c-Fos Proteasomal Degradation Is Activated by a Default Mechanism, and Its Regulation by NAD(P)H:Quinone Oxidoreductase 1 Determines c-Fos Serum Response Kinetics[▽]

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The short-lived proto-oncoprotein c-Fos is a component of the activator protein 1 (AP-1) transcription factor. A large region of c-Fos is intrinsically unstructured and susceptible to a recently characterized proteasomal ubiquitin-independent degradation (UID) pathway. UID is active by a default mechanism that is inhibited by NAD(P)H:quinone oxidoreductase 1 (NQO1), a 20S proteasome gatekeeper. Here, we show that NQO1 binds and induces robust c-Fos accumulation by blocking the UID pathway. c-Jun, a partner of c-Fos, also protects c-Fos from proteasomal degradation by default. Our findings suggest that NQO1 protects monomeric c-Fos from proteasomal UID, a function that is fulfilled later by c-Jun. We show that this process regulates c-Fos homeostasis (proteostasis) in response to serum stimulation, phosphorylation, nuclear translocation, and transcription activity. In addition, we show that NQO1 is important to ensure immediate c-Fos accumulation in response to serum, since a delayed response was observed under low NQO1 expression. These data suggest that *in vivo*, protein unstructured regions determine the kinetics and the homeostasis of regulatory proteins. Our data provide evidence for another layer of regulation of key regulatory proteins that functions at the level of protein degradation and is designed to ensure optimal formation of functional complexes such as AP-1.

The short-lived c-Fos protein is a component of the activator protein 1 (AP-1) transcription factor, a master switch that regulates numerous cellular processes such as proliferation, survival, differentiation, apoptosis, transformation, and tumor invasion (23). c-Fos is expressed at low or undetectable levels in most cell types but can be rapidly and transiently induced by many stimuli (23). As a component of the AP-1 complex, c-Fos is heterodimerized with members of basic region-leucine zipper (bZip) proteins from the Jun, activating transcription factor (ATF), and Maf families and binds to AP-1/TPA response element (TRE) and cyclic AMP response element (CRE) DNA motifs via a basic domain N-terminally adjacent to the leucine zipper domain (23). Although c-Fos accumulation is controlled by transcription induction, the rate of c-Fos translation, nuclear entry, and degradation is nevertheless important in determining c-Fos homeostasis (11, 16, 34, 44, 54).

The selective degradation of many short-lived proteins in cells is mediated via the ubiquitin-26S proteasome-dependent degradation (UDD) pathway (25, 27). However, proteins are also susceptible to ubiquitin-independent degradation (UID) that is mediated by the core 20S proteasomes (7, 31, 51) in the absence of 19S, a proteasomal regulatory complex, but in the presence or absence of REG γ , a 20S activator (36). Neither 20S nor REG γ recognizes ubiquitylated proteins, and therefore they selectively act on ubiquitylation-independent substrates.

Over the last 2 decades, experimental and theoretical evidence has accumulated demonstrating that many proteins con-

tain extensive disordered regions and that some proteins are even completely unstructured under physiological conditions (20). These proteins are termed natively unfolded (53), intrinsically disordered proteins (IDPs), intrinsically unstructured proteins (IUPs), or 4D proteins (51). Many IUPs are regulatory proteins and involved in transcriptional regulation and signal transduction (21, 49). New evidence raises the interesting possibility that intrinsically unstructured proteins are subjected to a UID pathway that does not require prior modification, a process we refer to as “degradation by default” (6, 52).

c-Fos is a short-lived protein with a half-life of approximately 1 h (32). Its instability explains why its physiological accumulation is transient. The c-Fos level is regulated by numerous and intricate transcriptional and posttranscriptional mechanisms. The major route of c-Fos protein degradation is mediated by the proteasome. 26S proteasome-dependent degradation of c-Fos has been shown to be ubiquitin dependent (44, 48). However, recent studies demonstrated that c-Fos is also degraded by the proteasome *in vivo* without requiring ubiquitination (11, 12). In the nucleus, c-Fos is subjected to UID, but in the cytoplasm, to UDD (44). At least the C-terminal half of c-Fos is intrinsically unstructured (14). This region is involved in UID (12). Fra-1, a related protein, appears to behave in a manner similar to that of c-Fos (11). Yet, the identity of the proteasome species mediating the UID of c-Fos has remained an open question. Also, given the expected differences between the kinetics of UDD and UID, it is possible that the balance between these processes may program c-Fos homeostasis.

As exemplified by p53, p73 α , and ornithine decarboxylase (ODC), ubiquitin-independent degradation by default is inhibited by NAD(P)H:quinone oxidoreductase 1 (NQO1) (6). NQO1 is a flavoenzyme that catalyzes two-electron reduction

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of various quinones, utilizing NADH or NAD(P)H (reviewed in reference 41). NQO1 is associated with the core 20S proteasome but not with the 26S proteasome (8). NQO1 physically interacts with and protects the IUPs, such as p53, p73 α , and ODC, from degradation (2, 8). The specific association of NQO1 with the 20S proteasomes and its ability to bind and to protect a subset of short-lived IUPs from 20S proteasomal degradation suggest that NQO1 is a proteasomal "gatekeeper" that inhibits degradation by default (6, 8). As the relative contribution of UID and UDD may program c-Fos homeostasis, it is important to know whether and how c-Fos UID is regulated by NQO1. The case of c-Fos is of particular interest since the degradation and nuclear entry of Yap4, a bZip yeast protein, is regulated by Lot6, the yeast NQO1 paralog (46, 47).

One of the major *cis*-acting elements regulating the basal and induced *nqo1* gene expression is the antioxidant response element (ARE) that contains AP-1 and AP-1-like recognition sequences and recruits several bZip transcription factors, such as Nrf1, Nrf2, Nrf3, c-Fos, Fra1, c-Jun, JunB, JunD, Bach1, and small Maf proteins (28). The positive regulation of *nqo1* expression is achieved mainly through Nrf2, which binds ARE as a heterodimer with small Maf proteins, as reviewed in reference 28. On the other hand, c-Fos plays a repressive role in the regulation of *nqo1* gene expression. First, c-Fos has been shown to bind the ARE of the *nqo1* gene (33). Second, overexpression of c-Fos represses ARE-mediated transcription (55). Finally, NQO1 protein levels and activity are increased in the tissues of mice lacking c-Fos compared to levels in the tissues of wild-type (WT) animals (55). The small Maf proteins, which also act in the transcriptional inhibitory mode on the *nqo1* promoter (19), were proposed to form the repressor complex with c-Fos (39). The regulatory module shared between c-Fos and *nqo1* and the fact that c-Fos level is likely to be regulated by the default mechanism prompted us to investigate regulation of the c-Fos level by NQO1.

