Src Subfamily Kinases Regulate Nuclear Export and Degradation of Transcription Factor Nrf2 to Switch Off Nrf2-mediated Antioxidant Activation of Cytoprotective Gene Expression

This article has been retracted by the publisher. Phillip M. Shelton agrees with the retraction. An investigation at the University of Maryland, Baltimore determined that bands depicted in several figures were digitally altered as follows. In Fig. 2, the LaminB immunoblot from “Src siRNA” was digitally mirrored and duplicated as LDH in the “YES siRNA” panel. Bands 4, 5, and 6 in the LDH panels of the Fyn and Lyn panels were duplicated. In Fig. 4A, in the Nrf2-V5 panel, bands 1 and 2 and bands 4 and 5 are identical. Similarly, in the IP:pTyr lane, bands 5 and 6 are identical. In Fig 4B, there is evidence of splicing in the Nrf2-V5 panel. In addition, in the FLAG panel, bands 1 and 2 and bands 4 and 7 are identical. In Fig. 5A, there is evidence of splicing in the Nrf2 panel. In addition, bands 1 and 2 in the Src panel are identical. In Fig. 5C, in the Nrf2 panel, lanes 1 and 2 and lanes 5 and 8 are identical. Similarly, in the Fyn panel, lanes 1 and 2 are identical. In addition, lanes 4 and 5 were duplicated and presented as mirror images of each other. In Fig. 5E, lanes 4 and 5 from the IP:V5 WB: pTyr panel were duplicated in Fig. 7A, right panel LDH, lanes 4 and 5. In Fig. 7A, the left Lyn panel and the right Yes panel were duplicated after resizing. In Fig. 7B, the left and right GSKβ panels were duplicated after resizing. In Fig. 8A, in the NQO1 panel, bands 2 and 6 are identical. In the HO-1 panel, bands 2 and 6 were resized and duplicated. In the GCLC panel, bands 1 and 6 were duplicated. In addition, the journal determined that lanes 2–4 of the HO-1 immunoblot from the “yes siRNA” panel from Fig. 1A was flipped horizontally and reused in lanes 2–4 of the Lyn immunoblot from the “Lyn siRNA” panel of the same figure. In Fig. 2, lanes 3 and 6 of the Src immunoblot from the “Src siRNA” panel were duplicated. In Fig. 5D, lanes 5 and 6 of the IP:V5 WB:pSer immunoblot from the right panels were duplicated. In Fig. 5D, lanes 1–4 of the Lamin B immunoblot from SYF−/− MEFs were duplicated in the LDH immunoblot from Fig. 7B, right panel. The LDH immunoblot from Fig. 7A, left panel, was duplicated as LDH in Fig. 7B, left panel. The right Fyn panel and the right Src panel were duplicated in Fig. 7A. In Fig. 7B, lanes 3 and 5 of the GSKβpY216 immunoblot, right panel, were duplicated.

The INrf2:Nrf2 complex serves as a sensor of chemical- and radiation-induced oxidative and electrophilic stress (1, 2). Nrf2 resides predominantly in the cytoplasm where it interacts with actin-associated cytosolic protein, INrf2 (inhibitor of Nrf2) or Keap1 (Kelch-like ECH-associated protein 1). INrf2 functions as a substrate adaptor protein for a Cul3-Rbx1-dependent E3 ubiquitin ligase complex that ubiquitinates and degrades Nrf2, activation, or subsequently prevent carcinogenic species from interacting with crucial cellular macromolecules, such as DNA, RNA, and proteins (21). A plausible mechanism by which blocking agents impart their chemopreventive activity is the induction of detoxification and antioxidant enzymes (22).

However, evidence also suggests that persistent accumulation of Nrf2 in the nucleus is harmful (1, 2). For example, INrf2 (Keap1)−− mice demonstrated persistent accumulation of...
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Nrf2 in the nucleus that led to postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and forestomach (23). Moreover, a reversal of this INrf2 deficiency phenotype has been achieved by breeding to Nrf2-null mice, suggesting tightly regulated negative feedback might be essential for cell survival (24). The systemic analysis of the INrf2 genomic locus in human lung cancer patients and immortal cancer cell lines showed that deletion, insertion, and missense mutations in functionally important domains of INrf2 results in reduction of INrf2 affinity for Nrf2 and elevated expression of cytoprotective genes, which resulted in drug resistance and cell survival in lung cancer cells (25, 26). Unrestrained activation of Nrf2 in cells increases a risk of adverse effects, including survival of damaged cells, tumorigenesis, and drug resistance (1). Therefore, it appears that cells contain mechanisms that autoregulate cellular abundance of Nrf2 (27, 28). Based on the reported studies, it is suggested that the Nrf2 up-regulation of ARE-mediated gene expression is an early response to antioxidants (1, 2). Subsequently, the late response of antioxidants appears to recruit negative factors, including Bach1-MafG, MafG/K/F-MafG/K/F, c-Jun-c-Fos, and c-Jun-Fra-1, to rapidly bring down the induced ARE-mediated gene expression to normal levels. Recently, studies have demonstrated that INrf2 is also localized in the nucleus presumably to degrade Nrf2 (29, 30).

