplemented with 5-IAF. The lysis buffer contained 25 mM sodium acetate (pH 5.0), 25 mM MES-NaOH (pH 6.0), 10% glycerol, 1% Triton X-100, catalase (1 μg/ml), 2 mM NaF, 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, leupeptin (4 μg/ml), and aprotinin (4 μg/ml), and was equilibrated overnight in an O_2-free atmosphere. $NaVO_3$ (2 mg/ml) was also included in the lysis buffer for experiments in which the lysates were subjected to immunoblot analysis with antibodies to phosphotyrosine. 5-IAF was freshly prepared as a 100× stock in dimethylformamide and added to the lysis buffer to a final concentration of 4–12 μM at the time of use. Cell lysates were rapidly frozen and stored at −70°C.

2.3. Detection of 5-IAF-labeled proteins by immunoblot analysis with antibodies to fluorescein

Frozen cell lysates were thawed rapidly, sonicated briefly on ice, incubated in a thermomixer at 37°C for 10 min, and microcentrifuged at top speed for 5 min in a cold room. After determination of protein concentration with the BCA assay (Pierce, Rockford, IL, USA), the resulting supernatants were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis with antibodies to fluorescein.

Alternatively, the supernatants were subjected to immunoprecipitation with antibodies to PTP1B or to PTP1D. The supernatants (500 μg of protein) were first pretreated with protein A-agarose beads (Pharmacia), neutralized, and diluted to a protein concentration of 1 μg/μl in Tris-HCl (pH 8 stock and final concentration 100 mM) containing leupeptin (4 μg/ml), aprotinin (4 μg/ml), and 0.2% NaN_3. After the addition of 6 μg of rabbit antibodies to PTP1B or to PTP1D, the resulting mixture (approximately 500 μl) was incubated with periodic rotation overnight at 4°C. Protein A-agarose beads (20 μl) were then added and the mixture was incubated for an additional 2 h. The beads were separated by brief centrifugation, washed five times with a washing buffer (50 mM Tris-HCl, 0.05% sodium deoxycholate, 0.1% Triton X-100, 1 mM NaF, 0.2 NaN_3, 2 μg/ml of leupeptin and aprotinin and 2 mg/ml of $NaVO_3$), resuspended in 50 μl of non-reducing SDS-PAGE sample buffer, and heated at 95°C for 5 min. The samples were then analyzed by SDS-PAGE and immunoblot analysis with antibodies to PTPs or to fluorescein, and immune complexes were detected by enhanced chemiluminescence (ECL) with a SuperSignal substrate system (Pierce).

3. Results and discussion

Because reactive cysteine residues with low pK_a values are the primary sites of reaction with either H_2O_2 or iodoacetamide, prior exposure of proteins with a reactive cysteine to H_2O_2 at neutral pH would be expected to block subsequent modification by iodoacetamide at the same pH. Taking advantage of the commercial availability of both 5-IAF, an iodoacetamide derivative containing a fluorescein moiety, and antibodies that recognize the fluorescein moiety, we investigated whether it was possible to detect proteins with a H_2O_2-sensitive cysteine by treatment with 5-IAF and immunoblot analysis with such antibodies. We first tested this approach with two purified proteins, papain and PTP1C, that contain a cysteine residue with a low pK_a at their active sites.

Papain, which contains an essential cysteine with a pK_a of 4.0 [12], was subjected to oxidation by 0.2 mM H_2O_2 at pH 5.0, 6.0, or 7.0, and then to modification by 5-IAF. Papain treated at each of the three pH values showed negligible reactivity with 5-IAF, compared with that of the protein not exposed to H_2O_2 (Fig. 1A), suggesting that the essential cysteine was almost completely oxidized at pH values of ≥5.0 and that the oxidized molecules do not react with 5-IAF. Immunoblot analysis with the ECL technique allowed us readily to detect 20 ng of 5-IAF-labeled papain (not shown). Substantial oxidation of PTP1C at pH 6.0 and 7.0 was detected with 2 mM H_2O_2 (Fig. 1B). The pK_a value for the active site cysteine of PTP1C is not known, but those for such cysteines of other mammalian PTP enzymes are generally above 5.0 [13–15].

