Phosphorylation and Dephosphorylation of Tyrosine 141 Regulate Stability and Degradation of INrf2

A NOVEL MECHANISM IN NRF2 ACTIVATION

Abhinav K. Jain, Shilpi Mahajan, and Anil K. Jaiswal

From the Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201

INrf2-Nrf2 proteins are sensors of chemical/radiation stress. Nrf2, in response to stresses, is released from INrf2. Nrf2 is translocated into the nucleus where it binds to the antioxidant response element and coordinately activates the expression of a battery of genes that protect cells against oxidative and electrophilic stress. An autoregulatory loop between INrf2 and Nrf2 regulates their cellular abundance. Nrf2 activates INrf2 gene expression, and INrf2 serves as an adapter for degradation of Nrf2. INrf2 is a pseudokinase that contains a unique model for nuclear/cytoplasmic collaboration. Under physiological conditions, phosphorylated INrf2 interacts with the nuclear export factor, CRM1, to export Nrf2 from the nucleus. Moreover, it has been proposed that result in liberation followed by stabilization of Nrf2. This mechanism leads to the activation of ARE-mediated gene expression. Several mechanisms both by modification of Nrf2 and/or INrf2 have been proposed that result in inhibition of ARE-containing defensive genes including NQO1 (NAD(P)H:quinone oxidoreductase 1).

Factors (1). Nrf2 heterodimerizes with small Mafs and/or c-Jun to induce the ARE-mediated gene expression. Several mechanisms both by modification of Nrf2 and/or INrf2 have been proposed that result in inhibition of ARE-containing defensive genes including NQO1 (NAD(P)H:quinone oxidoreductase 1). Nrf2 heterodimerizes with small Mafs and/or c-Jun to induce the ARE-mediated gene expression. Several mechanisms both by modification of Nrf2 and/or INrf2 have been proposed that result in inhibition of ARE-containing defensive genes including NQO1 (NAD(P)H:quinone oxidoreductase 1).

This article has been retracted by the publisher. In Fig. 3C, cytosol panels, lanes 5 and 6 were duplicated in the INrf2-V5 immunoblot. Additionally, lanes 5 and 6 were duplicated in the INrf2-Y141A-V5 immunoblot. In Fig. 3C, nucleus panels, lanes 1 and 2 of the INrf2-V5 immunoblot were duplicated. Additionally, the Lamin B immunoblot was duplicated in the top and bottom panels.

The abbreviations used are: ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase 1; PKC, protein kinase C; DM, double mutant; RIPα, radioimmune precipitation assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; IP, immunoprecipitation; BTB, brc-a-bric, tramtrack, broad complex.

RETRACTED
January 6, 2017
Phosphorylation and Dephosphorylation Control of INrf2

In this study, we demonstrate a novel mechanism of regulation of stability/degradation of INrf2 and its downstream gene expression. We show that INrf2 protein phosphorylated at tyrosine 141 is not yet known (18, 19).

In this study, we demonstrate a novel mechanism of regulation of stability/degradation of INrf2 and its downstream gene expression. We show that INrf2 protein phosphorylated at tyrosine 141 is not yet known (18, 19).

In related experiment, Hepa-1 cells were transfected with 0.5 μg of pcDNA or INrf2 or INrf2Y141A-V5 or INrf2Y208A-V5 or INrf2Y141A-Y208A-V5. 0.05 μg of Renilla luciferase was included as a control of transfection efficiency. The cells were harvested 36 h after transfection and analyzed by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody.

**Immunoprecipitation and Phosphorylation of Endogenous INrf2**—The cells either transfected or treated for appropriate times were washed twice with ice-cold PBS and harvested. The total cell lysates were prepared in RIPA buffer as described above. Five hundred micrograms of extract was used for immunoprecipitation. Briefly, lysate was incubated with 1× PBS and lysed in 1× passive lysis buffer from a dual luciferase reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (12).

In related experiment, Hepa-1 cells were transfected with 0.5 μg of pcDNA or INrf2 or INrf2Y141A-V5 or INrf2Y208A-V5 or INrf2Y141A-Y208A-V5. 0.05 μg of Renilla luciferase was included as a control of transfection efficiency. The cells were harvested 36 h after transfection and analyzed by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody.

**Immunoprecipitation and Phosphorylation of Endogenous INrf2**—The cells either transfected or treated for appropriate times were washed twice with ice-cold PBS and harvested. The total cell lysates were prepared in RIPA buffer as described above. Five hundred micrograms of extract was used for immunoprecipitation. Briefly, lysate was incubated with 1× PBS and lysed in 1× passive lysis buffer from a dual luciferase reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (12).