Src family kinases are frequently overexpressed and/or activated in human cancers and play key roles in cancer progression, metastasis, proliferation, survival, and apoptosis (31, 32). The Src family of tyrosine kinases contains two major subfamilies, including the Src subfamily and the Lyn subfamily members (31). Src subfamily has four members: Src, Fgr, Yes, and Fgr (31). Allosteric activation of Src family kinases is thought to be key to their function in physiological processes. Through well defined post-translational modifications in Src family members are also known to play important roles in human cancers (31). Recently, we demonstrated that phosphorylation and degradation of Nrf2 are involved in intriguing questions regarding the role of other members of the Src subfamily, including Src, Yes, and Fgr and Lyn subfamily in the control of Nrf2 and cytoprotective gene expression. In addition, the physiological role of alterations in Src subfamily members also remains unknown.

In this study, we demonstrate that all four members of the Src subfamily of kinases Src, Yes, Lyn, and Fgr but not Lyn phosphorylated Nrf2Tyr568 leading to nuclear export, ubiquitination, and degradation of Nrf2. This led to the switching off of antioxidant-mediated Nrf2 activation and down-regulation of cytoprotective gene expression to basal levels. Furthermore, we were able to demonstrate that antioxidant and oxidant both activated GSK3β, which phosphorylated Src kinases, leading to nuclear import/accumulation of Src kinases and phosphorylation of Nrf2Tyr568, followed by nuclear export and degradation of Nrf2. Studies on the physiological role of Src subfamily of kinases revealed that alterations in Src kinases were inversely related with nuclear accumulation of Nrf2 and cell survival. Together, our data suggest that the Src family kinases negatively regulate Nrf2 by enhancing nuclear export and degradation of Nrf2 to switch off Nrf2-mediated antioxidant activation of cytoprotective gene expression.

MATERIALS AND METHODS

Plasmid Construction and Cell Culture—Construction of Nrf2-V5 and Nrf2Y568A-V5 mutant plasmids and the luciferase plasmid harboring the human NQO1 gene are have been described previously (34). FLAG-tagged Fyn, Src, Yes, and Lyn plasmids were received as a generous gift from Dr. Shigeki Miyamoto (Dept. of Pharmacology, University of Wisconsin Medical School). The sequence accuracy of all constructs was confirmed by DNA sequencing using ABI-3700 capillary sequencer (Applied Biosystems, Foster City, CA).

Mouse hepatoma (Hepa-1) cells obtained from ATCC were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% l-glutamine (200 mM), penicillin (40 units/ml), and streptomycin (40 μg/ml). Src-, Yes-, and Fyn-positive (SYF+/+) and Src-, Yes+, and Fyn-null (SYF−/−) mouse embryonic fibroblasts were kindly provided by Dr. Ida S. Owens (National Institutes of Health) and grown in same DMEM as Hepa-1 cells. The cells were grown in monolayer in an incubator at 37 °C in 95% air and 5% CO2.

Transient Transfection and Luciferase Assay—Hepa-1 cells were plated in 6-well plates at a density of 1 × 10⁶ cells/plate 24 h prior to transfection. Cells were transfected with 1 μg of the reporter constructs and 0.5 μg of the siRNA transfection reagent according to the manufacturer’s instructions. After 48 h, cells were harvested, and cell-specific protein was assessed by Western blotting. For luciferase activity, 1 × 10⁶ cells were grown in monolayer culture dishes. After 12 h, cells were co-transfected with 0.1 μg of NQO1 promoter ARE-luciferase (ARE-Luc) reporter construct and 10 times less quantities of firefly Renilla luciferase encoded by plasmid pRL-TK. Renilla luciferase was used as the internal control in each transfection. After 24 h of transfection, the cells were washed with 1 × phosphate-buffered saline and lysed in 1 × Passive lysis buffer from the Dual-Luciferase® reporter assay system kit (Promega, Madison, WI). Similarly, SYF+/+ and SYF−/− mouse embryonic fibroblasts were transfected with 0.1 μg of NQO1 promoter ARE-Luc reporter constructs and 10 times less quantities of firefly Renilla luciferase encoded by plasmid pRL-TK. After 24 h of transfection, the cells were treated with DMSO or t-BHQ (50 μM) for 24 h. Cells were washed with 1 × phosphate-buffered saline and lysed in 1 × Passive lysis buffer. The luciferase activity was measured using the procedures described previously (7) and plotted.

siRNA Interference Assay—Fyn, Src, Yes, Lyn, and control siRNA were purchased from Dharmacon, and Fgr siRNA was from Ambion. Hepa-1 cells were transfected with 10, 50, and 100 nM Fyn, Src, Yes, Fgr, and Lyn siRNA and 100 nM control GAPDH siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Thirty hours after transfection, cells were harvested, and Fyn, Src, Yes, Fgr, and Lyn protein levels were analyzed by Western blotting. The siRNA-transfected cells were also analyzed for Nrf2 and Nrf2 downstream proteins, including NQO1, HO-1, and GCLC by Western blotting.

