c-Jun NH₂-terminal Kinases Target the Ubiquitination of Their Associated Transcription Factors

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Regulatory proteins are often ubiquitinated, depending on their phosphorylation status as well as on their association with ancillary proteins that serve as adapters of the ubiquitination machinery. We previously demonstrated that c-Jun is targeted for ubiquitination by its association with inactive c-Jun NH₂-terminal kinase (JNK). Phosphorylation by activated JNK protects c-Jun from ubiquitination, thus by prolonging its half-life. In the study reported here, we determined the ability of JNK to target ubiquitination of its other substrates (Elk1 and activating transcription factor 2 (ATF2)) and associated proteins (ATF2 and JunB). We demonstrate that phosphorylation by JNK protects ATF2, but not Elk1, from JNK-targeted ubiquitination. We also show that association of inactive JNK with JunB or ATF2 is necessary to target them for ubiquitination. Unlike its targeting of c-Jun, JNK requires additional cellular components, yet to be identified, to target the ubiquitination of ATF2. Elk1 is phosphorylated by JNK, but JNK neither associates with nor targets Elk1 for ubiquitination. The implications for the dual role of JNK in the regulation of ubiquitination and stability of c-Jun, ATF2, and JunB in normally growing versus stressed cells are discussed.

The cellular response to stress activates early response proteins by both transcription and post-translational modifications which dictate the cell’s ability to undergo cell cycle arrest for DNA damage repair or to initiate programmed cell death. Among the stress-modulated factors that contribute to the cell’s ability to cope with stress are c-Jun and ATF2, both of which are activated by their NH₂-terminal phosphorylation via stress-activated protein kinases (JNK) (1–3). JNKs are proline-directed serine/threonine kinases, which are activated by a wide variety of stimuli, including physical and chemical DNA-damaging agents and inhibitors of protein synthesis as well as heat and osmotic shock (reviewed in Kyriakis and Avruch (4)). Different forms of stress utilize alternate cellular pathways for JNK activation (5). For example, JNK activation by UV irradiation requires their phosphorylation by the upstream kinase, mitogen-activated protein-kinase kinase 4 (6), the association of JNK with p21(127) (7), the presence of nuclear DNA lesions (8, 9), and inactivation of a redox-sensitive inhibitor.²

JNK were first identified and named as a Jun-associated kinases (11), reflecting their strong hydrophobic interaction with c-Jun. JNK-c-Jun association is ATP-independent and is required for efficient ATP-dependent phosphorylation of c-Jun at flanking phosphoacceptor sites (Ser⁶³ and Ser⁷³). The mechanism by which JNK phosphorylation confers transcriptional activities of c-Jun remains largely unknown.

One of the key mechanisms for regulating protein’s activity is tight control of its stability. Many regulatory proteins are selectively degraded by the proteasome pathway at specific phases of cell growth. Polyubiquitination, i.e. covalent attachment of multiple ubiquitin residues to ε-lysyl amino groups of lysine, serves as a marker for proteasome recognition (reviewed in Hochstrasser (12)). The ubiquitination process is regulated by several mechanisms, including degradation of inhibitors, processing of inactive precursors, and stabilization of activated proteins. For example, activation of NFkB requires the ubiquitination and degradation of its inhibitor IκB as well as the processing of its precursor p105 (13). Conversely, it is the DNA damaged-induced stabilization of the tumor suppressor protein p53 that acquires its activities (14).

Central to JNK’s association with c-Jun is the δ domain of c-Jun (amino acids 30–57), which is deleted in its oncogenic counterpart v-Jun. The δ domain is also essential for c-Jun ubiquitination, which explains the mechanism underlying the greater stability of v-Jun as compared with its cellular homologue (15). Using an in vitro model system, we previously demonstrated that c-Jun is targeted for ubiquitination by association with JNK. However, phosphorylation of c-Jun on Ser⁷³ by JNK is sufficient to protect c-Jun from ubiquitination, resulting in a prolonged half-life (16). The dual activity of JNK in targeting c-Jun ubiquitination via physical association and in protecting it from entering this pathway via phosphorylation points to the role of JNK in controlling c-Jun’s stability in cells exposed to environmental stress or inflammatory cytokines. In light of finding phosphorylation-dependent targeting of c-Jun ubiquitination, in the present study, we have compared JNK target ubiquitination of its substrates and associated protein (ATF2, c-Jun) with nonassociated substrate (Elk1) and associated non-substrate (JunB). Our results provide the foundation for the model in which (i) JNK-targeted ubiquitination requires tight association and (ii) the degree of targeting is affected by the extent of phosphorylation on JNK-associated protein.