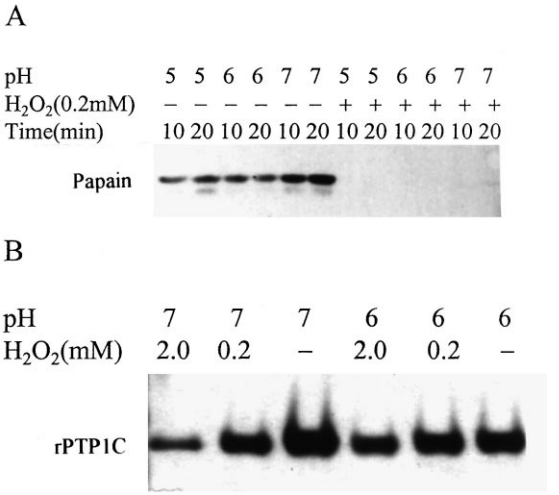

Fig. 1. Effect of prior incubation with H_2O_2 on the labeling of papain (A) and PTP1C (B) with 5-IAF at various pH values. Commercial papain was equilibrated with an equal molar amount of DTT before use. Papain (5 μg) or PTP1C (1 μg) was incubated for 5 min at 37°C in a final volume of 20 μl of 50 mM buffers at the indicated pH (sodium acetate for pH 5.0, MES for pH 6.0 and MOPS for pH 7.0) and concentration of H_2O_2. After the addition of 5-IAF (from freshly prepared stock) to a final concentration of 20 μM, the mixture was incubated for an additional 10 min at 37°C. The labeling reaction was stopped by the addition of 2×SDS sample buffer. Portions of the labeled protein (0.2 μg of papain, 0.1 μg of PTP1C) were subjected to SDS-PAGE on a 10% gel and immunoblot analysis with monoclonal antibodies to fluorescein. Immune complexes were detected by ECL.

To confirm the selectivity of 5-IAF for reactive sulfhydryl groups, PTP1C was mixed with a 10-fold molar excess of each of glutamine synthetase and bovine serum albumin (BSA), and then reacted with excess 5-IAF. Although glutamine synthetase and BSA both contain cysteine residue(s) that may become exposed to the solution, the extent of labeling of BSA was much less than that for PTP1C and no labeling of glutamine synthetase was detected in the absence of EDTA (not shown). Thus, labeling by 5-IAF appears specific for proteins that contain cysteine residues with low pK_a values.

To investigate whether H_2O_2 generated in response to EGF is able to oxidize proteinaceous sulfhydryl groups, we incubated A431 cells with EGF for 15 min or longer and then lysed the cells in an anaerobic chamber by exposure to a pH 5.5 buffer (a 1:1 mixture of pH 5.0 NaAc and pH 6.0 MES) containing up to 12 μM 5-IAF. The resulting cell lysates were subjected to immunoblot analysis with antibodies to fluorescein. The immunoblot shown in Fig. 2A represents optimal

exposure to X-ray film with regard to obtaining the greatest number of distinguishable bands. As the exposure time increased, the number of bands also increased until they became indistinguishable in the region of the gel corresponding to a molecular size of 50–80 kDa. The same cell lysates were also subjected to immunoblot analysis with antibodies to phosphotyrosine (Fig. 2B). The extent of labeling of several proteins with 5-IAF at pH 5.5 was decreased markedly as a result of prior stimulation of cells with EGF (Fig. 2A), suggesting that sulfhydryl groups of these proteins are susceptible to oxidation by H_2O_2 produced in response to EGF. Incubation of cells with DPI, an inhibitor of the flavoprotein component of NADPH oxidase, increased the extent of 5-IAF labeling of proteins, indicating that DPI inhibited the basal formation of H_2O_2 in unstimulated cells. Furthermore, DPI blocked the EGF-induced decrease in the extent of 5-IAF labeling of proteins, probably as a result of inhibition by DPI of the growth factor-induced production of H_2O_2. Stimulation of cells with EGF induced the tyrosine phosphorylation of several proteins, including the EGF receptor (Fig. 2B). DPI inhibited EGF-induced tyrosine phosphorylation, consistent with the notion that production of H_2O_2 is required for growth factor-induced increases in the extent of protein tyrosine phosphorylation [3]. DPI alone had no visible effect on protein tyrosine phosphorylation. Only a slight increase in protein tyrosine phosphorylation was observed at 200 μM H_2O_2. However, at 1 mM H_2O_2, a significant increase was apparent (not shown).