Subcellular Fractionation and Western Blotting—Hepa-1 or SYF+/+ and SYF−/− mouse embryonic fibroblast cells seeded
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in 100-mm plates and treated/transfected as displayed in the figures were washed twice with ice-cold phosphate-buffered saline, trypsinized, and centrifuged at 1500 rpm for 5 min. For making whole cell lysates, the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate) and supplemented with protease inhibitor mixture (1X) (Roche Applied Science). Cytoplasmic and nuclear biochemical fractionation of the cells was done using the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA) following the manufacturer’s protocol. The protein concentration was determined using the protein assay reagent (Bio-Rad). Sixty to eighty micrograms of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 3% nonfat dry milk in TBST. For immunoblotting, the following antibodies were used: anti-Fyn, anti-Src, anti-Fgr, anti-Lyn, and anti-GSK3β obtained from Cell Signaling; anti-GSK3β phospho-Y216 from BD Transduction Laboratories, and anti-Yes from Upstate Biotechnology, Inc. Anti-Nrf2 (H-300) and anti-HO-1 (H-105) antibodies were obtained from Santa Cruz Biotechnology, and anti-γ-glutamylcysteine synthetase (GCLC) antibody was from Abcam. Other antibodies such as anti-FLAG-HRP and anti-actin (Sigma), anti-V5 (Invitrogen), and anti-phosphoryrosine and anti-phosphoserine (StressGen) were obtained and used as per the manufacturer’s suggestions. The membranes were washed three times in TBST, and immunoactive bands were visualized using chemiluminescence ECL system (Amersham Biosciences). The intensity of protein bands after immunoblotting was quantified using the protein assay reagent (Bio-Rad). Sixty to eighty micrograms of extract from whole cell lysates were used for immunoprecipitation, immune complexes were immunoblotted with anti-V5 antibody, and after immunoprecipitation, immune complexes were immunoblotted with anti-ubiquitin antibody (Cell Signaling). Similarly, SYF+/+ and SYF−/− mouse embryonic fibroblasts cells were treated with DMSO or t-BHQ for 8 h, and endogenous Nrf2 was immunoprecipitated with anti-Nrf2 antibodies, and immune complexes were immunoblotted with anti-ubiquitin antibody.

RESULTS

siRNA-mediated Inhibition of Src Subfamily Kinases, but Not Lyn Kinase, Led to Stabilization and Nuclear Accumulation of Nf2 and Increased Downstream Gene Expression—Hepa-1 cells were transfected with varying concentrations of Fyn, Src, Yes, Fgr, and Lyn siRNA and immunoblotted for the various Src kinases, Nrf2, and Nrf2 downstream proteins (Fig. 1A). siRNA transfection led to 70–80% down-regulation of cellular levels...
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In similar experiments, siRNA-mediated down-regulation of Lyn kinase did not lead to an increase in Nrf2. In the same experiments, inhibition of Lyn kinase did not lead to an increase in Nrf2 downstream gene expression following the same pattern as Nrf2. The siRNA-mediated inhibition of Fyn, Src, Yes, and Fgr, but not Lyn, led to a significant increase in Nrf2 downstream gene expression (Fig. 1A). In related experiments, the siRNA-mediated inhibition of Fyn, Src, Yes, and Fgr, but not Lyn, showed a concentration-dependent increase in Nrf2 downstream gene expression (Fig. 1B). In related experiments, the siRNA-transfected cells were subcellularly fractionated to produce cytosolic and nuclear fractions that were immunoblotted for Src and Lyn kinases, Nrf2, and cytosolic and nuclear markers (Fig. 2). The results showed that siRNA inhibited both cytosolic and nuclear levels of Fyn, Src, Yes, Fgr, and Lyn in respective transfections. Interestingly, siRNA inhibition of Fyn, Src, Yes, Fgr, and Lyn, but not Lyn, led to nuclear accumulation of Nrf2 (Fig. 2). The cytosolic Nrf2 was more or less unchanged in these experiments (Fig. 2). Together, these results revealed that siRNA-mediated inhibition of Fyn, Src, Yes, and Fgr, but not Lyn, led to increased nuclear accumulation of Nrf2 and increased expression of Nrf2 downstream gene expression. The results also demonstrated that Fgr is a weak mediator of Nrf2 stabilization, as compared with other members of the Src subfamily of kinases.

