Human Ran Cysteine 112 Oxidation by Pervanadate Regulates Its Binding to Keratins*

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We used a proteomic approach to identify proteins that associate with keratins 8 or 18 (K8/K18) in a pervanadate-dependent manner. Pervanadate triggers Ran-K8/K18 binding and a gel-migration-shift of Ran from 25 to 27 kDa, which does not occur upon exposure to H2O2 or vanadate or if pervanadate is excluded during cell solubilization. Generation of 27-kDa Ran is not related to hyperphosphorylation, is heat-insensitive, but occurs upon conversion of Ran cysteines to cysteic acid. The pervanadate-mediated Ran cysteine → cysteic acid oxidation and its related gel migration shift affects other proteins including actin. Mutation of the three Ran cysteines (Cys-85, -112, and -120) showed that Ran Cys-112 oxidation generates 27-kDa Ran and accounts for its keratin binding. Proteasome inhibition accentuates Ran-keratin binding after cell exposure to pervanadate. Therefore, cell-free exposure to pervanadate causes cysteine to cysteic acid oxidation of Ran and several other proteins and Ran-K8/K18 association. In cells, stabilization of oxidized Ran by proteasome inhibition promotes Ran-keratin interaction. Keratin sequestration of oxidized Ran may provide a back-up protective mechanism in some cases of oxidative injury.

Epithelial cells express more than 20 keratin (K) gene products that are divided into type I (K9–K20) and type II (K1–K8), which exist as obligate noncovalent heteropolymers and serve as epithelial cell-specific markers (1, 2). The keratin complement of epithelial cells is unique depending on the cell type, as exemplified by glandular epithelia that express primarily K8/K18 with variable levels of K7/K19/K20 and by epidermal epithelia that preferentially express K5/K14 basally or K1/K10 suprabasally. The tissue specialization of keratin and other intermediate filament (IF) proteins is highly relevant as reflected by a broad range of tissue-specific diseases that are caused by IF gene mutations (3).

IF regulation and function are modulated in large part by phosphorylation and a relatively small number of characterized IF-associated proteins (4–6). Keratin phosphorylation occurs primarily on serine residues, but tyrosine phosphorylation of K8 and K19 was demonstrated when cells were cultured in the presence of the protein tyrosine phosphatase (PTP) inhibitor pervanadate (PV) (7). The use of Ser/Thr phosphatase inhibitors such as okadaic acid (OA) has been valuable in helping identify K8/K18 interaction with 14-3-3 proteins and Raf-1 kinase (8, 9). However, whether cell exposure to PV and consequent modulation of tyrosine phosphorylation could regulate keratin interaction with an associated protein is unknown.

Protein tyrosine phosphorylation is pivotal for regulating a variety of fundamental cellular processes (10, 11). The established PTP inhibitors vanadate and PV (vanadate mixed with H2O2 generate PV) are commonly used to study a broad range of biologic processes. PV is significantly more effective than vanadate in increasing cellular tyrosine phosphorylation (12, 13), and PV treatment of cells leads to tyrosine hyperphosphorylation of many proteins (14–16). Although the precise details of the inhibitory effect of PV on phosphatases are not completely understood, PTP oxidation is a likely key mechanism as demonstrated for the irreversible inhibition of PTP1B by PV via oxidation of its catalytic cysteine residue (17).

Oxidative stress has been associated with several human degenerative disorders (18–20), as supported by the accumulation of oxidized cellular proteins due to increased protein oxidation or and reduced elimination of the modified proteins. For example, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is modified to cysteic acid in the brains of patients with Alzheimer and Parkinson diseases, suggesting a link between oxidative damage of neuronal proteins and the pathogenesis of Alzheimer and Parkinson diseases (21, 22). Also, DJ-1, the mutation of which causes autosomal recessive Parkinson disease (23), oxidizes its Cys-106 to cysteic acid upon the exposure of umbilical vein endothelial cells to H2O2 (24). Interestingly, DJ-1 Cys-106 plays an important neuroprotective role in the response to oxidative stress (25). Thus, oxidative injury and consequent cysteine to cysteic acid oxidation may be involved in the pathogenesis of several human disorders.