In related experiment, Hepa-1 cells were transfected with 0.5 μg of pcDNA or INrf2 or INrf2Y141A-V5 or INrf2Y208A-V5 or INrf2Y141A-Y208A-V5. 0.05 μg of Renilla luciferase was included as a control of transfection efficiency. The cells were harvested 36 h after transfection and analyzed by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody.

**Immunoprecipitation and Phosphorylation of Endogenous INrf2**—The cells either transfected or treated for appropriate times were washed twice with ice-cold PBS and harvested. The total cell lysates were prepared in RIPA buffer as described above. Five hundred micrograms of extract was used for immunoprecipitation. Briefly, lysate was incubated with 1× PBS and lysed in 1× passive lysis buffer from a dual luciferase reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (12).

In related experiment, Hepa-1 cells were transfected with 0.5 μg of pcDNA or INrf2 or INrf2Y141A-V5 or INrf2Y208A-V5 or INrf2Y141A-Y208A-V5. 0.05 μg of Renilla luciferase was included as a control of transfection efficiency. The cells were harvested 36 h after transfection and analyzed by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody.

**Immunoprecipitation and Phosphorylation of Endogenous INrf2**—The cells either transfected or treated for appropriate times were washed twice with ice-cold PBS and harvested. The total cell lysates were prepared in RIPA buffer as described above. Five hundred micrograms of extract was used for immunoprecipitation. Briefly, lysate was incubated with 1× PBS and lysed in 1× passive lysis buffer from a dual luciferase reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (12).
Phosphorylation and Dephosphorylation Control of INrf2

added and boiled, and immunoprecipitates were resolved on a 10% SDS-PAGE followed by immunoblotting with anti-V5-HRP, anti-Tyr(P), or anti-FLAG-HRP antibodies. FLAG immunoprecipitation was done using the anti-FLAG-M2 agarose beads (Sigma).

In related experiments, 1.0 mg of Hepa-1 cell total lysate was immunoprecipitated with either IgG (control) or mouse anti-INrf2 antibody (Santa Cruz, CA). The immunoprecipitate was analyzed by Western blotting and probing with anti-phosphotyrosine antibody. In reverse IP, Hepa-1 total cell lysate was immunoprecipitated with anti-phosphotyrosine antibody followed by Western analysis with anti-INrf2 antibody.

INrf2 Phosphorylation Analysis in Transfected Cells—Hepa-1 cells transfected with INrf2-V5 or INrf2Y141A-V5 or with INrf2Y208A-V5 or INrf2Y141A-Y208A-V5 double mutant or treated with cycloheximide + H2O2 ± genistein were lysed in RIPA buffer supplemented with tyrosine phosphatase inhibitor mixture and protease inhibitor mixture. 1 mg of total cell lysate was used to immunoprecipitate with anti-V5 or anti-Tyr(P) antibodies as described above. The input and immunoprecipitates were boiled in SDS sample dye and resolved on 10% SDS-PAGE and immunoblotted with respective antibodies.

Degradation of INrf2—Hepa-1 cells were grown in 6-well tissue culture plates and were transfected with 0.5 μg of either pcDNA-INrf2-V5 or pcDNA-INrf2Y141A-V5 plasmids. Twenty-four h after transfection, the cells were pretreated with either Me2SO or MG132 (20 μM) for 8 h. The cells were washed twice with ice-cold PBS and lysed in RIPA buffer as described above. For different time points (1, 3, 6, or 8 h) the treated or anti-INrf2 antibody. 50 μg of total cell lysate was used for immunoprecipitation with anti-V5 or anti-Tyr(P) antibodies as described above. The input and immunoprecipitates were boiled in SDS sample dye and resolved on 10% SDS-PAGE and immunoblotted with respective antibodies.

Pulse-Unless Chase—Hepa-1 cells were transfected with INrf2-V5 or INrf2Y141A-V5. 24 h after transfection cells were incubated with methionine-deficient Dulbecco’s modified Eagle’s medium (Sigma) for 30 min. The cells were then labeled with methionine-deficient Dulbecco’s modified Eagle’s medium containing ~200 μCi of [35S]methionine mixture (Exper35S35S; PerkinElmer Life Sciences), for 1 h at 37 °C (Pulse). After rinsing with normal culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum), the cells were chased by normal culture medium supplemented with 100 μg/ml L-methionine for 1, 3, 6, and 8 h. MG132 (20 μM) was added wherever indicated. The cells were rinsed once with PBS and lysed in RIPA buffer for 30 min. Insoluble cellular debris was cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. After centrifugation, the supernatants were used for immunoprecipitation with anti-V5 antibody as described earlier. Immunoprecipitates were boiled in 1× SDS buffer and resolved on 10% SDS gel. The gel was treated with Amplify solution to enhance the 35S signal, dried, and autoradiographed.