Here, we demonstrate that NQO1 protects c-Fos from UID under overexpression conditions and in its normal physiological setting to program c-Fos rapid accumulation. We show that the NQO1 protective function becomes dispensable upon formation of an AP-1 complex by mechanisms that involve the leucine zipper and nuclear translocation. We also demonstrate that NQO1 and c-Fos are interconnected by a feedback regulatory loop that may control the intracellular levels of the c-Fos proto-oncogene.

MATERIALS AND METHODS

Tissue culture, transfections, and reagents. The cell lines used were 293 human embryonic kidney (HEK293) cells, A31N-ts20 cells, a BALB/c mouse cell line that harbors a temperature-sensitive E1 ubiquitin-activating enzyme (17, 43), human breast adenocarcinoma cell line MCF-7, human non-small-cell lung carcinoma cell line H1299, and p53-null human colon carcinoma HCT116 cells (13). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 8% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin and cultured at 37°C in a humidified incubator with 5% CO₂. H1299 cells were cultured under the same conditions with RPMI medium (Gibco). A31N-ts20 cells were cultured at 32°C. To inactivate the E1 ubiquitin-activating enzyme, A31N-ts20 cells were transferred to 39°C for 24 h (17). Transient transfections of HEK293 cells were carried out by the calcium phosphate method, and transient transfections of A31N-ts20, MCF-7, and HCT116 cells were done with jetPEI (Polyplus Transfection). Transfected A31N-ts20 cells were cultured for 4 h at 32°C and then transferred to 39°C for another 24 h. For serum stimulation, cells were washed twice, cultured for 24 h in serum-free

DMEM, and then challenged with 10% FBS. To establish a stable NQO1 knock-down cell line, MCF-7 and A31N-ts20 cells were transduced with lentiviral particles containing NQO1-targeting or control short hairpin microRNA (shRNA_{Amir}) (Open Biosystems), according to the manufacturer's instructions. The cells were then selected with puromycin.

Plasmids. The plasmids used were pEFIREs-NQO1 encoding human NQO1, pSUPER-NQO1 expressing NQO1 shRNA (4), and pEFIREs-Flag-p73 α and -Flag-p73 β encoding simian Flag-p73 α and Flag-p73 β (8). The coding region of human c-Fos was cloned into pCDNA vector without or with the addition of the Flag tag at the N terminus. A leucine zipper mutant of c-Fos defective in heterodimerization with c-Jun (with L179V, L186A, and L193V) (45) was cloned by site-directed mutagenesis, resulting in pCDNA-Flag-c-Fos (VAV). The c-Fos mutant truncated before the leucine zipper domain (harboring the first 164 amino acids) was cloned based on pCDNA-Flag-c-Fos. The coding region of human c-Jun was cloned into pCDNA or used in a hemagglutinin (HA)-tagged version in S3H c-Jun plasmid (18). The wild-type mouse c-Fos (pCEFL-AU5-c-Fos) and the mutant containing simultaneous alanine replacements on T-232, T-325, T-331, and S-374, a gift from J. S. Gutkind (37), were cloned into pCDNA vector with the addition of the Flag tag at the N terminus. The lysineless Myc₆K/R-c-FosK/R mutant and the phosphomimetic Fos mutants (with S362D and S374D or T232D, T325D, and T331D) in pCDNA are gifts from M. Piechaczyk (24).

The coding region of human REG γ (provided by B. W. O'Malley) was cloned into pEFIREs expression vector with the addition of the Flag tag at the N terminus (pEFIREs-Flag-REG γ). The expression vector for p21^{Cip1} (pCS2-p21^{Cip1}) was provided by M. Pagano. The expression vector for human Maff (pEGFP-Maff) was a gift from Y. Yarden.

Proteasome purification and *in vitro* protein degradation assays. 20S proteasomes from mouse livers were purified as previously described (8). For the *in vitro* degradation assay, c-Fos was *in vitro* translated and labeled with [³⁵S]methionine by using the TNT coupled reticulocyte lysate system (Promega). The *in vitro* degradation assay of *in vitro*-translated c-Fos with 2 μ g of purified 20S proteasome was carried out as described previously (8).

Immunoblot analysis. Cell extracts and immunoblot analyses were carried out as described previously (3). The antibodies used were anti-NQO1 C-19 and H90, anti-c-Fos H-125, anti-c-Jun H-79, anti-p21 F5 and C-19 (Santa Cruz Biotechnology), an anti-C9 (PSMA4) subunit of the 20S proteasome (35), anti- β -tubulin, and anti-Flag M5 antibody (Sigma). Coimmunoprecipitation experiments were carried out as previously described using anti-Flag agarose beads (Sigma) (5). Alkaline phosphatase treatment of cell extracts was done using 30 μ g of protein in 10 μ l radioimmunoprecipitation assay (RIPA) buffer incubated with 30 units of calf intestine phosphatase (CIP; New England BioLabs) for 45 min at 30°C.

Fractionation studies. A subcellular fractionation procedure was performed with HEK293 cells 24 h following transfection with pCDNA-c-Fos with or without pEFIREs-NQO1 and pCDNA-c-Jun. To extract cytosol, phosphate-buffered saline (PBS)-washed cells (10⁷) were incubated for 1.5 min on ice in 10 mM hypotonic HEPES-KOH buffer (pH 7.9) containing 10 mM KCl, 0.34 M sucrose, 10% glycerol, 1.5 mM MgCl₂, 0.2% NP-40, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Sigma) (cytosolic extraction buffer). The samples were centrifuged at 1,000 $\times$ g for 5 min at 4°C to separate nuclei from the cytosol. Cytosolic fraction (C) was clarified by centrifugation at 10,000 $\times$ g for 15 min at 4°C. Nuclei were washed three times in the above-mentioned buffer but devoid of the detergent, and the nuclear proteins were extracted for 10 min on ice in the nuclear extraction buffer (10 mM HEPES-KOH, pH 7.9, containing 400 mM NaCl, 25% glycerol, 1.5 mM MgCl₂, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT, and protease inhibitor cocktail). The samples were centrifuged at 10,000 $\times$ g for 15 min at 4°C to separate the soluble nuclear fraction (N) and the insoluble materials. Equivalent amounts of the fractions were subjected to immunoblot analysis.