Ran is an abundant and highly conserved GTPase of the Ras superfamily and is essential for viability in every tested orga...
FIG. 1. Association of K8/K18 with Ran is induced after pervanadate treatment. A, HT29 cells were treated with OA, PV, An, or Me2SO alone as a control (C) followed by solubilization with Nonidet P-40 (NP40) and then precipitation using anti-K8/K18 or nonspecific IgG. Immunoprecipitates (lanes 1-8) and Nonidet P-40 fractions (lanes 9-12) from the cell treatments were separated by SDS-PAGE followed by blotting with anti-Ran Ab. B, HT29 cells were cultured with 1 mM OA, H2O2, or PV and then solubilized with 1% Nonidet P-40 in phosphate-buffered saline containing 0.1 mM OA, H2O2, or PV in parallel to the original treatments. Nonidet P-40 fractions were prepared from total cell lysates (Total) by centrifugation. A portion of the Nonidet P-40 lysates was used for precipitation of K8/K18 followed by blotting using anti-Ran Ab. C, HT29 cells were treated with PV for the indicated times and then solubilized with 1% Nonidet P-40 in phosphate-buffered saline (+0.1 mM PV) followed by K8/K18 precipitation (left panel). Similarly treated duplicate cells were immediately solubilized with hot SDS-containing Laemmli buffer without PV (right panel). Samples were blotted with anti-Ran Ab, and the area highlighted by broken lines (right panel) corresponds to the position of the 27-kDa Ran seen in the left panel. D, HT29, AsPC1, and Huh7 cells were processed as follows: (i) untreated cells + hot sample buffer (lanes 1, 5, and 9); (ii) cells + PV followed by washing and then by adding hot sample buffer (lanes 2, 6, and 10); (iii) cells + PV followed by washing and then by solubilizing with 1% Nonidet P-40 + PV (i.e. cell-free exposure to PV) (lanes 3, 7, and 11); (iv) cells + PV and then washing and solubilizing with 1% Nonidet P-40 without PV (lanes 4, 8, and 12). The asterisk indicates the position of the Ab band.

Keratins Bind to Oxidized Ran

Keratins, a family of low-molecular-weight keratins (26, 27). They shuttles between the nucleus and cytoplasm, plays an important role in nucleocytosol transport (28), and is implicated in several nuclear functions including transport, cell cycle control, and post-mitotic nuclear assembly (29, 30). We hypothesized that tyrosine phosphorylation may regulate keratin interaction with hitherto uncharacterized binding proteins and utilized PV treatment of cells as a handle to study such potential interactions. We show that PV induces K8/K18-Ran binding, which is associated with the slower migration of Ran on SDS-PAGE. These findings are not caused by tyrosine hyperphosphorylation but by oxidation of an exposed Ran cysteine residue. Our results suggest that the cytoprotective effects of keratins can be related, in part, to sequestration of damaged oxidized proteins during conditions when the proteinome system is overwhelmed or inhibited.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human (h) HT29 (colon), Huh-7 (liver), and AsPC1 (pancreas) cells and human Ran cDNA were obtained from American Type Culture Collection. PV was prepared by mixing 1% vanadate and 30% hydrogen peroxide (5:1, v/v). After 5 min (22 °C), the generated PV was added to cell cultures (1 mM final) or to solubilization buffers (0.1 mM). The antibodies (Abs) used included those directed to: h-Ran amino acids 7–171 (BD Transduction Laboratories) or 207–216 (Sigma); actin (Calbiochem); pET protein expression system (Novagen), and QuickChange site-directed mutagenesis kit (Stratagene).32P-labeled γ-ATP/GTP and α-GTP were purchased from PerkinElmer Life Sciences.

Immunoprecipitation, Gel, and Protein Analysis—HT29 cells were treated with PV (1 mM, 90 min), OA (1 µg/ml, 2 h), anisomycin (An, 10 µg/ml, 20 h), or carrier (0.1% Me2SO for 20 h; used as solvent for OA/An). Cells were solubilized (2 h, 4 °C) with 1% Nonidet P-40 in phosphate-buffered saline containing 0.1 mM OA, H2O2, or PV in parallel to the original treatments. Pakistan P-40 fractions were prepared from total cell lysates (Total) by centrifugation. A portion of the Nonidet P-40 lysates was used for precipitation of K8/K18 followed by blotting using antiserum (C). HT29 cells were treated with PV for the indicated times and then solubilized with 1% Nonidet P-40 in phosphate-buffered saline (+0.1 mM PV) followed by K8/K18 precipitation (left panel). Similarly treated duplicate cells were immediately solubilized with hot SDS-containing Laemmli buffer without PV (right panel). Samples were blotted with anti-Ran Ab, and the area highlighted by broken lines (right panel) corresponds to the position of the 27-kDa Ran seen in the left panel. D, HT29, AsPC1, and Huh7 cells were processed as follows: (i) untreated cells + hot sample buffer (lanes 1, 5, and 9); (ii) cells + PV followed by washing and then by adding hot sample buffer (lanes 2, 6, and 10); (iii) cells + PV followed by washing and then by solubilizing with 1% Nonidet P-40 + PV (i.e. cell-free exposure to PV) (lanes 3, 7, and 11); (iv) cells + PV and then washing and solubilizing with 1% Nonidet P-40 without PV (lanes 4, 8, and 12). The asterisk indicates the position of the Ab band.