In Vitro Binding—The in vitro transcription/translation of the plasmids encoding INrf2-V5, INrf2Y141A-V5, and FLAG-Nrf2 were performed using the Tnt-coupled rabbit reticulocyte lysate system (Promega). Redivue 1-[35S]methionine (Amersham Biosciences) substituted methionine in the reactions to radiolabel the translated proteins. The plasmid encoding luciferase provided in the kit was used as a control for the transcription/translation reaction. After the coupled transcription/translation, the proteins were checked for their correct size by SDS-PAGE and autoradiography. All of the in vitro transcribed/translated proteins gave the expected size bands. An in vitro binding assay was performed as described earlier (13). Briefly, 5 μl of each in vitro translated protein (INrf2-V5 + FLAG-Nrf2 or INrf2Y141A-V5 + FLAG-Nrf2) in protein binding buffer (1 M Tris, pH 7.5, 2 M NaCl, 10% glycerol, 10% Nonidet P-40, 1 M sodium vanadate supplemented with proteinase inhibitors) were mixed and incubated at 37 °C for 30 min. This was followed by the addition of 2.5 μg of anti-V5 antibody and sufficient protein A binding buffer to make the volume 100 μl and incubated overnight at 4 °C with shaking. After incubation, 10 μl of protein A beads (Santa Cruz Bio- technologies Inc., CA) were added and incubated for 1 h at 4 °C. The beads were washed twice with the protein binding buffer. In a similar binding experiment, protein mixtures were immunoprecipitated with either IgG (control) or mouse anti-FLAG-M2 beads (Sigma). Finally, the beads were washed twice with SDS sample dye and analyzed by SDS-PAGE as described above.

In Vivo Dimerization—The in vivo transcription/translation of the plasmids encoding INrf2-V5 and INrf2Y141A-V5 was performed as described above. 5 μl of each in vitro translated protein was incubated with 0.005, 0.01, or 0.02% glutaraldehyde for 30 min at room temperature. The reaction was terminated by adding SDS sample dye. The samples were resolved on SDS-PAGE and autoradiographed.

In Vivo Dimerization—Hepa-1 cells were cultured in 6-well plates and transfected with 0.5 μg of INrf2-V5 or INrf2Y141A-V5. 24 h after transfection the cells were washed and incubated with fresh medium containing 0.005, 0.01, or 0.02% glutaraldehyde at room temperature for 30 min. The cells were washed twice with PBS and lysed with RIPA buffer as described above. 50 μg of total cell lysate was immunoblotted with anti-V5-HRP and anti-actin antibodies.

RESULTS

Tyrosine 141 Mutation Renders INrf2 Protein Unstable—Mouse INrf2 protein consists of five discrete domains (Fig. 1A). These include the N-terminal region, the bric-a-bric, tramtrack, broad complex (BTB), the intervening region, the diglycine repeats, and the C-terminal regions. Several cysteine residues have been identified in the BTB domain that are crucial for dimerization and function of INrf2. The amino acid sequence of mouse INrf2 protein was aligned with human, rat, Xenopus, and zebrafish INrf2 sequences to identify conserved domains of putative significance. Two putative tyrosine phos-
Phosphorylation and Dephosphorylation Control of INrf2

INrf2Y141A, INrf2Y208A, and double mutant INrf2Y141A-Y208A. Western analysis of Hepa-1 cells transfected with INrf2-V5 and mutant-V5 plasmids revealed expression of comparable amounts of INrf2-V5 and mutant INrf2-Y208A-V5 proteins (Fig. 2A, lanes 2 and 4). Interestingly, the transfection of mutants INrf2Y141A-V5 and INrf2DM-V5 plasmids showed significantly lower amounts of proteins (Fig. 2, lanes 3 and 5). In the same experiment, the treatment of transfected cells with proteasome inhibitor MG132 significantly increased the mutant proteins INrf2Y141A and INrf2DM (Fig. 2A, compare lanes 7 and 9 with lanes 3 and 5). However, the treatment with MG132 had no effect on INrf2-V5 and mutant INrf2Y208A-V5 (compare lane 2 with lane 6 and lane 4 in lane 8). Similar results were observed in HepG2 cells transfected with wild type and mutant INrf2 plasmids (data not shown). These results indicated that mutation of tyrosine 141 causes INrf2 protein to degrade because the protein stabilized after inhibition of protein degradation by MG132. Next, Hepa-1 cells were transfected with varying concentrations of plasmids encoding wild type INrf2 and mutant INrf2Y141A to determine the fold difference in the stability of wild type and mutant INrf2 proteins. The analysis revealed that 0.05 μg of wild type INrf2 plasmid produced an equivalent amount of protein to that observed in cells transfected with 0.5 μg of mutant INrf2Y141A plasmid (Fig. 2B). This indicated a 10-fold difference in the stability of two proteins (Fig. 2B). The plasmids encoding wild type and mutant proteins were in vitro transcribed and translated in presence of protease inhibitors (Fig. 2C). All four plasmids encoded wild type and mutant proteins (Fig. 2C). In other words, wild type and mutant plasmids upon transcription and translation in an in vitro cell-free system produced similar amounts of the respective proteins (Fig. 2C). This indicated that all four plasmids encoding wild type and mutant INrf2 proteins are phosphorylation sites (Tyr141 and Tyr208) that are conserved among the various species and are shown in Fig. 1B.