Expression of Fyn, Src, and Yes, but Not Lyn, Induced Nuclear Export and Degradation of Nrf2—Hepa-1 cells were co-transfected with varying amounts of FLAG-Fyn, FLAG-Src, FLAG-Yes, or FLAG-Lyn with fixed amounts of Nrf2-V5 to determine the role of Src kinases in nuclear export and degradation of Nrf2. One set of transfected cells was treated with DMSO and another set with proteasome inhibitor MG132 for 8 h. The cells were lysed and immunoblotted for the various Src kinases and Nrf2-V5 (Fig. 3A). The results showed that overexpression of Fyn, Src, and Yes led to a dose-dependent decrease in Nrf2-V5 (Fig. 3A, left upper three panels). However, the overexpression of Lyn had no effect on Nrf2-V5 (Fig. 3A, left lower panel). The results also demonstrated that post-treatment of cells with MG132 after overexpression of Src kinases stabilized Nrf2-V5 (Fig. 3A, right panels). In related experiments, overexpression of Fyn, Src, and Yes, but not Lyn kinase, led to dose-dependent inhibition of ARE-mediated luciferase expression (Fig. 3B). In total, these results revealed that overexpression of Fyn, Src, and Yes, but not Lyn kinase, led to increased nuclear export and degradation of Nrf2.

Fyn, Src, and Yes, but Not Lyn Kinase, Phosphorylated Nrf2 Tyr568, Leading to Nuclear Export, Ubiquitination, and Degradation of Nrf2—Hepa-1 cells were co-transfected with FLAG-Fyn, FLAG-Src, FLAG-Yes, or FLAG-Lyn, and Nrf2-V5 or mutant Nrf2Y568A-V5. The transfected cells were lysed and analyzed for phosphorylation, nuclear export, and ubiquitination of Nrf2 and mutant Nrf2Y568A-V5 (Fig. 4). Forward and reverse immunoprecipitation of wild type and mutant Nrf2...
from Hepa-1 cells overexpressing FLAG-Fyn, FLAG-Src, or FLAG-Yes showed phosphorylation of Nrf2Tyr568 but not mutant Nrf2Y568A (Fig. 4A, middle and right panels). Overexpression of FLAG-Lyn in the same experiment failed to phosphorylate both Nrf2Tyr568 and mutant Nrf2Y568A (Fig. 4A, middle and right panels). In similar experiments, overexpression of FLAG-Fyn, FLAG-Src, and FLAG-Yes that led to phosphorylation of Nrf2Tyr568 also led to nuclear export and ubiquitination of Nrf2 (Fig. 4B). Interestingly, overexpression of FLAG-Lyn that failed to phosphorylate Nrf2 (Fig. 4A) also failed
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**FIGURE 4. Overexpression of Fyn, Src, and Yes kinases but not Lyn kinase phosphorylates Nrf2 Tyr568 leading to nuclear export and degradation of Nrf2.** A, left panel, Western blotting (WB). Hepa-1 cells were co-transfected with pcDNA constructs and pcDNA or FLAG-tagged Lyn, Src, Yes, and Fyn kinase constructs for 24 h. Sixty micrograms of lysates were immunoprecipitated (IP) with anti-V5 and anti-FLAG antibodies, and then immunoblotted with anti-phosphotyrosine and anti-V5 antibodies. Right panels, effect of Src kinases on Nrf2 ubiquitination. Hepa-1 cells were co-transfected with Nrf2-V5 or mutant Nrf2Y568A-V5 constructs and pcDNA or FLAG-tagged Fyn, Src, Yes, and Lyn kinase constructs. One mg of whole cell extracts was immunoprecipitated (IP) with anti-V5 antibody and immunoblotted with anti-ubiquitin (Ub) antibody. Ubiquitination of Nrf2-V5 was increased by 1.7-, 2.0-, and 1.9-fold when cells were transfected with FLAG-tagged Fyn, Yes, and Src kinases, respectively, compared with FLAG-Lyn or pcDNA-transfected cells (upper right panel, lanes 3–5). B, left panels, Nuclear extracts were prepared using Active motif kit. Sixty micrograms of nuclear extracts were immunoblotted with anti-V5, anti-FLAG, and anti-lamin-B antibodies. Right panels, effect of Src kinases on phosphorylation of Nrf2 Tyr568. Hepa-1 cells were co-transfected with Nrf2-V5 or mutant Nrf2Y568A-V5 constructs and pcDNA or FLAG-tagged Lyn, Src, Yes, and Fyn kinase constructs. One mg of lysates from the same time points of exposure as used in the earlier experiments was immunoprecipitated (IP) with anti-V5 and anti-phospho-tyrosine antibodies (Fig. 5, B and D). The results demonstrated that antioxidants/oxidant and UV light during early response induced nuclear localization of Nrf2. This was followed by nuclear import of Src kinases that led to nuclear export of Nrf2.