Mass Spectrometry and Amino Acid Composition—Keratin-associated proteins were purified from PV-treated HT29 cells by K8/K18 co-immunoprecipitation. As a specificity control, precipitates were also prepared using protein A-Sepharose beads alone. After gel separation, trypsin was used for in-gel-digestion followed by peptide extraction and then identification of co-precipitated proteins by mass spectrometry-
immunoprecipitates (solubilized with Nonidet P-40 (a)
ble Nonidet P-40 fractions of HT29 cells were mixed with 50
mM, 60 min, 4 °C). Labeling was quenched by adding sample buffer (5
mM, 95 °C) followed by SDS-PAGE, transfer to a membrane, autoradiography, and then anti-Ran blotting. Ran was purified by immuno-
precipitation from HT29 cells that were cultured and solubilized (b)
and then incubated with ±5 units of alkaline phosphatase (60 min,
37 °C). Inhibition of proteasomal degradation was done by culturing
cells (8 h) with lactacystin (20 μM) or N-acetyl-l-leucinyl-l-leucinyl-
orn-leucinyl (100 μM) or 0.1% MeSO (vehicle control). PV (1 mM, 1.5 h) without changing the medium. Cells were then solu-
bilized (−PV) followed by K8/K18 precipitation, blotting with anti-Ran Ab, and then Coomassie Blue staining of the membrane. The arrowhead indicates position of normal nonoxidized 25-kDa Ran.

were mixed with 300 μl of bacterially expressed wild type (WT) or
mutant Ran in buffer B containing 0.1 mM PV (4 °C, 16 h). The beads
were washed, and bound proteins was eluted from the beads with Laemmli sample buffer and then analyzed by blotting.

RESULTS AND DISCUSSION

Identification of Ran as a Novel Keratin-associated Protein—To identify K8/K18-binding proteins that may be regulated
by tyrosine phosphorylation, K8/K18 immunoprecipitates were obtained from HT29 cells that were treated and solubil-
in the presence or absence of PV. One major protein that specifically associated with K8/K18 precipitates isolated from
PV-treated cells was identified by mass spectrometry as the GTP-binding protein Ran (not shown). This was confirmed by
immunoblot analysis of K8/K18 precipitates obtained from cells

Expression of Ran Mutants and Reconstitution Experiments—h-Ran
cDNA was amplified from a pCMV-Sport6-Ran vector followed by
subcloning into pET23A (+) and then generation of four Ran mutants
(C85F, C112F, C120A, and triple C85F,C112F,C120A mutant), which
were confirmed by DNA sequencing. Ran was extracted from the bac-
eria using lysozyme and freeze-thawing. For the in vitro reconstitution
assays, equal amounts of washed Sepharose-Ab-K8/K18 beads (30 μl)

were prepared from HT29 cells that were solubilized with Nonidet P-40 (−/−PV; lanes 1 and 2). A duplicate of the
immunoprecipitates in lane 2 was incubated with alkaline phosphatase (AP) (lane 3) followed by blotting with anti-Ran Ab.

Nonidet P-40 fractions of HT29 cells were mixed with [γ-32P]GTP, [γ-32P]GTP, or [α-32P]GTP (+−PV). Protein lysates were then transferred to a mem-

brane followed by autoradiography and then blotting with anti-Ran Ab. No [32P]Po labeling is found where normal and shifted Ran are predict-
able located (area inside broken lines). Arrows highlight [32P]-labeled proteins whose identities are not known. C, HT29 cell Nonidet P-40
lysates were stored at 4 °C or heated (90 °C) for 5 min. The lysates were then further incubated at 4 °C (+−PV) followed by blotting with anti-
Ran Ab. D, lanes 1−4, K8/K18 or control (C) precipitates bound to Sepharose beads were incubated with r-Ran +−PV. The beads were then washed and analyzed by blotting using anti-Ran Ab. Lanes 5 and 6, r-Ran was incubated +−PV followed by blotting.