Recombinant plasmids were generated that upon transfection in Hepa-1 cells expressed V5-tagged INrf2 and mutants NQO1 gene expression. Hepa-1 cells were transfected with wild type and mutant INrf2. The cells were lysed and analyzed by SDS-PAGE, Western blotting, and probing with NQO1 antibody.

FIGURE 1. Schematic presentation of INrf2 domains. A, five discrete domains of the mouse INrf2 protein are designated as the N-terminal region (NTR), BTB domain, linker region (IVR), Kelch domain/diglycine repeats (DGR), and C-terminal region (CTR). The locations of conserved tyrosine residues in BTB domain are indicated. B, the sequence of human and mouse INrf2 protein regions 133–149 and 197–209 containing conserved tyrosine residues are aligned with other species for comparison. * conserved tyrosine residues Tyr141 and Tyr208 are highlighted.

FIGURE 2. INrf2Y141A mutant is unstable. A, expression analysis. Hepa-1 cells were transfected with empty vector pcDNA, INrf2-V5, INrf2Y141A-V5, INrf2Y208A-V5, or INrf2Y141AY208A-V5 DM. 24 h after transfection, the cells were treated with Me2SO or MG132 (20 μM) for 8 h. 50 μg of total cell lysate was loaded and immunoblotted with anti-V5 and anti actin antibodies. B, Hepa-1 cells were transfected with different amounts of INrf2-V5 or INrf2Y141A-V5 plasmids, and the expression pattern was analyzed by immunoblotting as in A. C, in vitro transcription and translation. Wild type and mutant INrf2 plasmids were in vitro transcribed and translated in presence of [35S]methionine. 2 μl of the protein lysate was resolved on SDS-PAGE and autoradiographed. D, phosphorylation analysis of endogenous INrf2. Hepa-1 cell lysates were immunoprecipitated with mouse INrf2 antibody. The immunoprecipitate was analyzed by SDS-PAGE, Western blotting (WB), and probing with anti-phosphotyrosine antibody (left panel). In reverse IP, Hepa-1 cell lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by Western analysis with anti-INrf2 antibody. E, phosphorylation analysis. 1 mg of lysates from Hepa 1 cells transfected with wild type and mutant INrf2 plasmids were used to immunoprecipitate with anti-Tyr(P) or anti-V5 antibodies, and the immune complexes were immunoblotted with anti-V5 and anti-Tyr(P) antibodies, respectively. F, ARE-luciferase assay, Hepa-1 and HepG2 cells were co-transfected with hNQO1 ARE-luciferase, Renilla luciferase, and the indicated plasmids. 24 h later the cells were harvested, lysed, and analyzed for luciferase activity. The results are presented as the means ± S.E. of three independent experiments, and each experiment was done in triplicate. G, Western analysis of endogenous NQO1 gene expression. Hepa-1 cells were transfected with wild type and mutant INrf2. The cells were lysed and analyzed by SDS-PAGE, Western blotting, and probing with NQO1 antibody.

JUNE 20, 2008 • VOLUME 283 • NUMBER 25 • 17715
Phosphorylation and Dephosphorylation Control of INrf2

A. Cells were seeded in 6-well plates and transfected with 0.5 μg either INrf2-V5 or mutant INrf2Y141A-V5 after transfection, the cells were pretreated with either dimethyl sulfoxide (DMSO) or MG132 for different time points (1, 3, 6, 8 hr). The cells were then harvested, and whole cell lysate was prepared. 50 μg of protein lysates were resolved on 10% SDS-PAGE and immunoblotted with anti-V5, anti-β-actin, and anti-Lamins antibody. Both immunoprecipitation with either anti-V5 followed by Western analysis with anti-β-actin antibody and reverse IP analysis revealed that endogenous INrf2 protein is phosphorylated at tyrosine residue Y141 (Fig. 2A). In a related experiment, Hepa-1 cells expressing V5-tagged wild type and mutant INrf2 proteins were lysed and immunoprecipitated with either anti-V5 followed by Western analysis with anti-β-actin antibody. Reverse immunoprecipitation with anti-β-actin antibody was also performed followed by Western blot with anti-V5 antibody. The results revealed that wild type and mutant INrf2Y208A proteins were phosphorylated at tyrosine residues (Fig. 2E). However, tyrosine phosphorylation was absent in mutant INrf2Y141A and INrf2DM bearing Y141A mutation (Fig. 2E). These results suggested that phosphorylation of tyrosine 141 stabilizes INrf2 protein.