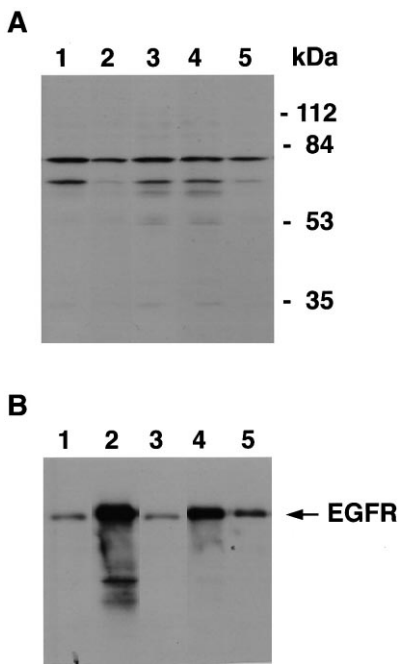

Fig. 2. Effects of exposure of A431 cells to EGF, DPI, or H_2O_2 on the labeling of proteins by 5-IAF (A) and on protein tyrosine phosphorylation (B). Serum-deprived A431 cells were incubated in the absence (lanes 1) or presence of EGF (500 ng/ml) for 20 min (lanes 2), 50 μM DPI for 30 min (lanes 3), 50 mM DPI for 30 min and then with EGF (500 ng/ml) for 20 min (lanes 4), or 200 μM H_2O_2 for 8 min (lanes 5), and were then lysed in an oxygen-free lysis buffer containing 12 μM 5-IAF (A). An aliquot of the lysate was saved in 2 mg/ml of $NaVO_3$ (B). The resulting lysates (25 μg of protein per lane) were fractionated by SDS-PAGE on a 10% Tris-glycine gel and subjected to immunoblot analysis with rabbit antibodies to fluorescein (A) or monoclonal antibodies to phosphotyrosine (B). The positions of prestained molecular size markers (in kDa) are indicated on the right of A, and that of the EGF receptor (EGFR) is shown on the right of B.

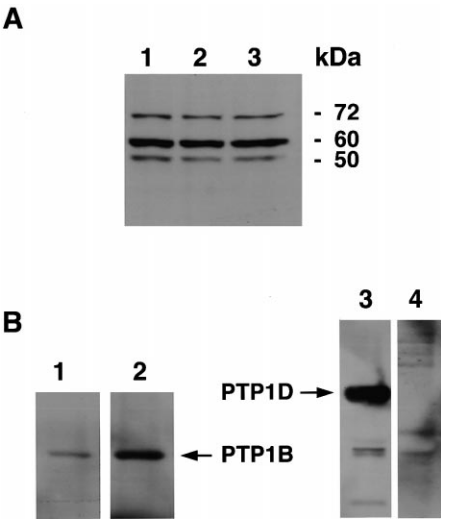

Fig. 3. Effects of stimulation of A431 cells with EGF or H_2O_2 on the labeling of cellular proteins by a reduced concentration of 5-IAF (A) and identification of a 5-IAF-labeled protein as PTP1B (B). A: Serum-deprived A431 cells were incubated in the absence (lane 1) or presence of EGF (500 ng/ml for 20 min) (lane 2) or H_2O_2 (0.2 mM for 2 min) (lane 3), and then lysed in a buffer containing 4 μM 5-IAF. The resulting lysates (25 μg of protein per lane) were subjected to SDS-PAGE on a 10% gel and immunoblot analysis with rabbit antibodies to fluorescein. B: Serum-deprived A431 cells were lysed in a buffer containing 4 μM 5-IAF, and the resulting lysates were subjected to immunoprecipitation with antibodies to PTP1B (lanes 1 and 2) or to PTP1D (lanes 3 and 4). The immunoprecipitates were then subjected to SDS-PAGE on a 10% gel and immunoblot analysis with antibodies to PTP1B (lane 1), or with antibodies to fluorescein (lanes 2 and 4) or to PTP1D (lane 3). The positions of PTP1B and PTP1D are indicated.