For fractionation studies on a glycerol gradient, HEK293 cells were transfected with pCDNA-c-Fos with or without pEFIREs-NQO1 for 24 h. Cells were lysed in RIPA buffer and loaded onto 10 ml of a 5 to 20% glycerol gradient built in 20 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl. The samples were ultracentrifuged at 96,000 $\times$ g for 16 h. Fractions (1 ml/tube) were collected, and samples (10 μ g) were analyzed by Western blot analysis.

RT-PCR analysis. Total RNA was extracted from cells using Tri-Reagent (Promega). Reverse transcription (RT) was carried out from total RNA using the Reverse-iT 1st-strand synthesis kit (ABgene, United Kingdom). PCRs were done using Taq polymerase master mix from Promega.

Transactivation assay. For transactivation assays, cells were transfected with the pColl-73-Luc or pHV-nqo1-Luc reporter plasmid, the pEF1Renilla plasmid as a normalization control, pCDNA-c-Fos, S3H HA-c-Jun, pEFIREs vector or pEFIREs-NQO1, pEGFP or pEGFP-Maff, and pSUPER empty vector or

FIG. 1. c-Fos undergoes degradation by default. (A) Structure of c-Fos protein. Numbers indicate the amino acid positions. DBD, DNA binding domain; LZ, leucine zipper domain; transcription activation domain (TAD). Regions characterized by intrinsic disorder (14) are underlined. Major phosphorylation sites recognized by the respective kinases (10, 38, 44) are shown. (B) *In vitro*-translated ^{35}S -labeled c-Fos or PCNA was incubated with purified 20S proteasomes at 37°C for the indicated times. The products of the degradation reactions were separated by SDS-PAGE and visualized by autoradiography. In this experiment, c-Fos was readily degraded, whereas PCNA was stable up to 180 min. (C) ^{35}S -labeled c-Fos was treated as described for panel B, but CIP was added to detect the c-Fos phosphorylated form. (D) A31N-ts20 cells transfected with pCDNA-Flag-c-Fos at 39°C for 24 h were cultured in the presence of proteasome inhibitor MG132 (2 μM) or the vehicle (dimethyl sulfoxide [DMSO]) for 5 h, harvested, and analyzed by Western blotting. Under this condition, c-Fos accumulated by MG132 provides a reliable indication for c-Fos UID. (E) To examine the possibility that Reg γ regulates c-Fos degradation, HEK293 cells were cotransfected with pCDNA-c-Fos and pCS2-p21^{Cip1} with or without pEFIREs-Flag-REG γ . Twenty-four hours after transfection, the cells were treated with 25 μM MG132 or DMSO for 5 h and analyzed as described for panel D.

pSUPER-NQO1shRNA, as appropriate. Luciferase activity was examined 24 or 48 h following transfection, using a Modulus microplate luminometer (Turner Biosystems).

Immunofluorescence staining. HEK293 cells were seeded on glass coverslips precoated with 0.1% gelatin. Twenty-four hours following transfection with pCDNA-c-Fos with or without pEFIREs-NQO1 and pCDNA-c-Jun, the cells were washed and fixed in 4% paraformaldehyde for 30 min. Fixed cells were then permeabilized with 0.5% (vol/vol) Triton X-100 for 25 min and washed and blocked with fetal calf serum (FCS) containing 10% skim milk and 0.2% Tween 20 for 1 h. Cells were then incubated for 1 h with a mixture of mouse monoclonal anti-Flag M5 antibody, rabbit anti-c-Jun H-79 antibody, and goat anti-NQO1 C19 and H90 antibodies in PBS containing Tween 20 (PBS-T) with 10% milk. The cells were washed and incubated with a mixture of Cy3-conjugated donkey anti-mouse antibody, fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody, and Cy5-conjugated donkey anti-goat antibody (Jackson Immuno Research Laboratories) for 1 h in PBS-T with 10% milk. The washed cells were stained with Hoechst 33342 (Molecular Probes) and mounted in Aqua-Poly/Mount (Polysciences). Microscopic images were obtained with a Nikon Eclipse TE300 microscope (60 $\times$ /1.4 numerical aperture [oil] objective) equipped with a Radiance 2100 confocal laser scanning system (Bio-Rad) and imaged by Laser Sharp 2000 software (Zeiss). Photoshop (Adobe) was used to make combinatory figures.

RESULTS

c-Fos undergoes degradation by default. Proteins bearing intrinsically unstructured regions (IUPs) are prone to degradation *in vitro* by the 20S proteasome (51). Also, *in vivo*, these proteins can undergo degradation in a ubiquitin-independent fashion by a “degradation-by-default” mechanism (6). The C-

terminal half of c-Fos is intrinsically unstructured (Fig. 1A) (14) and involved in UID of c-Fos (12). To examine the behavior of c-Fos as an IUP, we incubated ^{35}S -labeled c-Fos with purified 20S proteasomes and revealed that c-Fos was efficiently degraded, whereas the structured protein PCNA was resistant (Fig. 1B). Under this condition, c-Fos was phosphorylated, as detected by incubation with CIP (Fig. 1C). The 20S proteasome susceptibility was not affected by phosphorylation. These results demonstrate that c-Fos, an IUP, can be degraded by the 20S proteasomes and therefore is likely to undergo *in vivo* degradation by default.