In related experiment, we analyzed the effects of wild type and mutant INrf2 proteins on ARE-luciferase expression in HeP-1 and HepG2 cells (Fig. 2F). The transfection of cells with wild type INrf2 and mutant INrf2Y208A resulted in 5-fold repression in ARE-luciferase activity, as compared with vector control pcDNA transfected cells. On the other hand, the overexpression of mutant INrf2Y141A and INrf2DM failed to repress ARE-luciferase gene expression, indicating that tyrosine 141 phosphorylation might be critical for normal functioning of INrf2. Analysis of Hepa-1 cells overexpressing cDNA-derived INrf2, INrf2Y141A, INrf2Y208A, and double mutant INrf2Y141A-Y208A revealed similar effects on endogenous expression of Nrf2 downstream NQO1 gene expression (Fig. 2G). Overexpression of wild type INrf2 and mutant INrf2Y208A significantly repressed NQO1 gene expression. However, in the same experiment, overexpression of mutant INrf2Y141A and double mutant INrf2Y141A-Y208A failed to repress NQO1 gene expression (Fig. 2G). It is noteworthy that INrf2Y141 and INrf2Y208 were also mutated to phenylalanine. INrf2Y141F, INrf2Y208F, and the double mutant demonstrated similar results as alanine mutants (data not shown).

Mutant INrf2Y141A Is Rapidly Degraded as Compared with Wild Type INrf2—The rate of degradation of wild type INrf2 and mutant INrf2Y141A proteins was examined in the presence of protein synthesis inhibitor cycloheximide. Hepa-1 cells transfected with wild type INrf2-V5 and mutant INrf2Y141A-V5 were pretreated with MG132 followed by cycloheximide. The cells were harvested at different time intervals of cycloheximide treatment, and INrf2 and mutant proteins were analyzed by Western blotting and probed with anti-V5 antibody. The results indicated that INrf2Y141A degraded more rapidly as compared with wild type INrf2 (Fig. 3A). Similar results were also obtained in a pulse-chase experiment involving radiolabeled methionine (Fig. 3B). Contrary to the stability of wild type INrf2, a majority of mutant INrf2Y141A degraded within 8 h of cycloheximide treatment. These results indicated that mutation of tyrosine 141 causes the INrf2 to degrade rapidly. Interestingly, further experiments revealed rapid degradation of mutant INrf2Y141A in both cytosolic and nuclear compartments (Fig. 3C). But the wild type INrf2 protein was stable in both cytosolic and nuclear compartments. These results together indicated that INrf2Y141A degrades much faster than wild type INrf2 irrespective of subcellular distribution.
Phosphorylation and Dephosphorylation Control of INrf2

Mutant INrf2Y141A, Like INrf2, Forms Dimers—INrf2 is known to form dimers in vitro and in vivo (20). Dimerization and multimerization of INrf2 are required for function, and the BTB domain is required for dimerization (20). Interestingly, the BTB domain is required for dimerization of INrf2 (20). Interestingly, tyrosine 141 is crucial for INrf2 function. In vitro and in vivo glutaraldehyde cross-linking experiments were performed to test the potential of mutant INrf2Y141A to form di- and multimers. The results are shown in Fig. 4 (A and B). In the presence of glutaraldehyde, INrf2Y141A formed di- and multimers similar to wild type INrf2 in in vitro (Fig. 4A) as well as in vivo experiments (Fig. 4B). In related experiments, Hepa-1 cells were co-transfected with FLAG-INrf2 and INrf2-V5 or mutant INrf2Y141A-V5 plasmids to obtain further evidence that mutant INrf2Y141A is capable of dimerization with INrf2 (Fig. 4C). The transfected cells were lysed and immunoprecipitated with either anti-V5 or anti-FLAG antibodies. The immunoprecipitate was analyzed by immunoblotting with anti-V5 and anti-FLAG antibodies. Anti-V5 antibody immunoprecipitated INrf2-V5 and mutant INrf2-V5. FLAG-INrf2 co-precipitated with both INrf2-V5 and INrf2Y141A-V5. Similarly, in a reverse immunoprecipitation experiment, anti-FLAG antibody immunoprecipitated FLAG-INrf2. Both INrf2-V5 and INrf2Y141A-V5 co-precipitated with FLAG-INrf2. The results indicated direct interaction of wild type INrf2-V5 and mutant INrf2Y141A-V5 with FLAG-INrf2. Taken together, these results demonstrated that mutant INrf2Y141A, like wild type INrf2, is capable of forming di- and multimers. In other words, the mutation of tyrosine 141 to alanine had no effect on INrf2 dimerization.