based methods (peptide mass mapping and de novo sequencing whenever necessary). For amino acid composition, acid hydrolysis of proteins was carried out in constantly boiling HCl followed by analysis with a Beckman System 6300 High Performance Analyzer.
treated with PV, OA, An, or Me₂SO (Fig. 1A). The analysis of K8/K18 precipitates obtained from cells treated with OA or An was included as "non-Tyr" phosphorylation controls since such treatments induce Ser/Thr keratin hyperphosphorylation due to Ser/Thr phosphatase inhibition (OA) or induction of apoptosis (An) (4). Notably, there was a Ran-Ab-reactive 27-kDa (in addition to the normal 25 kDa) species seen only in the presence of PV (Fig. 1A and B). Generation of the slower migrating 27-kDa Ran species and Ran association with K8/K18 were confirmed using a second anti-Ran Ab that recognizes an independent Ran epitope (not shown). The migration shift of Ran occurred only after treatment with PV but not with vanadate or H₂O₂ alone (Fig. 1B). Therefore, the altered migration of Ran suggests a post-translational modification in response to PV but not vanadate or H₂O₂.

The PV-induced 27-kDa Ran Is Due to Cysteine Oxidation but Is Not Due to Hyperphosphorylation—We hypothesized that the SDS-PAGE migration shift in Ran may be due to a phosphorylation event since PV is a highly effective PTP inhibitor, and retarded migration of many proteins is frequently related to phosphorylation (14–16, 31). However, this hypothesis became suspect when formation of the slower migrating 27-kDa Ran species and its association with K8/K18 were noted to be independent of the exposure time of cells to PV (Fig. 1C, left panel). Also, if PV is excluded during solubilization, only the 25-kDa Ran species is seen despite cell exposure to PV prior to their lysis (Fig. 1C, right panel). Formation of 27-kDa Ran was not unique to HT29 cells, as it was noted in two other tested cell lines but only when PV was added during solubilization (Fig. 1D).
We then used several approaches to exclude cell-free in vitro phosphorylation or another enzymatic process as the cause of 27-kDa Ran formation. First, 27-kDa Ran is not affected by treatment with alkaline phosphatase (Fig. 2A). Second, incubation of a Nonidet P-40 lysate with various donor phosphates ([γ-32P]ATP/GTP or [α-32P]GTP) in the presence or absence of PV does not generate a labeled Ran-like species although 27-kDa Ran is the primary formed species in the presence of PV (Fig. 2B, lanes 2, 4, and 6), whereas other in vitro phosphorylated proteins are generated (Fig. 2B, arrows). Third, formation of 27-kDa Ran was heat-insensitive (Fig. 2C) and occurs by simply mixing PV with r-Ran (Fig. 2D, compare lanes 5 and 6). Furthermore, association of Ran with K8/K18 can be reconstituted in vitro using r-Ran and K8/K18 immunoprecipitates but only in the presence of PV (Fig. 2D, lanes 1–4). Taken together, these results indicate that an enzymatic modification of Ran is unlikely to account for its binding to K8/K18 or formation of 27-kDa Ran.

The above findings, coupled with the previous report that inhibition of PTP-1B by PV was caused by cysteine oxidation of the phosphatase (17), raised the possibility that Ran oxidation is the trigger for its migration shift and association with K8/K18. Support for this possibility was obtained after amino acid analysis of native and PV-treated r-Ran, which showed that all three cysteines of Ran become oxidized to cysteic acid in the presence of PV (Supplemental Table 1).

PV Induces Generalized Protein Cysteine Oxidation and SDS-PAGE Migration Shifts—We tested the effect of PV on actin and Gβ. Actin was selected for this analysis because it is an abundant Cys-containing protein. Gβ was examined because it undergoes in vitro phosphorylation by [γ-32P]GTP (32), and the in vitro labeling by [γ-32P]GTP in the absence of PV (Fig. 2B, lane 3) highlighted a major species of 36 kDa (likely corresponding to Gβ) that was almost completely abolished with generation of a faint slower migrating species in the presence of PV (Fig. 2B, lane 4). Cell-free PV treatment of Nonidet P-40 lysates, isolated from HT29 cells, also showed the SDS-PAGE migration of actin and Gβ (Fig. 3A) but did not affect the migration of two unknown species that cross-reacted with anti-Gβ Ab (Fig. 3A, arrows). Treatment of purified actin with PV followed by amino acid analysis confirmed that all five cysteines of actin become oxidized to cysteic acid (Supplemental Table 2). Thus, PV-induced protein oxidation on cysteine residues appears to be a generalized occurrence that affects several Cys-containing proteins.