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JNK Targets Ubiquitination of Its Associated Proteins

Materials and Methods

Plasmin and Recombinant Proteins—Constructions encoding c-Jun(15), c-JunΔN(15), E1k1α(17) were previously described. JunB open reading frame was amplified by PCR using the wild type JunB mammalian expression vector (18) as a template and cloned into the pET15b vector (Novagen) at the NdeI site. A BamHI digest of the same amplification product has been cloned into pET15b at the BamHI site, providing the JunBα construct, which lacks the first 44 amino acids (JunB1–44). Full-length ATF2 as well as ATF2 with mutated JNK phosphoacceptor sites Thr—Ala(49) and Ala(45) open reading frames were amplified by PCR using pECE-ATF2 plasmids (4, 19) as templates followed by unidirectionally cloning them into pET15b at NdeI/BamHI sites, resulting in ATF2α(15) and ATF2β(15) constructs, respectively. The ATF2 mutant lacking JNK binding site (ATF2α(15)D) has been created using a QuickChange site-directed mutagenesis kit (Stratagene). An HA-tagged ubiquitin encoding construct was generated by PCR-mediated cloning. The sequence encoding ASYPYDVDPYASLSR followed by phosphoacceptor sites Thr—Ala were expressed in the BL21 (DE3)pLysS strain and purified by affinity chromatography using nickel resins under denaturing conditions, as recommended by the manufacturer (Qiagen). Proteins attached to the beads were refolded by excessive (20 volumes) column washings with mixtures of 8 M urea in sodium phosphate buffer (pH 8.0) and renaturation buffer (Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1 M phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40) to block the remaining nonoccupied nickel binding sites, which are not protected by bound proteins. Bead-bound proteins were then resuspended in 50 mM glycine and stored at 20 °C.

HA-tagged ubiquitin (Ub-HA)-bound beads were washed three more times (rather than resuspended in glycerol) with thrombin cleavage buffer (40 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2.5 mM CaCl2) and incubated in 10 volumes of the same buffer with thrombin (Sigma; 2 units/mg of recombinant protein) for 16 h at 20 °C. Resins were pelleted, and the supernatant containing Ub-HA was incubated at 90 °C for 15 min, chilled on ice, and cleared by centrifugation at 15,000 × g for 20 min at 4 °C. The resulting supernatant was concentrated and washed in double distilled water using Ultra-free-15 centrifugation units (Sigma) with 5000 cutoff membranes to obtain pure Ub-HA (verified by silver staining of SDS-PAGE gel and immunoblotting with antibodies against ubiquitin and HA).

Cells, Lysates, and JNK Purification—4A-3T3 and NIH-3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Life Technologies, Inc.) and antibiotics at 37 °C and 5% CO2. Calpain inhibitor (LLM, Sigma) and proteinase inhibitor MG132 (Pep tide International Co.) were added to the cells at 50 and 10 μM (respectively) in 1× serumless (less than 0.1% of medium) volume 16 h before harvesting. UV exposure (50 J/m2) was performed on ice with bacterially expressed substrates (1–5 μg) bound to nickel beads for 45 min. After extensive washes (four times with 1 ml of kinase buffer) (16), the substrate-bound beads were equilibrated with 1× ubiquitination buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.5 mM dithiothreitol, and 33% RL (v/v) in a total volume of 30 ml at 30 °C for 5 min. Ubiquitin-aldehyde, synthesized as described previously (21), was added at 1 μM final concentration as indicated under “Results.” The reaction was stopped by adding 0.5 ml of 8% urea in sodium phosphate buffer (pH 6.3) with 0.1% of Nonidet P-40. The beads were washed three times with the stop-buffer and once with phosphate-buffered saline supplemented with 0.5% of Triton X-100, and the protein moiety was eluted at 100°C in a sample buffer containing 8% SDS-PAGE and electrophoresed onto a nitrocellulose filter. When anti-ubiquitin antibody was used, a polyvinylidene difluoride membrane served as a filter. Nitrocellulose filters were boiled in double distilled water for 10 min, blocked with 5% nonfat milk, and probed with HA11 antibody. After their detection with chemiluminescence (ECL, Amersham Corp.) the blots were stripped and reprobed with antibody against the specific substrate, followed by alkaline phosphatase detection to ensure equal loading of the substrate.