**Antioxidant, Oxidant, and UV Light-Induced Tyrosine Phosphorylation during Delayed/Late Phase of Induction Led to Nuclear Accumulation of Src Kinases and Nuclear Export of Nrf2 Tyr568, but Not Mutant Nrf2Y568A—**We first determined the effect of exposure to antioxidant (t-BHQ), hydrogen peroxide, and ultraviolet radiation on the nuclear import and export of Src kinases and Nrf2. Hepa-1, cells were exposed to t-BHQ, hydrogen peroxide, and UVB radiation for different time intervals, and subcellular localization of Src kinases and Nrf2 was analyzed by immunoblotting (Fig. 5, A–C). The results demonstrated that t-BHQ led to a 4-fold increase in nuclear Nrf2 within 4 h of treatment (Fig. 5A). The nuclear level of Nrf2 significantly declined at 8 h after t-BHQ treatment. Interestingly, t-BHQ led to a time-dependent increase in nuclear Fyn, Src, and Yes kinases (Fig. 5A). The increase in nuclear Src kinases was minimal at 1 h and reached their highest level at 8 h (Fig. 5A). In other words, the nuclear Src kinases levels were minimal during the early phase of Nrf2 nuclear import/activation but increased in nuclear localization with longer exposure to stress, reaching the highest nuclear levels during delayed/late phase that promoted nuclear export of Nrf2. In agreement, a similar pattern of results was observed with hydrogen peroxide (Fig. 5B) and UV light (Fig. 5C). These results demonstrated that antioxidant/oxidant and UV light during early response induced nuclear localization of Nrf2. This was followed by nuclear import of Src kinases that led to nuclear export of Nrf2.

Previous studies have shown that chemical/radiation exposure induces Nrf2Ser40 phosphorylation, leading to nuclear import of Nrf2, and coordinated activation of gene expression (1, 2). This is followed by Nrf2Tyr568 phosphorylation in the nucleus, leading to nuclear export and degradation of Nrf2 (1, 2). Therefore, we reasoned that chemicals and radiation induce Nrf2Ser40 phosphorylation during the early phase of exposure, with a subsequent increase in Nrf2Tyr568 phosphorylation during delayed/late phase of induction. We tested this by using the same time points of exposure as used in the earlier experiments. Hepa-1 cells were co-transfected with Nrf2-V5 or mutant Nrf2Y568A-V5, and transfected cells were treated with DMSO (8 h), antioxidant t-BHQ, hydrogen peroxide, or UV light for different time intervals as shown in the figures. Cells were lysed, and cell lysates were immunoprecipitated with IgG (control) or anti-V5 and immunoblotted with anti-V5, anti-phosphoserine, and anti-phosphotyrosine antibodies (Fig. 5, D and E). The results revealed an increase in Nrf2 serine phosphorylation at 1 and 4 h of t-BHQ exposure (Fig. 5D, left panel). The
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FIGURE 5. Exposure to antioxidant/oxidant or UV light triggers nuclear import of Src kinases leading to nuclear export of Nrf2. A–C, antioxidant/oxidant and UV-mediated nuclear import of Src kinases. Hepa-1 cells were transfected with Nrf2-V5 and mutant Nrf2Y568A (D), or exposed to 2.5 mM H2O2 (E). Hepa-1 cells were transfected with Nrf2-V5 (D) or mutant Nrf2Y568A-V5 (E). In related experiments, SYF fibroblasts (MEFs) expressing Fyn, Src, Yes, and Fgr (SYF) were used to demonstrate phosphorylation of Nrf2 (D) and mutant Nrf2Y568A (E). Hepa-1 cells were transfected with Nrf2-V5 (D) or mutant Nrf2Y568A-V5 (E) and SYF fibroblasts (MEFs) expressing Fyn, Src, Yes, and Fgr (SYF). A–C, time-dependent nuclear import of Src kinases leading to nuclear export of Nrf2. Nrf2 was phosphorylated at Tyr568 in SYF cells expressing Fyn, Src, Yes, and Fgr (Fig. 5D). However, SYF-/- cells deficient in Src subfamily members showed absence of phosphorylation in both SYF+/+ and SYF/-/- cells indicating that Src subfamily members phosphorylated Nrf2 at the Tyr-568 site (Fig. 5C). In related experiments, we followed t-BHQ-dependent Fyn localization in the nucleus and nuclear export of Nrf2 in SYF+/+ and SYF/-/- cells (Fig. 6D). Antioxidant t-BHQ in SYF+/+ cells led to nuclear import of Nrf2 at 1 h, with a nuclear export between 4 and 8 h after nuclear accumulation of Fyn (Fig. 6D). Interestingly, SYF/-/- cells demonstrated t-BHQ time-dependent nuclear accumulation of Nrf2 that maximized at 8 h after exposure. In further experiments, SYF/-/- cells failed to show increased ubiquitination of Nrf2 and also had higher basal expression and t-BHQ induction of ARE-luciferase gene expression, as compared with SYF+/+ cells (Fig. 6E and F). The results together provided additional support to conclusions from Hepa-1 cells and demonstrated that Src subfamily members Fyn, Src, Yes, and Fgr phosphorylated Nrf2 and impaired nuclear accumulation of Nrf2.