We then tested whether oxidized actin or Gβ also binds to keratins. As shown in Fig. 3B, the oxidized form of Gβ (Gβ contains multiple cysteines) co-immunoprecipitates with K8/K18 when isolated from PV-treated cells and not from untreated cells. However, actin was not detected using similar K8/K18 immunoprecipitation conditions (not shown). This suggests that some but not all oxidized proteins can bind to keratins, which is likely related to the context of the oxidized epitope within the protein backbone.

Oxidized Ran Is Rapidly Degraded in Vivo by the Proteasome—The potentially deleterious effects of oxidation and generation of reactive oxygen species can be overcome by several cellular defense mechanisms including superoxide dismutase and catalase. However, oxidized proteins often loose their function and are potentially toxic if allowed to accumulate (33, 34). Oxidized proteins are susceptible to rapid degradation by the proteasome, which provides a likely protective mechanism to prevent cellular accumulation of potentially toxic damaged proteins (34). Thus, we tested whether proteasome inhibition helps to detect the 27-kDa Ran and its K8/K18 association in vivo. Oxidized Ran co-immunoprecipitated with K8/K18 when cells are cultured in the presence of PV and proteasome inhibitors but not in the presence of PV alone (Fig. 3C). This explains why PV-oxidized Ran is readily seen in cell-free systems (Fig. 1) but is difficult to detect in cells. Notably, calpain (PD150606) and lysosome (pepsstatin/leupeptin) inhibitors had a minimal effect on 27-kDa Ran turnover (not shown). These findings are consistent with earlier studies that demonstrate accumulation of oxidized proteins and a concomitant decline in proteolytic activity with age (35, 36).

PV-induced Oxidation of Ran Cys-112 Accounts for Formation of 27-kDa Ran and Keratin-Ran Association—We hypothesized that oxidation of one or more of the Ran cysteines may account for generation of 27-kDa Ran and for Ran-keratin binding. To test this hypothesis, we generated individual or combined human Ran cysteine mutants and demonstrated their expression in *Escherichia coli* (Fig. 4, A and B). We then examined the effect of Cys mutation on the ability to form 27-kDa Ran and showed that mutation of the three Ran cysteines or of Ran Cys-112 alone ablated the altered Ran gel migration after exposure to PV, whereas the Ran Cys-85 or Cys-120 mutants had no effect (Fig. 4C). Similarly, reconstitution of Ran-keratin binding using WT or mutant bacterially expressed Ran showed that Cys-112 is the critical cysteine that, when oxidized to cysteic acid, causes Ran-keratin binding (Fig. 4D).

Our overall findings led to two important conclusions. First, PV-mediated oxidation involved not only Ran but also actin and Gβ and was likely to affect many other cyst-containing proteins by converting cysteine to cysteic acid. This PV-induced oxidation resulted in SDS-PAGE retardation (Fig. 5, inset panel) that mimicked migration changes caused by phosphorylation. Thus, caution should be used in assigning such shifts to phosphorylation, particularly since PV indirectly also increases tyrosine phosphorylation of many proteins. Second, we propose a potential unique role of keratins in sequestering oxidized damaged proteins, such as Ran, when proteasome function is inhibited or overwhelmed (Fig. 5). Such a role meshes well with the known function of K8/K18 in cytoprotection, as demonstrated particularly in the liver (37). It will be important to determine whether keratin sequestration of oxidized proteins extends to other keratins or other IF proteins and the scope of other non-Ran oxidized proteins that can be involved in such binding. Keratin binding to oxidized proteins does involve other non-Ran proteins, as demonstrated herein for Gβ (Fig. 3B). A data bank search of the Ran sequence surrounding Cys-112 did not show homology with other non-Ran proteins. This, coupled with the ability of K8/K18 to bind oxidized Ran and Gβ, suggested the importance of nonlinear, and likely charged, epitopes in such binding. Interestingly, the Ran crystal structure (38) supports the importance of oxidized Ran Cys-112 in binding to K8/K18 since Cys-112 is exposed on the surface of the Ran molecule in contrast to Ran Cys-85 and Cys-120, which are more hidden. It remains to be determined whether Ran Cys-112 oxidation interferes with any of its nucleo-cytoplasmic functions.

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