Next, we used the A31N-ts20 BALB/c mouse cell line harboring the temperature-sensitive ubiquitin-activating enzyme E1, whose ubiquitin-dependent degradation (UDD) is massively repressed under a restrictive temperature (39°C) (43). Previously, using this cell line, we found that at 39°C the UDD of p53 is blocked, giving rise to p53 accumulation (5). c-Fos accumulated in response to treatment with the proteasome inhibitor MG132 in A31N-ts20 cells grown at 39°C (Fig. 1D), suggesting that c-Fos is subjected to proteasomal UID, as previously reported (12, 44). To explore whether the proteasome activator REG γ is involved in the UID of c-Fos, we examined c-Fos protein levels under REG γ overexpression. In contrast to p21^{Cip1}, a known substrate of REG γ -regulated proteasomal degradation (36), c-Fos protein was REG γ refrac-

FIG. 2. NQO1 inhibits c-Fos degradation by default. (A) HEK293 cells were transiently transfected with pCDNA-c-Fos with increasing amounts of pEFIREs-NQO1. Twenty-four hours after transfection, cells were lysed, and cell extracts were analyzed by Western blotting. An arrow marks the phosphorylated upshifted band. (B) MCF-7 cells were transfected with pCDNA-Flag-c-Fos alone or cotransfected with pSUPER empty vector or pSUPER-NQO1 expressing NQO1-specific shRNA. After 48 h, cell extracts were analyzed as described for panel A. (C) MCF-7 cells were treated as described for panel B, but 48 h after transfection, MG132 (10 μM) was added for 3 h. (D) HEK293 cells were transfected with the indicated plasmids. Twenty-four hours later, the cells were treated with 20 μM MG132 for 4 h, as indicated. Cells were labeled for 5 min with [³⁵S]methionine, and samples were immunoprecipitated with anti-Flag antibody and subjected to autoradiography (IP) or immunoblotted (total). (E) HEK293 cells were transiently transfected with pCDNA-Flag-c-Fos without or with pEFIREs-NQO1. After 24 h, cells were treated with 20 μg/ml CHX for 3 h, and the extracts were analyzed as described for panel A. (F) MCF-7 cells were transfected with pCDNA encoding Flag-c-Fos together with pSUPER-NQO1shRNA or pSUPER vector. After 24 h, cells were starved for serum for 24 h and challenged with serum for 1 h, and the extracts were analyzed as described for panel A. (G) HEK293 cells were transiently transfected with pCDNA encoding either the wild type or the phosphorylation-deficient mutant (T232, T325, T331, S374A) (A mutant) without or with pEFIREs-NQO1. After 24 h, the samples were treated as described for panel A. (H) MCF-7 cells were transfected with pCDNA encoding either the wild type or the phosphorylation-deficient A mutant together with pSUPER-NQO1shRNA or pSUPER vector. After 48 h, the samples were treated as described for panel A. (I) To demonstrate that NQO1 protects c-Fos UID, we used A31N-ts20 cells stably overexpressing pEFIREs-NQO1 and the parental cell line stably transfected with the empty vector pEFIREs. WT c-Fos, a lysineless mutant (with no lysine residue even in the Myc tag) that is unable to undergo ubiquitination, and a phosphomimetic mutant (with S362D and S374D) (D mutant) were all accumulated by NQO1. Under the restrictive temperature of 39°C (24 h), only the UID takes place; hence, the accumulation of c-Fos by NQO1 provides strong evidence for NQO1 blocking c-Fos UID. The fold increase in Fos protein levels caused by NQO1 overexpression was quantified by the LAS-4000 software and is shown relative to the corresponding control sample.

tory (Fig. 1E). These results suggest that c-Fos is an IUP that may undergo 20S proteasomal degradation by default.

NQO1 promotes accumulation of c-Fos by inhibiting its UID. NQO1 prevents proteasomal degradation by default of certain proteins (6). We examined whether the c-Fos level is regulated by NQO1. A significant dose-dependent increase in c-Fos protein level was observed in the presence of increasing amounts of NQO1 in HEK293 cells (Fig. 2A). Similar results

were obtained in MCF-7 and HCT116 p53-null cells (not shown). In a reciprocal experiment, NQO1 knockdown resulted in a marked reduction in the level of the transfected c-Fos protein (Fig. 2B), provided that no proteasome inhibitor MG132 was added (Fig. 2C), suggesting that NQO1 inhibits c-Fos proteasomal degradation.

Next, HEK293 cells were transfected with Flag-c-Fos with or without NQO1 to measure the c-Fos translation rate. After

FIG. 3. Role of c-Jun in the regulation of c-Fos degradation by default. (A) To examine the role of c-Jun in regulating c-Fos UID, MCF-7 cells were transfected with pCDNA-Flag-c-Fos alone or cotransfected with pCDNA-c-Jun. Twenty-four hours after transfection, cell extracts were analyzed by Western blotting. (B) To confirm that the upshifted band is the phosphorylated c-Fos, we used CIP. Cell extracts from the experiment described for panel A were incubated with CIP and analyzed by Western blotting. An arrow marks the phosphorylated upshifted band. (C) To demonstrate that c-Jun blocked c-Fos degradation by interfering with the UID process, A31N-ts20 cells were transfected with pCDNA-Flag-c-Fos without or with pCDNA-c-Jun at 39°C for 24 h, and the extracts were processed as described for panel A. (D) To examine the stability of c-Fos, we monitored the reduction level of the protein up to 6 h after blocking protein synthesis by CHX. HEK293 cells were transfected with pCDNA-Flag-c-Fos with or without pCDNA-c-Jun. After 24 h, the cell extracts were analyzed as described for panel A. (E) NQO1 is important for c-Jun to increase c-Fos accumulation. MCF-7 cells were transfected with pCDNA-Flag-c-Fos or pCDNA-c-Jun, alone or cotransfected with pSUPER-NQO1shRNA or pSUPER vector. After 48 h, cell extracts were analyzed as described for panel A. The level of c-Fos accumulation is much lower under NQO1 knockdown than in the presence of NQO1.

24 h, cells were MG132 treated for 4 h and then labeled for 5 min with [35 S]methionine. The labeled c-Fos was visualized by autoradiography after immunoprecipitation (IP) (Fig. 2D). NQO1 had a minor effect on c-Fos translation, because more labeled c-Fos was detected with NQO1 coexpression, even in the presence of MG132. The selective effect of NQO1 on c-Fos stability was verified by utilizing the protein synthesis inhibitor cycloheximide (CHX). As expected, the level of c-Fos was markedly attenuated by CHX but not under NQO1 overexpression (Fig. 2E), suggesting that NQO1 prolonged the c-Fos half-life. Furthermore, in the presence of NQO1, the full-length c-Fos was upshifted (Fig. 2A), possibly due to its phosphorylation.