Mouse Embryonic Fibroblasts Deficient in Src Subfamily Members Fyn, Src, Yes, and Fgr Lack Nrf2 Tyrosine Phosphorylation and Nuclear Export of Nrf2

The Src subfamily members Fyn, Src, Yes, and Fgr were shown to be involved in the phosphorylation of Nrf2 (Fig. 6B). These results indicated that the Src subfamily members phosphorylated Nrf2, which was maximized during delayed/late phase of t-BHQ treatment. In similar experiments, mutant Nrf2Y568A showed absence of phosphorylation in both SYF+/+ and SYF/-/- cells indicating that Src subfamily members phosphorylated Nrf2 at the Tyr-568 site (Fig. 6C). In related experiments, we followed t-BHQ-dependent Fyn localization in the nucleus and nuclear export of Nrf2 in SYF+/+ and SYF/-/- cells (Fig. 6D). Antioxidant t-BHQ in SYF+/+ cells led to nuclear import of Nrf2 at 1 h, with a nuclear export between 4 and 8 h after nuclear accumulation of Fyn (Fig. 6D). Interestingly, SYF/-/- cells demonstrated t-BHQ time-dependent nuclear accumulation of Nrf2 that maximized at 8 h after exposure. In further experiments, SYF/-/- cells failed to show increased ubiquitination of Nrf2 and also had higher basal expression and t-BHQ induction of ARE-luciferase gene expression, as compared with SYF+/+ cells (Fig. 6E and F). The results together provided additional support to conclusions from Hepa-1 cells and demonstrated that Src subfamily members Fyn, Src, Yes, and Fgr phosphorylated Nrf2 and impaired nuclear accumulation of Nrf2.
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GSK3β Is Upstream to Src Subfamily Kinases in Control of Nrf2—GSK3β have recently been reported to phosphorylate Nrf2 in its Neh6 domain on a serine leading to Nrf2 degradation by the SCFβ-TrCP complex, mostly in the cytosol (35). In addition, we have reported that GSK3β regulates Fyn, leading to nuclear localization of Fyn, and phosphorylation of Nrf2 (34). In this study, we examined the role of GSK3β in the antioxidant t-BHQ-mediated nuclear import of Src kinases and nuclear export of Nrf2 (Fig. 7). We used siRNA and LiCl to inhibit GSK3β and determine its effect on nuclear import of Fyn, Src, Yes, and Lyn kinases and nuclear export of Nrf2 (Fig. 7, A and B). The treatment of untransfected Hepa-1 cells with antioxidant t-BHQ had no effect on nuclear levels of GSK3β (Fig. 7A, left panel). On the contrary, the treatment of Hepa-1 with t-BHQ led to time-dependent nuclear accumulation of Fyn, Src, and Yes, but not Lyn, in a reciprocal pattern to the nuclear import and then export of Nrf2 (Fig. 7A, left panel). siRNA-mediated inhibition of GSK3β blocked the nuclear accumulation of Src kinases and led to nuclear accumulation of Nrf2 (Fig. 7A, right panel). In related experiments, the treatment of Hepa-1 cells with t-BHQ at 8 h and hydrogen peroxide at 4 h both led to an increase in phospho-Y216GSK3β (activated GSK3β), nuclear accumulation of Src kinases, and nuclear export of Nrf2 (Fig. 7B). Interestingly, inhibition of GSK3β activity using lithium chloride led to a significant decrease in nuclear accumulation of Fyn, Src, Yes, and a corresponding increase in nuclear accumulation of Nrf2 because of decreased nuclear export (Fig. 7B). These results suggest that GSK3β is upstream to Src kinases that regulate nuclear export of Nrf2.

Physiological Significance of Src Kinase Family-mediated Regulation of Nrf2—Two different but complementary systems were used to determine the physiological significance of Src kinases in control of Nrf2. The first system used Hepa-1 cells that were transfected with pcDNA or Nrf2-V5, or Nrf2-V5 in combination with FLAG-tagged Src kinases Fyn, Src, Yes, or Lyn. Immunoblotting was performed to analyze the transfected cells for the overexpression of FLAG-tagged kinases, Nrf2-V5, and Nrf2 downstream gene expression. The transfected cells were also analyzed for etoposide-induced apoptotic cell death.

basal and t-BHQ-induced expression of Nrf2 downstream genes.