Reducing the concentration of 5-IAF in the lysis buffer from the 12 μM used for the experiments shown in Fig. 2A resulted in the detection of fewer 5-IAF-labeled proteins by immunoblot analysis with monoclonal antibodies to fluorescein. At a 5-IAF concentration of 4 μM, only three 5-IAF-labeled bands, with apparent molecular sizes of 76, 60, and 50 kDa, were detected (Fig. 3A). The intensities of these three bands were all decreased after stimulation of cells with EGF or H_2O_2. It may be necessary to point out that oxidation of the 60-kDa band is not clear on this particular blot because its signal is saturated when that of the 50- and 76-kDa bands are optimal for viewing. Because the sizes of the 76- and 50-kDa proteins are similar to those of PTP1D and PTP1B, respectively, we investigated whether the two bands corresponded to the two tyrosine phosphatases. 5-IAF-treated cell lysates were subjected to immunoprecipitation with antibodies specific for PTP1B or for PTP1D, and the resulting immunoprecipitates were subjected to immunoblot analysis with antibodies to fluorescein as well as with corresponding anti-PTP antibodies (Fig. 3B). The 50-kDa protein immunoprecipitated by antibodies to PTP1B was recognized by both antibodies to fluorescein and antibodies to PTP1B, suggesting that the 50-kDa band in Fig. 3A is likely PTP1B. However, the protein specifically immunoprecipitated with antibodies to PTP1D was recognized by antibodies to PTP1D but not by antibodies to fluorescein. This observation suggests that the 76-kDa band

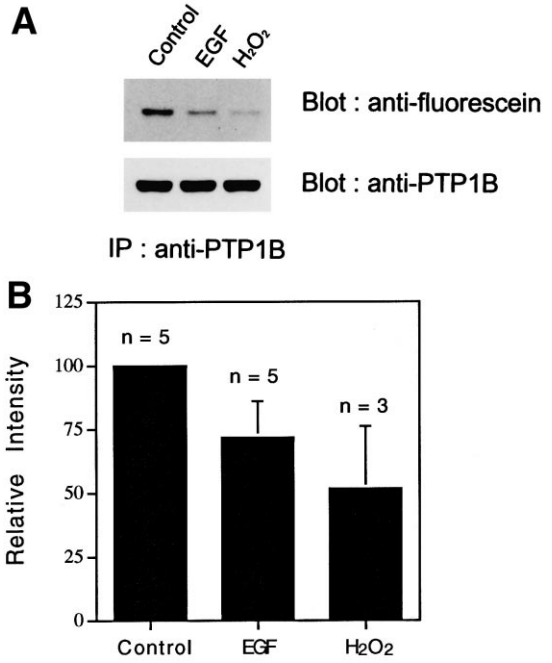

Fig. 4. Effects of stimulation of A431 cells with EGF or H_2O_2 on the labeling of PTP1B with 5-IAF. A: Serum-deprived A431 cells were stimulated and lysed as described in the legend to Fig. 3A. The resulting lysates were subjected to immunoprecipitation (IP) with antibodies to PTP1B, and the immunoprecipitates were subjected to SDS-PAGE on a 10% gel and immunoblot analysis with antibodies to fluorescein (upper panel). The same blot was stripped and re-probed with monoclonal antibodies to PTP1B (lower panel). B: The relative intensity of the fluorescein moiety (Fig. 3A) vs. that of the PTP1B protein (Fig. 3B) was quantitated with a LKB Ultroscan laser densitometer. Data are expressed as a percentage of the value for immunoprecipitates derived from unstimulated A431 cells, and are means ± S.E.M. from five (EGF) or three (H_2O_2) independent experiments.

in Fig. 3A does not contain PTP1D, and that no cysteine residue of PTP1D was available for modification by 5-IAF.

Although PTP1B was shown to be readily modified by 5-IAF, the decrease in the intensity of the 50-kDa 5-IAF-labeled band from A431 cells treated with EGF or H_2O_2 was not necessarily fully attributable to PTP1B alone. To verify that PTP1B was indeed oxidised, we stimulated A431 cells with EGF or H_2O_2, lysed the cells in the presence of 4 μM 5-IAF, subjected the lysates to immunoprecipitation with antibodies to PTP1B, and analyzed the immunoprecipitates by immunoblotting with antibodies to fluorescein (Fig. 4A). The intensity of the 5-IAF-labeled PTP1B band from cells treated with EGF or H_2O_2 was markedly reduced compared with that of the corresponding band from control cells. Data from several independent experiments revealed that the extent of the decrease in 5-IAF labeling of PTP1B from EGF-treated or H_2O_2-treated cells was 28 ± 14% and 53 ± 24%, respectively (Fig. 4B). These results indicate that a cysteine residue of PTP1B is oxidized in response to stimulation of cells with EGF or H_2O_2. Recently, we detected the EGF-dependent oxidation of the active site cysteine of PTP1B using [^{14}C]iodoacetic acid [16]. However, the procedure was insensitive, expensive and cumbersome.