It is well documented that c-Fos accumulates in response to serum induction. Transcription induction is a major mechanism, but we hypothesized that blocking c-Fos UID by default is also important. Interestingly, under NQO1 knockdown conditions, Flag-c-Fos accumulation in response to serum treatment was abolished (Fig. 2F). In a reciprocal experiment, NQO1 overexpression resulted in enhanced and faster accumulation of c-Fos in response to serum (data not shown). These data suggest that NQO1 is required for serum-induced c-Fos accumulation (also see below). To examine whether c-Fos phosphorylation is important in NQO1-dependent stabilization, we utilized a c-Fos mutant unable to undergo the major phosphorylation events (T232A, T325A, T331A, and S374A) (A mutant) (37) and obtained similar results with overexpres-

sion of NQO1 (Fig. 2G) and NQO1 knockdown (Fig. 2H). Finally, to demonstrate that NQO1 regulates c-Fos degradation via inhibiting UID, we used A31N-ts20 cells stably overexpressing NQO1 and revealed that NQO1 provoked a significant accumulation of the WT c-Fos, a lysineless (K/R) mutant, and phosphomimetic (D) mutants (with S362D and S374D [Fig. 2I] or T232D, T325D, and T331D [data not shown]) at the restrictive temperature. These results suggest that c-Fos UID is blocked by NQO1 and that protection by NQO1 is independent of c-Fos phosphorylation.

c-Jun blocks c-Fos degradation by default. As a component of AP-1, c-Fos acts as an obligatory heterodimer with basic region-leucine zipper (bZip) proteins (23). We hypothesized that NQO1 might protect monomeric c-Fos before it forms functional interactions with other bZip proteins and DNA. One of the main transcriptional partners of c-Fos is c-Jun, a member of the bZip family. Coexpression of c-Jun together with c-Fos in MCF-7 or HEK293 cells led to a significant increase in the steady-state level of c-Fos and also in the appearance of a slower-migrating band of c-Fos (Fig. 3A and B). The upshifted band appeared to be phosphorylated c-Fos, since treatment of cell lysates with alkaline phosphatase severely diminished its appearance (Fig. 3B). To determine whether c-Jun protects c-Fos from UID, both were coexpressed in A31N-ts20 cells, and the stabilizing effect of c-Jun on c-Fos was reproduced under 39°C (no ubiquitination) (Fig. 3C). Using CHX, we revealed that coexpression with c-Jun

FIG. 4. Role of the c-Fos leucine zipper in degradation by default. (A) To examine possible complex formation between NQO1 and c-Fos, HEK293 cells were transiently transfected with pCDNA-Flag-c-Fos, pEFIREs-Flag-p73α, or pEFIREs-Flag-p73β, together with pEFIREs-NQO1 (TOTAL). The p73α and p73β constructs were used as positive and negative controls, respectively. Immunoprecipitation of Flag-tagged proteins was performed with anti-Flag antibody-coupled beads (IP: Flag). The data clearly indicate that NQO1 is in association with c-Fos. (B) To examine whether NQO1 generates a tripartite complex with c-Fos and c-Jun, HEK293 cells were transiently transfected with pCDNA-Flag-c-Fos and pEFIREs-NQO1 without (–) or with (+) S3H HA–c-Jun (TOTAL). Immunoprecipitation of Flag–c-Fos was performed with anti-Flag beads (IP: Flag). The obtained data revealed that c-Jun interferes with NQO1–c-Fos complex formation. (C) HEK293 cells transfected with overexpressing c-Fos without or with NQO1 were lysed in RIPA buffer and loaded onto a 5 to 20% glycerol gradient. Afterward, ultracentrifugation fractions were collected and analyzed. The box with the dashed border shows the proteins shifted by NQO1 overexpression (lower panel) toward heavier complexes. (D) To assess the requirement of the c-Fos leucine zipper in NQO1 interaction, HEK293 cells were transiently transfected with pEFIREs-NQO1 with either pCDNA-Flag-c-Fos, the pCDNA-Flag-c-Fos VAV leucine zipper mutant, or pEFIREs-Flag-p73β (TOTAL). We performed immunoprecipitation of Flag-tagged proteins with anti-Flag beads (IP: Flag). The conclusion is that an intact leucine zipper is important for c-Fos–NQO1 complex formation. (E) To test the prediction that NQO1 will not support the c-Fos leucine zipper mutant, which does not associate with NQO1, MCF-7 cells were transfected with either pCDNA-Flag-c-Fos or the pCDNA-Flag-c-Fos VAV leucine zipper mutant alone or were cotransfected with pSUPER-NQO1shRNA or pSUPER vector. After 48 h, cell extracts were analyzed by Western blot analysis. Data show that the capacity of NQO1 to give rise to c-Fos accumulation is compromised once the leucine zipper is defective. The fold decrease in Fos protein levels caused by NQO1 knockdown was quantified by the LAS-4000 software and is shown relative to the corresponding control sample.

dramatically increased the c-Fos level. The accumulated c-Fos had an extended half-life, in particular the phosphorylated upshifted form, whose level even increased with time (Fig. 3D).

Two distinct mechanisms protect c-Fos from UID. It is possible that these mechanisms do not overlap but are sequentially functional; namely, NQO1 protects newly synthesized c-Fos until it interacts with the second stabilizer, c-Jun. This model was tested by knocking down the endogenous NQO1. Under this condition, not only the amounts of native and phosphorylated c-Fos were reduced, but also the ability of c-Jun to stabilize c-Fos was compromised (Fig. 3E). These data suggest that both NQO1 and c-Jun protect c-Fos degradation by default (UID) and that NQO1 functions upstream of c-Jun in supporting c-Fos accumulation.