FIGURE 6. Mouse embryonic fibroblasts deficient in Src subfamily kinase (MEF SYF/−/−) express higher accumulation of Src kinases and demonstrate reduced phosphorylation/nuclear accumulation of Nrf2 and decreased ubiquitination. A, western blot (WB) analysis of MEF SYF/−/− showing absence of Src subfamily kinases Src, Yes, Fyn, and Fgr. Cell lysates (80 μg) from SYF/−/−, SYF/−/−, and SYF/++ were immunoblotted with Nrf2, Fyn, Src, Yes, Fgr, and actin antibodies. B, Nrf2 tyrosine phosphorylation analysis. SYF/−/− and SYF/++ were treated with DMSO or t-BHQ for 1–8 h. Cells were harvested, and nuclear extracts were prepared using Active Motif kit. Five hundred micrograms of nuclear lysates were immunoprecipitated (IP) with control IgG or anti-Nrf2 antibody or anti-phosphotyrosine antibody and immunoblotted with anti-phosphotyrosine antibody or anti-ubiquitin antibody, respectively. *, unspecific bands. C, tyrosine phosphorylation of Nrf2 and mutant Nrf2Y568A-V5 for 24 h and cells were treated with DMSO or t-BHQ. Whole cell lysates (80 μg) and nuclear extracts were prepared by Active Motif kit. Eighty micrograms of proteins were immunoblotted with antibodies against Nrf2, Fyn, lamin-B, and LDH. D, ubiquitination analysis of Nrf2 in SYF/−/− and SYF/++ MEFs. SYF/−/− and SYF/++ MEFs were treated with DMSO or t-BHQ for 1–8 h. Cells were harvested, and nuclear extracts were prepared using Active Motif nuclear extract kit. Eighty micrograms of proteins were immunoblotted with anti-Nrf2 antibody or anti-phosphotyrosine antibody or anti-ubiquitin antibody, respectively. E, whole cell lysate. SYF/−/− and SYF/++ MEFs were treated with DMSO or t-BHQ, respectively (compare lanes 2–5). Whole cell lysate. Ubiquitination (Ub) of endogenous Nrf2 was 2.5- and 1.9-fold more in SYF/−/− and SYF/++ MEFs, SYF/−/− and SYF/++ MEFs, respectively. F, luciferase assay. SYF/−/− and SYF/++ MEFs were treated with DMSO or t-BHQ for an additional 24 h. Cells were harvested, and luciferase activity was measured and plotted. The data shown are mean ± S.D. of three independent transfection experiments. All experiments were performed three times, and one set of data was shown.
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FIGURE 7. Inhibition of GSK3β decreases antioxidant/oxidant-induced nuclear import of Src subfamily kinases and nuclear accumulation of Nrf2. A, siRNA-mediated inhibition of GSK3β. Hepa-1 cells were treated with DMSO or t-BHQ (50 μM) for 2–8 h (left panel). Another set of cells was transfected with control siRNA (50 nM) or GSK3β siRNA (50 nM) for 24 h, and cells were then treated with t-BHQ (50 μM) for 2–8 h (right panel). Nuclear extracts were prepared using Active Motif kit, and 80 μg of nuclear extracts were immunoblotted with GSK3β, Fyn, Src, Yes, Lyn, Nrf2, lamin-B, and LDH antibodies. B, LiCl inhibition of GSK3β. Hepa-1 cells were treated with DMSO or t-BHQ (50 μM) for 4–8 h (left panel) or H2O2 (2.5 mM) for 2–4 h (right panel). Another set of cells was pretreated with LiCl (50 mM) for 2 h, and cells were further treated with t-BHQ (50 μM) for 2–8 h (left panel) or H2O2 (2.5 mM) for 2–4 h (right panel) in the presence of LiCl. Nuclear extracts were prepared using Active Motif kit, and 80 μg of nuclear extracts were immunoblotted with GSK3β, GSK3β phosphotyrosine 216, Fyn, Src, Yes, Lyn, Nrf2, lamin-B, and LDH antibodies. All experiments were performed three times, and one set of data is presented.

DISCUSSION

Nrf2 is a master regulator of 200+ cytoprotective gene expression (1, 2). These cytoprotective genes include chemical/drug-detoxifying enzymes, antioxidants, proteasomes, drug transporters, and anti-apoptotic proteins (1, 2). Exposure to chemical and radiation stressors stabilizes Nrf2, leading to coordinated activation of cytoprotective gene expression and cell survival. Nrf2 is a double edge sword that is required for cellular protection, but accumulation of nuclear Nrf2 also leads to problems associated with decreased apoptosis/increased survival of damaged cells and drug resistance. Therefore, Nrf2-mediated transcription has to be tightly regulated by positive and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A). Etoposide-mediated apoptotic cell death and cell survival of damaged cells and drug resistance. Therefore, Nrf2-mediated transcription has to be tightly regulated by positive and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A). Etoposide-mediated apoptotic cell death and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A). Etoposide-mediated apoptotic cell death and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A). Etoposide-mediated apoptotic cell death and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A). Etoposide-mediated apoptotic cell death and cell survival (Fig. 8). Immunoblotting results demonstrated that overexpression of Fyn, Yes, and Src, but not Lyn, significantly decreased cellular Nrf2 and Nrf2 downstream gene expression (Fig. 8A).
and negative factors. Specifically, Nrf2 must be switched off once it has done its cellular protective function. The positive factors that control Nrf2 dissociation from INrf2, its subsequent nuclear translocation, and the coordinated activation of Nrf2 downstream gene expression have been well characterized (1, 2). Studies have shown that stress-induced modification of INrf2- and PKCα-mediated Nrf2Ser40 phosphorylation leads to the release of Nrf2 from INrf2, stabilization, and nuclear translocation of Nrf2 and activation of Nrf2 downstream gene expression leading to cellular protection.

This study employed siRNA knockdown of the individual Src kinase family members, as well as their overexpression in Hepa-1 cells and Src kinase null cell systems, to investigate the role of Src kinases in the control of Nrf2. The positive factors that control the dissociation from INrf2, its subsequent nuclear translocation, and the coordinated activation of Nrf2 downstream gene expression have been well characterized (1, 2). Studies have shown that stress-induced modification of INrf2- and PKCα-mediated Nrf2Ser40 phosphorylation leads to the release of Nrf2 from INrf2, stabilization, and nuclear translocation of Nrf2 and activation of Nrf2 downstream gene expression leading to cellular protection.