Mutant INrf2Y141A Binds to Nrf2 but Lacks Capacity to Degrade Nrf2—INrf2 is known to bind to Nrf2 and function as an adapter for Cul3/Rbx1-mediated ubiquitination and degradation of Nrf2. We performed in vitro and in vivo experiments to determine the effect of Y141A mutation on INrf2 binding with Nrf2 and degradation of Nrf2. Luciferase (Luc control), INrf2-V5, INrf2Y141A-V5, and FLAG-Nrf2 all were successfully in vitro transcribed and translated (Fig. 5A, left panel). [35S]Methionine was included during translation to radiolabel the proteins. The translated and 35S-labeled proteins were mixed in combinations as shown in Fig. 5A (middle and right panels), incubated and immunoprecipitated with mouse IgG (control), anti-V5 antibody (middle panel), or anti-FLAG antibody (right panel), and analyzed by autoradiography.

The results demonstrated that both mutant INrf2Y141A-V5 and wild type INrf2-V5 interacted with FLAG-Nrf2 because anti-V5 antibody immunoprecipitated INrf2, INrf2Y141A and FLAG-Nrf2 were co-precipitated with them (Fig. 5A, middle panel). Similarly, in reverse immunoprecipitation experiment, anti-FLAG antibody immunoprecipitated FLAG-Nrf2, and INrf2-V5 and INrf2Y141A-V5 were co-precipitated in the complex (Fig. 5A, right panel). In related experiment, Hepa-1 cells were co-transfected with INrf2-V5 or mutant INrf2Y141A-V5 with FLAG-Nrf2, and total cell lysates were immunoprecipitated with anti-V5 or anti-FLAG antibodies in separate experiments (Fig. 5B). The immunoprecipitates were analyzed by SDS-PAGE, Western blotting, and probing with anti-V5 and anti-FLAG antibodies. The FLAG-Nrf2 was co-precipitated with both INrf2-V5 and mutant INrf2Y141A-V5. In reverse immunoprecipitation experiment, both INrf2-V5 and mutant INrf2Y141A-V5 co-precipitated with FLAG-INrf2. These results demonstrated that both INrf2 and INrf2Y141A could interact with Nrf2 in in vitro and in vivo experiments.

Next, we compared the capacities of wild type INrf2 and mutant INrf2Y141A to degrade Nrf2 (Fig. 5C). Hepa-1 cells were co-transfected with 0.5 μg of FLAG-Nrf2 and increasing amounts of INrf2-V5 (Fig. 5C, left panel) or INrf2Y141A-V5 (Fig. 5C, right panel). Overexpression of wild type INrf2 caused INrf2 dose-dependent degradation of Nrf2. In contrast, mutant INrf2Y141A failed to degrade Nrf2 in the same experiment. This indicated that mutant INrf2Y141A lacks the capacity to degrade Nrf2. Taken together, these results indicate that tyrosine 141 is crucial for INrf2 function.
Hydrogen Peroxide Induces Degradation of INrf2 by Modification of INrf2Y141—Hydrogen peroxide, an oxidizing agent, was used to study in vivo relevance of phosphorylation of tyrosine 141 in INrf2. Hepa-1 cells were transfected with INrf2-V5 and treated with cycloheximide (Fig. 6A, left panel) or hydrogen peroxide and cycloheximide in the absence (Fig. 6A, middle panel) or presence (Fig. 6A, right panel) of the tyrosine kinase inhibitor genestein for different time intervals. The amount of INrf2-V5 was analyzed by Western blotting of total cell lysates obtained from these cells as shown in Fig. 6. INrf2-V5 protein more or less did not degrade during 8 h of cycloheximide treatment, indicating that INrf2-V5 is a stable protein (Fig. 6A, left panel). Interestingly, the treatment with hydrogen peroxide resulted in degradation of INrf2-V5 (Fig. 6A, middle panel). More than 50% of INrf2 protein was degraded 6 h after cycloheximide and hydrogen peroxide treatment, as compared with less than 10% degradation in cells treated with cyclo-
**Phosphorylation and Dephosphorylation Control of INrf2**

![Model demonstrating the role of phosphorylation of tyrosine 141 under oxidative stress.](image)

The above results suggested that the initial response to oxidative stress can cause activation of Nrf2 by destabilizing INrf2 during the experiment, INrf2Y141A mutant failed to demonstrate tyrosine phosphorylation and rapid degradation of INrf2 in presence of hydrogen peroxide. The rapid degradation of INrf2 after hydrogen peroxide treatment (Fig. 6B, right panel). In a similar experiment, INrf2Y141A mutant failed to demonstrate tyrosine phosphorylation in the absence or presence of hydrogen peroxide (data not shown). The above results suggested that mutation of Tyr141 and inhibition of INrf2 phosphorylation both render the INrf2 protein susceptible to degradation. The rapid degradation of INrf2 in presence of hydrogen peroxide implies involvement of an unknown tyrosine phosphatase that can cause activation of Nrf2 by destabilizing INrf2 during the initial response to oxidative stress.