The role of the c-Fos leucine zipper in degradation by default. The protective effect of NQO1 on p53, p73α, and ODC depends on their physical association (2, 4, 8). To examine whether NQO1 and c-Fos physically interact, we performed coimmunoprecipitation experiments. p73α, which interacts with and is protected by NQO1, and p73β, which neither binds to nor is protected by NQO1 (8), were used as references for specificity. Western blot analysis demonstrated that NQO1 coimmunoprecipitated with Flag–c-Fos and with Flag-p73α but not with Flag-p73β (Fig. 4A), suggesting that c-Fos specifically binds NQO1. Interestingly, the binding of NQO1 to c-Fos was severely reduced in the presence of c-Jun (Fig. 4B). Next, we separated the complexes on a glycerol gradient and revealed that under NQO1 overexpression, mostly c-Fos was

coshifted with NQO1 to the heavier fractions (Fig. 4C). This result does not support the formation of the NQO1–c-Fos–c-Jun triple complex.

The negative effect of c-Jun on the c-Fos–NQO1 interaction led us to hypothesize that NQO1 could bind to c-Fos via its leucine zipper. Indeed, the c-Fos leucine zipper VAV mutant poorly bound NQO1 (Fig. 4D). In addition, we found that c-Fos that is truncated just before the leucine zipper domain [Fos(1–164)] is also defective in NQO1 binding (not shown). Given the fact that binding to NQO1 is required for escaping degradation (8), the level of such a c-Fos mutant should not be much affected by NQO1. To this end, we knocked down NQO1 and compared the levels of WT c-Fos and the VAV c-Fos mutant. The steady-state level of the VAV c-Fos mutant was substantially lower than that of WT c-Fos (Fig. 4E), in agreement with the inability of the VAV c-Fos mutant to bind NQO1. Consistently, knocking down the endogenous NQO1 was more effective in destabilizing WT c-Fos than in destabilizing the mutant. These data suggest that the leucine zipper domain of c-Fos plays a role in protecting c-Fos prior to its engagement with c-Jun.

Subcellular localization of c-Fos is determined by the nature of the interacting partner. Our data are consistent with the possibility that NQO1 protects the newly synthesized c-Fos up to its interaction with functional partners such as c-Jun to form AP-1. We further tested this hypothesis by examination of the intracellular localization of c-Fos protein. c-Fos transfected alone was detectable mostly in the cell nuclei (Fig. 5A, lanes 1 and 2). Overexpression of c-Jun resulted in a massive accumulation of c-Fos in the nuclear fraction (lanes 5 and 6). These observations reflect the reported importance of c-Jun in the nuclear translocation and retention of c-Fos (16, 34). However, under NQO1 overexpression, which also led to a pronounced accumulation of c-Fos, a significant fraction was always detected in the cytoplasm (lanes 3 and 4), where the majority of NQO1 is found (56). Remarkably, the combined effect of NQO1 and c-Jun led to the localization of all the accumulated c-Fos exclusively in the nucleus (lanes 7 and 8). These observations correlated well with the results from the immunofluorescence studies of cells by confocal microscopy (Fig. 5B). These findings support the model that NQO1 physically interacts with the newly synthesized c-Fos in the cytoplasm and keeps it protected from degradation by default until c-Fos is engaged in the formation of the AP-1 complex and localizes to the nucleus.

NQO1 regulates c-Fos protein level in response to serum induction. c-Fos is rapidly and transiently induced by a variety of factors, including serum (26). We examined the effect of NQO1 on the serum induction of endogenous c-Fos. NQO1 overexpression increased the level of endogenous c-Fos protein induced in response to serum and resulted in its earlier appearance in HCT116 cells (Fig. 6A). Control RT-PCR experiments demonstrated that the effect of NQO1 on c-Fos was not exerted at the transcriptional level (data not shown). Next, we established an MCF-7 cell line that stably expresses NQO1-specific shRNAmir. c-Fos induction by serum was considerably compromised in these cells compared to that in the control cell line (Fig. 6B). Interestingly, under this condition, the peak level of c-Fos was delayed from 1 h to 2 h, suggesting that not only the level of c-Fos but also the rate of c-Fos accumulation

is lower. These data, therefore, suggest that NQO1 plays an important role in the physiological induction of endogenous c-Fos.

Next, we asked whether the serum-stimulated c-Fos undergoes degradation under conditions whereby the process of polyubiquitination is inactive. ts20 cells under the restrictive temperature were starved and serum stimulated. Under this condition, the endogenous c-Fos was expressed to reach a peak at 1 h poststimulation (Fig. 6C). Beyond this peak, a clear reduction in the level of c-Fos was observed, suggesting that c-Fos can undergo ubiquitin-independent degradation. We next knocked down NQO1 and found that the level of c-Fos in response to serum stimulation was compromised, reaching a peak only after 2 h. The level of c-Fos mRNA was monitored by RT-PCR and found to be refractory to the level of NQO1 (Fig. 6D). These data suggest that NQO1 inhibits ubiquitin-independent c-Fos degradation during serum stimulation, and therefore, NQO1 is important for rapid c-Fos induction and in reaching a maximal level.

NQO1 and c-Fos are interconnected by a feedback regulatory loop. We next asked whether the NQO1-mediated accumulated c-Fos is functional in supporting transcription of its target genes. To address this question, we measured the transactivation ability of c-Fos as a function of NQO1 levels by using the luciferase reporter gene under the control of an AP-1-responsive (TRE) collagenase promoter. NQO1 overexpression induced the TRE-collagenase reporter in a dose-dependent manner (Fig. 7A). In contrast, knocking down NQO1 led to a decrease in collagenase promoter activity in MCF-7 cells (Fig. 7B), suggesting that the NQO1-mediated c-Fos accumulation is functional in supporting c-Fos target gene transcription.

Several lines of evidence strongly indicate that c-Fos plays a repressive role in the regulation of *nqo1* gene expression (55) via the antioxidant response element (ARE), which contains AP-1 and AP-1-like recognition sequences (29). Thus, in light of our findings, it is possible that NQO1 and c-Fos are regulated by a negative feedback loop. c-Fos attenuated the activity of the luciferase reporter gene under the control of the native *nqo1* promoter (Fig. 7C). Coexpression of c-Fos together with NQO1 resulted in a further repression of *nqo1* promoter activity. In regulating transcription, c-Fos acts as an obligatory heterodimer, and the small Maf family members have been proposed to form the repressor complexes with c-Fos (39). Therefore, we examined the operation of the loop between NQO1 and c-Fos in the presence of MafF. Indeed, we found that the inhibitory effect on *nqo1* transcription imposed by c-Fos, protected from degradation by NQO1, was significantly augmented in the presence of MafF (Fig. 7C). These data demonstrate that stabilization of c-Fos by NQO1 is under negative feedback regulation.