Although we did not identify the specific thiol group (or groups) of PTP1B that was modified by 5-IAF, the active site cysteine (Cys^{215}) is the most likely target because labeling of recombinant human PTP1B with 5-IAF was accompanied by irreversible inhibition of enzyme activity (not shown). Other sulfhydryl modifying reagents such as iodoacetic acid have been shown to modify various PTPs at their active site cysteines, resulting in enzyme inactivation [13–15]. Furthermore, oxidation of protein tyrosine phosphatases by H_2O_2 has been shown to prevent modification of the catalytic cysteine by iodoacetic acid [17]. All PTPs contain a catalytically essential cysteine residue in the signature active site motif HCXXGXXR(S/T). The pK_a values of these active site cysteines are generally between 5.0 and 6.0 [18], indicating that they exist as thiolate anions at physiological pH. The low pK_a values of the active site cysteines are attributable to the fact that the thiolate anion is stabilized by a salt bridge to the conserved arginine residue in the active site motif [19].

PTP1D was not modified by 5-IAF, probably because its active site cysteine is located inside a pocket with restricted access. The peroxidase 1-Cys peroxiredoxin contains a highly reactive cysteine in its active site pocket that is readily oxidized by H_2O_2 at neutral pH, but which is not susceptible to sulfhydryl modifying reagents such as 5-IAF and Ellman's reagent because the diameter of the entrance to the pocket is only 4 Å [20]. It is possible that the active site cysteine of PTP1D is not available for modification by 5-IAF but may be susceptible to oxidation by the smaller H_2O_2. Unresponsiveness of certain PTPases like PTP1D to inactivation by H_2O_2 might explain why protein tyrosine phosphorylation was insensitive to the added H_2O_2, whereas protein cysteine modification was decreased significantly in Fig. 2.

A physiological role for H_2O_2 and other cellular oxidants as inhibitors of PTPs has been proposed on the basis of the observation that the extent of protein tyrosine phosphorylation is increased in cells treated with exogenous oxidants. It has been thought that inhibition of PTPs by oxidants in cells might render the ligand-independent, basal activity of PTKs sufficient to increase the extent of protein tyrosine phosphorylation. Our current data indicate that H_2O_2 generated in response to EGF is sufficient to cause inactivation of a PTP1B. This result, together with the previous observation that growth factor-induced protein tyrosine phosphorylation requires H_2O_2 generation [1,3], indicates that both the activation of PTKs as well as the inhibition of PTPs through H_2O_2 production might be necessary for growth factors to increase the extent of protein tyrosine phosphorylation in cells.

We have developed a procedure to detect proteins that might be readily susceptible to oxidation at cysteine residues by H_2O_2 in cells. This method is based on the fact that, at neutral pH, the thiolate anions derived from cysteine residues with a low pK_a are likely susceptible to both alkylation by 5-IAF and oxidation by H_2O_2. Thus, prior oxidation of a protein by H_2O_2 will reduce the amount of 5-IAF that can subsequently be incorporated. Given that the incubation with 5-IAF is performed at pH 5.5, only those proteins that contain cysteine residues with low pK_a values are labeled. Although iodoacetamide also reacts with histidine at basic pH [21], albeit more slowly than it alkylates cysteine, the short incubation time (10 min) minimizes the labeling of residues other than cysteine. The fact that most of the 5-IAF-labeled proteins from A431 cells were sensitive to H_2O_2 suggests that the major site of labeling was indeed cysteine. Not every protein with a low-pK_a cysteine is likely to be detected by this method: because of the bulkiness of 5-IAF, certain such cysteines

located inside small pockets would not be labeled. By limiting the concentration of 5-IAF in the lysis buffer, we were able to reduce the number of 5-IAF-labeled proteins to three. One of these three proteins, PTP1B, is relatively abundant (0.1% of total soluble protein in A431 cells) and contains a low-pK_a cysteine. The other two labeled proteins are therefore likely also to be abundant and to contain a cysteine with a low pK_a.

Whereas the signaling role of H_2O_2 is now widely accepted, the intracellular targets of this molecule have remained obscure. With the procedure described here, it is now possible to identify potential intracellular targets of H_2O_2. Furthermore, it should be possible to enrich or purify such target proteins by labeling with 5-IAF and affinity chromatography based on monoclonal antibodies to fluorescein.

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