**DISCUSSION**

INrf2-Nrf2 protein complex is known to serve as sensors of oxidative and electrophilic stresses (1). Mechanisms have been reported that modify INrf2 cysteines and/or Nrf2 serine residues in response to signals from xenobiotics, chemicals, and UV radiation (1). This leads to dissociation of Nrf2 from INrf2, nuclear translocation of Nrf2 and coordinated activation of a battery of chemoprotective/defensive genes. The coordinated induction of Nrf2 downstream gene expression is critical for cellular protection and cell survival.

Nrf2 is a short lived protein (1). Once Nrf2 function inside the nucleus is over, it is exported out and degraded (13, 14). On contrary, INrf2 is a stable protein with a half-life in days (17). Multiple mechanisms and/or multiple steps in a mechanism are expected to modify INrf2-Nrf2 leading to the dissociation of Nrf2 from INrf2. This is because INrf2-Nrf2 are capable of receiving signals from a variety of diverse stressors.

The studies in this report present evidence that phosphorylation and dephosphorylation of tyrosine 141 controls stability and degradation of INrf2 respectively. This has provided new insights into the regulation of Nrf2-mediated antioxidant protection and cell survival. Tyrosine 141 is highly conserved in INrf2 from the various species. De novo synthesized INrf2 protein is phosphorylated at tyrosine 141, suggesting this phosphorylation is required for the stability of the INrf2 protein. Mutation of tyrosine 141 resulted in the loss of INrf2 phosphorylation and rapid degradation of INrf2 protein. The studies also revealed a novel mechanism of the oxidative/electrophilic stress-mediated stabilization of Nrf2 by dephosphorylation and degradation of INrf2. This was evident from oxidative/electrophilic stress-mediated dephosphorylation of tyrosine 141 followed by rapid degradation of INrf2. The decrease in INrf2 led to stabilization of Nrf2 and activation of Nrf2-regulated gene expression. Interestingly, mutant INrf2Y141A protein was capable of binding to Nrf2 but failed to catalyze degradation of Nrf2. Nrf2 functions as an adapter for Cul3-mediated ubiquitination and degradation of Nrf2 (5, 21, 22). It is possible that INrf2Y141A mutant had lost its capacity to interact with Cul3 and/or lost the property to properly present Nrf2 to Cul3 for degradation and remains to be investigated.

The cytochrome 273 and 288 in INrf2 are reported to be required for ubiquitination of Nrf2 and cysteine 151 is required for INrf2 dependent degeneration of Nrf2 by sulforaphane (16). However, the studies have also suggested that modifying specific cysteines of the electrophile-sensing INrf2 is insufficient to disrupt binding to Nrf2-derived Neh2 (23). Nrf2 will not degrade if it cannot bind to INrf2 (23). It is unclear at present whether mechanism involving dephosphorylation of tyrosine 141 functions independently or is in concert with cysteine modification mechanism. In other words it is possible that tyrosine 141 dephosphorylation mechanism functions independent of cys-
Phosphorylation and Dephosphorylation Control of INrf2

teines modification or modification of cysteine is followed by dephosphorylation of INrf2Y141. In either case, the INrf2 modification(s) leads to degradation of INrf2 and stability of Nrf2. The PKC modification of Nrf2 might take place at the same time as tyrosine dephosphorylation or following tyrosine dephosphorylation and remains to be determined. The above events result in nuclear translocation of Nrf2 and activation of ARE-mediated gene expression. The tyrosine kinase that phosphorylates tyrosine 141 of INrf2 is not known. The protein phosphatase that can act on phospho-INrf2 to remove the phospho group also remains unknown. Based on our data, it is suggested that the phosphatase involved in this pathway might be the one that can respond to oxidative/electrophilic stress.

A model depicting phosphorylation/dephosphorylation control of INrf2 is proposed in Fig. 7. Under normal conditions, de novo synthesized INrf2 is phosphorylated at tyrosine 141 by an unknown kinase and forms homodimers at the BTB domain. INrf2-INrf2 homodimers then hold Nrf2 in cytoplasm and by interacting with Cul3 ubiquitine ligase initiates ubiquitination and degradation of Nrf2. In response to oxidative/electrophilic stress, INrf2 and Nrf2 are modified in either independently or sequentially in a single mechanism. INrf2 modifications include electrophiles binding with cysteines and tyrosine 141 dephosphorylation, and Nrf2 modification is phosphorylation of serine 40 by PKC. The modification of cysteines presumably initiates structural alterations in INrf2 followed by tyrosine 141 modification(s) leads to degradation of INrf2 and stability of Nrf2. PKC-mediated serine 40 phosphorylation in Nrf2 releases Nrf2 from INrf2. These events result in nuclear translocation of Nrf2 and activation of chemoprotective and defensive genes. Nrf2 translocates inside the nucleus, binds to the ARE, and up-regulates the expression of chemoprotective genes.