DISCUSSION

The unstable nature of the c-Fos protein is crucial for quick-turnoff AP-1 activity and endows this transcription factor with a tight control mechanism protecting the cell from transformation and tumorigenesis. c-Fos degradation is regulated by both UDD and UID mechanisms (30). The present study demonstrates that c-Fos is a potential substrate of the 20S proteasome

FIG. 5. NQO1 and subcellular localization of c-Fos. (A) To determine protein subcellular localization, we first performed extract fractionation experiments. HEK293 cells were transfected with pCDNA-c-Fos alone or together with pEFIREs-NQO1 and pCDNA-c-Jun. Twenty-four hours after transfection, the cells were fractionated as described in Materials and Methods, and the equivalent amounts of the cytosolic (C) and soluble nuclear (N) fractions were submitted to immunoblot analysis. The localization of actin and β -lamin was analyzed to validate the fractionation procedure. The data show that nuclear accumulation of c-Fos is positively regulated by NQO1 and c-Jun. (B) Subcellular localization was visualized by confocal microscopy. HEK293 cells were transfected with pCDNA-Flag-c-Fos alone or together with pEFIREs-NQO1 and pCDNA-c-Jun. After 24 h, cells were fixed for immunofluorescence analysis with the indicated antibodies. Nuclear staining is indicated by using Hoechst 33342 stain.

in an NQO1- but not REG γ -dependent manner. An increasing number of proteins were reported to undergo efficient degradation by the core 20S proteasome, without requiring polyubiquitination (51). A common feature of these 20S proteasome-susceptible substrates appears to be the presence of IUP regions. In c-Fos, the large C-terminal transcriptional activation domain of the protein is unstructured and highly mobile, which could contribute to functional diversity by permitting formation of a variety of protein complexes (14). This could

explain c-Fos recognition by the 20S proteasome and its degradation by the “default” mechanism. We provide evidence that *in vivo* c-Fos undergoes degradation by default, i.e., UID, and that this process is blocked by NQO1, a gatekeeper of the 20S proteasome (7). First, in agreement with previous reports (11, 12), we found that c-Fos undergoes degradation under conditions whereby the process of polyubiquitylation is not functional. Second, we showed that NQO1 overexpression gives rise to significant accumulation of

FIG. 6. NQO1 is required for efficient c-Fos serum induction. (A) To examine whether NQO1 overexpression affects the kinetics of endogenous c-Fos induction in response to serum induction, HCT116 p53^{-/-} cells stably transfected with pEFIRE5-NQO1 or with the empty vector were starved for serum for 24 h. The cells were then induced with serum, and the cell extracts were analyzed by Western blotting. c-Fos accumulation was more profound in HCT116 NQO1-overexpressing cells than in NQO1-deficient cells. (B) To estimate the kinetics of c-Fos serum induction under NQO1-deficient conditions, MCF-7 cells stably expressing NQO1 shRNA or control shRNA were starved for serum for 24 h. The cells were then induced with serum and analyzed as described for panel A. Under a low NQO1 level, c-Fos induction by serum was compromised, with a much lower rate of reaching a peak. (C) Serum induction of c-Fos was monitored under conditions devoid of ubiquitination and NQO1 knockdown. A31N-ts20 cells stably expressing NQO1 shRNA or the control shRNA were starved for serum for 24 h at 39°C. The cells were then incubated with serum for the indicated time points, lysed, and analyzed by Western blotting. Note the delay in appearance of the c-Fos peak from 1 to 2 h. (D) RT-PCR analysis on total RNA extracted from cells was done as in the experiment described for panel C. The level of c-Fos mRNA was found to be refractory to the level of NQO1.

both ectopically and endogenously expressed c-Fos. Experiments were conducted to demonstrate that the obtained c-Fos accumulation is largely the result of protein stability. Third, in reciprocal experiments, NQO1 knockdown resulted in poor c-Fos accumulation. Finally, we demonstrated that stabilization of c-Fos by NQO1 requires physical interaction between the proteins. Consequently, the steady-state level of the c-Fos leucine zipper mutant, which only poorly binds NQO1, is NQO1 refractory. In this regard, the c-Fos degradation process is similar to other reported cases of regulation of protein stability by NQO1 (reviewed in reference 6). Unlike p73 α , p73 β does not bind NQO1 and its degradation is not regulated by NQO1, thereby showing a correlation between physical interaction and escape from degradation by default (8). The behavior of c-Fos lends further support to this model.

Interestingly, a mechanism similar to that of c-Fos degradation was reported for yeast. *Saccharomyces cerevisiae* Lot6p is the ortholog of mammalian NQO1 with flavin cofactor (46). Lot6 binds to the 20S proteasome and forms a ternary complex with Yap4p, a member of the yeast activator protein family of transcription factors (47). The AP-1-like bZip transcription factor Yap4p plays a role in response to hyperosmotic stress. Interestingly, under this condition, degradation of Yap4p by the 20S proteasome is prevented. Formation of this ternary protein complex also influences the localization of Yap4p. In unstressed yeast cells, Yap4p is present in the cytosol, whereas under oxidative stress, it relocates to the nucleus. Apparently, oxidation of the flavin cofactor of Lot6p results in the dissoci-

ation and concomitant relocation of the released transcription factor to the nucleus, where expression of stress-related genes then occurs.