This study employed siRNA knockdown of the individual Src kinase family members, as well as their overexpression in Hepa-1 cells and Src kinase null cell systems, to investigate the role of Src kinases in the control of Nrf2. The results present clear evidence that all the four Src subfamily members, including Fyn, Src, Yes, and Fgr, follow Nrf2 in the nucleus, phosphorylate Nrf2Tyr568 that leads to nuclear export, ubiquitination/degradation of Nrf2, and down-regulation of Nrf2 downstream gene. Notably, the Lyn kinase belonging to the Lyn subfamily of Src kinases did not participate in the control of Nrf2. The results also showed that stress-induced activation of GSK3β in response to stressors regulated the nuclear accumulation of Src kinases in the nucleus leading to phosphorylation of Nrf2Tyr568 and nuclear export/degradation of Nrf2. The stress-responsive kinase(s) involved in phosphorylation of GSK3βY216 leading to activation of GSK3β remains unknown. The results also showed that in response to stress, the kinetics of the Nrf2 cycle have two prominent phases (Fig. 10). The first phase, or early response to stressors, spans 0–4 h. During this time, Nrf2 is activated in response to stress, imported in the nucleus, forms heterodimers with its partners, and binds to ARE in promoter regions of cytoprotective genes leading to coordinated activation of their expression. Subsequently, early response phase is followed by another 4 h (5–8 h) of delayed/late response phase to stressors. Activated GSK3β phosphorylates Src subfamily members, which signals their translocation into the nucleus to switch off Nrf2 activation. This is achieved by Src subfamily member-mediated phosphorylations of Nrf2Tyr568 in the nucleus, which leads to nuclear export, ubiquitination, and degradation of Nrf2. The sites of phosphorylation of Src kinases by GSK3β remain unknown. In addition, the mechanism of nuclear import of Src subfamily of kinases also remains unknown and is a subject of ongoing investigation. This is especially significant because Procite analysis of Fyn, Src, Yes, and Fgr showed absence of putative nuclear import signals. Therefore, the nuclear import of these kinases likely involves the use of yet to be identified carrier protein(s).

3 Phillip Shelton and Anil K. Jaiswal, unpublished data.
Current studies also demonstrate that alterations in Src subfamily kinases adversely affect Nrf2 degradation and Nrf2 downstream gene expression. Overexpression of Src subfamily kinases led to down-regulation of Nrf2 downstream cytoprotective proteins, increased apoptosis, and decreased cell survival. Therefore, overexpression of Src kinases has an adverse impact on normal cell survival. It is noteworthy that many cancers, including prostate, melanoma, pancreatic, glioma, and chronic myelogenous leukemia, either overexpress or have constitutively activated Src kinases (32). Our results suggest that the Src kinase regulations of Nrf2 might have contributed to the development of these cancers because overexpression/activated Src kinases could significantly reduce Nrf2 in the nucleus leading to the loss of cytoprotection. Our results also indicated that SYF<sup>−/−</sup> cells, in comparison with SYF<sup>+/+</sup> cells, contain higher nuclear Nrf2 and express higher levels of Nrf2 downstream gene expression, demonstrate decreased apoptosis, and increased survival when exposed to DNA damaging agents, such as etoposide. This result, along with our observation that GSK3β controls nuclear localization of Src kinases, suggests that defective nuclear localization of Src kinase might lead to accumulation of Nrf2 in the nucleus and increased survival of damaged cells. In addition, PTEN-deficient tumors are known to have increased activation of the PI3K/Akt pathway (36). Activated Akt is known to phosphorylate GSK3βS9 leading to its inactivation. Thus, PTEN-deficient tumors containing higher inactive GSK3β are expected to have reduced accumulation of Src kinases in the nucleus, leading to nuclear accumulation of Nrf2 and increased cell survival. Therefore, the loss of Src kinase-mediated control of Nrf2 is expected to have contributed to the development of PTEN-deficient tumors. Taken together, alterations in Src kinases have adverse effects on Nrf2 and downstream cytoprotective proteins with implications in cancers with overexpressed/activated Src kinases and/or loss of PTEN.

In summary, we have shown that the Src subfamily members Fyn, Src, Yes, and Fgr are all substrates of stress-activated GSK3β and accumulate in the nucleus. The nuclear accumulation of the Src kinases results in the phosphorylation of Nrf2<sup>Tyr568</sup>, which leads to nuclear export and degradation of Nrf2. In summary, Src kinase-mediated degradation of Nrf2 is important in switching off the Nrf2 downstream cytoprotective gene expression that was coordinately activated because of stress-induced nuclear import of Nrf2. The alterations in Src kinases lead to alterations in cytoprotection, apoptosis, and cell survival. The role of Src kinases in the regulation of the Nrf2...
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pathway might have contributed to development of cancers overexpressing Src kinases and/or deficient in PTEN. Together, this implicates Src kinases as being an important part of the Nrf2 cycle that provides protection to cells against chemical/radiation exposures.

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