In conclusion, we demonstrate that phosphorylation of tyrosine 141 of INrf2 regulates its stability. Tyrosine 141-phosphorylated INrf2 is ubiquitinated and unbiiquitinate and degrade Nrf2. Oxidative/electrophilic stress-mediated elimination of the phosphate group from tyrosine 141 in INrf2 results in instability and loss of function of INrf2. The resultant dephosphorylated INrf2 can dimerize but is highly unstable and nonfunctional. Taken together, these results demonstrate the presence of a unique model of Nrf2 activation via de-activation of Nrf2 inhibitor, INrf2 by post-translational modifications.

Acknowledgments—We thank our colleagues for their help and discussions. The contributions of Kanan Sankaranaryanan and Ying Li in some initial work is acknowledged and appreciated.

REFERENCES
1. Jaiswal, A. K. (2004) Free Rad. Biol. Med. 36, 1199–1207
2. Breimer, L. H. (1990) Mol. Carcinog. 3, 188–197
3. Breimer, L. H. (2000) Mol. Carcinog. 30, 191–197
4. Dhakshinamoororthy, S., and Jaiswal, A. K. (2001) Oncogene 20, 3906–3917
5. Cullinan S. B., Gordan J. D., Jin J., Harper J. W., and Diehl J. A. (2004) Mol. Cell. Biol. 24, 8477–8486
6. Bloom, D. A., and Jaiswal, A. K. (2003) J. Biol. Chem. 278, 44675–44682
7. Huang, H. C., Nguyen, T., and Pickett, C. B. (2002) J. Biol. Chem. 277, 42769–42774
8. Buckley, B. J., Marshall, Z. M., and Whorton, A. R. (2003) Biochem. Biophys. Res. Commun. 307, 973–979
9. Zipper, L. M., and Mulcahy, R. T. (2000) Biochem. Biophys. Res. Commun. 278, 484–492
10. Balogun, E., Hoque, M., Tong, P., Killeen, E., Green, C. J., Forresti, R. Alam, I., and Motterlini, R. (2005) Mol. Carcinog. 44, 10941–10953
11. Kobayashi, A., Kang, M. J., Okawa, H., Ohtsuji, M., Zeneca, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004) Mol. Cell. Biol. 24, 7130–7139
12. Zhang, D. D., Cross, J. V., Templeton, D. J., and Hannink, M. (2005) J. Biol. Chem. 280, 30091–30099
13. Igarashi, K., and Yamamoto, M. (1999) Mol. Cell. Biol. 19, 8477–8486
14. Jain, A. K., Bloom, D. A., and Jaiswal A. K. (2005) J. Biol. Chem. 280, 8137–8151
Src subfamily kinases regulate nuclear export and degradation of transcription factor Nrf2 to switch off Nrf2-mediated antioxidant activation of cytoprotective gene expression.
Suryakant K. Niture, Abhinav K. Jain, Phillip M. Shelton, and Anil K. Jaiswal

Prothymosin-α mediates nuclear import of the INrf2/Cul3-Rbx1 complex to degrade nuclear Nrf2.
Suryakant K. Niture and Anil K. Jaiswal

Phosphorylation and dephosphorylation of tyrosine 141 regulate stability and degradation of INrf2. A novel mechanism in Nrf2 activation.
Abhinav K. Jain, Shilpi Mahajan, and Anil K. Jaiswal

The publisher of the Journal of Biological Chemistry is issuing this Expression of Concern to notify readers that questions have been raised with the corresponding author’s institution regarding some of the data and the conclusions in the articles listed above.

This Expression of Concern is solely intended to notify readers for informational purposes. It is not a statement regarding the validity of the data. The Journal of Biological Chemistry will provide additional information as it becomes available.

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Phosphorylation and Dephosphorylation of Tyrosine 141 Regulate Stability and Degradation of INrf2: A NOVEL MECHANISM IN NRF2 ACTIVATION
Abhinav K. Jain, Shilpi Mahajan and Anil K. Jaiswal

J. Biol. Chem. 2008, 283:17712-17720.
doi: 10.1074/jbc.M709854200 originally published online April 23, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M709854200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 23 references, 16 of which can be accessed at
http://www.jbc.org/content/283/25/17712.full.html#ref-list-1