The studied substrates of the degradation-by-default pathway all appear to be at least partially unstructured, a feature that may explain the proposed susceptibility to the 20S proteasome both *in vitro* and *in vivo*. However, many unstructured proteins undergo folding transitions upon binding to their biological targets (21). Under this condition, they resist 20S proteasomal degradation (51). Here, we consistently show that c-Jun, one of the major interaction partners of c-Fos within the AP-1 complex, protects c-Fos from degradation by default, despite the fact that under this condition no c-Fos interaction with NQO1 was observed. The involvement of c-Jun in the degradation of c-Fos has been previously examined. It has been shown that c-Fos degradation *in vitro* is targeted by a phosphorylation-dependent signal on c-Jun (40) and that the addition of c-Jun increases ubiquitination and subsequent degradation of c-Fos *in vitro* (48). Another study demonstrated that degradation of c-Fos by the 26S proteasome *in vitro* is accelerated by c-Jun upon addition of three protein kinases (50). The apparent discrepancy between these observations and our findings indicates that the mechanisms whereby c-Fos is degraded by the 20S and 26S proteasomes appear to be, in principle, distinct (12, 44).

c-Fos is phosphorylated by multiple kinases. These modifications often lead to c-Fos stabilization and to an increase in transcription activation (11, 37, 38, 44). We observed that over-

FIG. 7. NQO1-mediated c-Fos accumulation participates in a negative feedback loop. (A) Effect of NQO1 on the AP-1-responsive gene transcription. HEK293 cells were transfected with a luciferase reporter plasmid under the control of the collagenase promoter, together with pCDNA-c-Fos, pEFIREs-NQO1, or pEFIREs empty vector, as indicated. (B) MCF-7 cells were transfected with a luciferase reporter plasmid under the control of the collagenase promoter, together with pCDNA-c-Fos, pSUPER-NQO1shRNA, or empty pSUPER vector, as indicated. (C) NQO1 and c-Fos are interconnected by a feedback regulatory loop. H1299 cells were transfected with a luciferase reporter plasmid under the control of the *nqo1* promoter, together with pCDNA-c-Fos, pEFIREs-NQO1, or pEGFP-MafF, as indicated. Luciferase activities were normalized by the transfection efficiency (shown as the means $\pm$ standard deviations), and the statistical significance (indicated by *P* values) was tested by the *t* test.

expression of either NQO1 or c-Jun resulted in a massive accumulation in the level of phosphorylated c-Fos. This event is physiological, since knocking down the endogenous NQO1 leads to a marked reduction in the amount of the phosphorylated c-Fos. This effect of NQO1 and c-Jun might be an outcome of increasing the c-Fos intracellular pool. The fact that both wild-type c-Fos and the mutant, which is unable to undergo the major known phosphorylation events, are similarly sensitive to NQO1 levels favors this model. In addition, NQO1 may also be involved in the activation of other protein kinases that phosphorylate c-Fos, as recently demonstrated using NQO1-null cells stimulated by TNF (1).

Serum supplementation is a well-known physiological inducer of c-Fos transcription, modification, and transient stabilization. Ectopically expressed c-Fos accumulates in response to serum treatment, uncoupling transcription induction from stabilization. Interestingly, we found that the accumulation of both endogenous and overexpressed c-Fos is NQO1 dependent. NQO1 overexpression supports serum-induced c-Fos accumulation, and conversely, NQO1 knockdown leads to a marked reduction in the c-Fos level and substantially compromises the induction of the endogenous c-Fos protein in serum-challenged cells. Remarkably, these experiments revealed that c-Fos degradation by default and its inhibition by NQO1 de-

termine the temporal rate of c-Fos accumulation as well. Under low NQO1 levels, c-Fos reached the peak an hour slower than it did under higher NQO1 levels. Thus, NQO1 has an important role in controlling c-Fos physiological induction. The fact that NQO1 increases the phosphorylated c-Fos fraction in the cells lends further support to this assumption. Furthermore, we found that stabilization of c-Fos by NQO1 is important in the elevation of biological activity of c-Fos as a transcription factor. Moreover, given the negative effect of c-Fos on the transcription of the *nqo1* gene, our data describe a new feedback regulatory loop.

The subcellular localization of c-Fos is controlled by its import from the cytoplasm to the nucleus and its retention in the nuclear compartment. Nuclear localization and retention are dependent on protein domains within c-Fos (15), interaction with Jun family members (34), and the appropriate signaling (42, 44). Here, we show that NQO1 increases c-Fos levels in both cytosolic and nuclear compartments. NQO1 is found mainly in the cytoplasm (22), with a small amount in the nucleus (56). We show here that in the cytoplasm, NQO1 interacts with the "free," possibly newly synthesized c-Fos, a process that protects it from degradation by default. Nuclear entry and retention of c-Fos are greatly stimulated by the Jun family members (34). As we have shown, the interaction of

FIG. 8. Steps in c-Fos expression; a simplified model to highlight our findings. According to our model, the newly synthesized c-Fos (arrow 1) is subjected to degradation by the 20S proteasome (arrow 2). NQO1 physically interacts with c-Fos to inhibit its degradation by default (arrow 3). Next, the AP-1 complex is formed (arrow 4), translocates to the nucleus, and is functional in DNA binding (arrow 5). At this stage, c-Fos degradation is regulated by ubiquitination and 26S-dependent proteasomal degradation (arrow 6).

c-Fos with c-Jun and NQO1 is mutually exclusive. Moreover, binding of c-Jun to c-Fos has a dominant character, leading to complete localization of the protected c-Fos to the nucleus. We propose that the above-described characteristics of interactions between c-Fos, NQO1, and Jun are responsible for the following logical chain of events during the c-Fos life cycle (Fig. 8). The free, newly synthesized c-Fos monomers bearing accessible unstructured regions (14) are susceptible to degradation by default via a UID mechanism by the 20S proteasome. Transient binding to NQO1, the 20S gatekeeper, is needed to protect c-Fos from degradation by default. The very high affinity of binding of c-Jun to c-Fos through their leucine zipper regions displaces NQO1 from c-Fos when it becomes part of a functional AP-1 complex. c-Fos, upon interaction with its transcriptional partners, is expected to adopt much lower conformational freedom, and the unstructured domain(s) may be masked. This explains c-Fos protection from degradation via the UID mechanism, as well as its “safe” nuclear translocation. When the c-Fos-mediated transcription event must be shut down, a different degradation system seems to operate in the cell. Under this condition, the mechanism of ubiquitination, which may be targeted by the associated c-Jun (40), is needed to downregulate the c-Fos level.

Many processes regulate protein homeostasis, or proteostasis (9). Temporal expression of c-Fos was thoroughly investigated with the assumption that the rate of accumulation is defined by the balance between synthesis (transcription and translation) and proteasomal degradation. Here, we provide evidence for the complexity of the degradation process that is designed not only to determine the c-Fos rate of elimination but, unexpectedly, the rate of accumulation as well. This is possible because of the susceptibility of c-Fos to proteasomal degradation by default.

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