In vitro Binding Assay—To analyze the association of JNK with JunB and JunBΔN(15). 250 μg of lysates obtained from UV-irradiated 4A-3T3 cells were incubated with NTG bead-bound JunB protein for 45 min on ice. After four washes with kinase buffer, proteins were eluted by boiling in Laemmli sample buffer, separated on 10% SDS-PAGE, and transferred onto a nitrocellulose filter. The filter was probed with antibodies to JNK (clone 666, PharmMingen) and reprobed with polyclonal anti-JunB (Santa Cruz) antibody.

In vitro Ubiquitination Assay—Previously we established an in vitro system for studying the ubiquitination of c-Jun (16). In this system, after preincubation of recombinant c-Jun with whole cell extract, the bound proteins are washed off and the targeting effects of c-Jun-bound proteins on its ubiquitination by rabbit RL are monitored. The extent of ubiquitination is reflected by the intensity of the multi-ubiquitin chain that appears as a smear of ubiquitin immunoreactive material produced from the position of the substrate to the top of the gel. Using this system, we demonstrated that binding of cellular proteins from rat fibroblasts to recombinant c-Jun increases its ubiquitination.

Immunodepletion of JNK reduced the targeting activity of protein extracts, which could be restored by adding immunopurified JNK (16). However, in the absence of any fibroblast proteins, the purified form of JNK was capable of mediating only a marginal increase in c-Jun ubiquitination. The latter has been attributed to low sensitivity of our detection system which relied on antibodies to ubiquitination.

To improve the sensitivity of the ubiquitination assay, we generated a construct that allows expression and purification of Ub-HA. The NH2-terminal fusion of HA peptide was shown not to interfere with formation of polyubiquitin chains in vivo (15), and yet it enabled tracking ubiquitination of different substrates with highly specific and sensitive anti-HA antibody.

Fifty micrograms of whole cell lysates from NIH-3T3 cells (immuno depleted with normal rabbit serum or antibody against JNK as described previously (16)) were incubated with purified Ub-HA bound to nickel beads (Sigma) with 5000 cutoff membranes to obtain pure Ub-HA (verified by silver staining of SDS-PAGE gel and immunoblotting with antibodies against ubiquitin and HA).

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RESULTS

JNK Targeting of c-Jun Ubiquitination and Stability—Pre-incubation of c-Junhis with JNK purified from 4A-3T3 mouse fibroblasts led to substantial increase in c-Junhis ubiquitination by RL immunodepleted of JNK (Fig. 1; compare lanes 1 and 2). A further increase was noted when the whole cellular extract, immunodepleted with normal rabbit serum, was added as a source of targeting proteins (Fig. 1, lane 3). Immunodepletion of whole cellular extract with antibody to JNK substantially decreased its ability to target c-Junhis ubiquitination; adding purified JNK to the JNK-depleted protein extract restored the original level of c-Jun ubiquitination (Fig. 1). The addition of ubiquitin aldehyde to the ubiquitination reaction did not change the pattern of results (data not shown), suggesting that addition and removal of JNK affected the conjugation of ubiquitin rather than isopeptidase activity. As negative control nickel resin beads were incubated with protein lysates of uninduced bacterial strain BL21(D30E)pLysS and purified, as c-Jun, rather than ubiquitin, was the substrate for protein conjugation. The position of the ubiquitin conjugates is marked on right lane. Lower panel, the blot was reprobed with polyclonal antibody against c-Jun followed by alkaline phosphatase detection. The position of c-Jun is indicated.

Since the physical association between JNK and c-Jun targets the latter for ubiquitination, their in vitro interaction is expected to be unstable because of c-Jun degradation. To modulate the steady state level of the c-Jun-JNK complex, mouse fibroblasts were pretreated with potent proteasome inhibitor MG132 (20). Fig. 2A demonstrates that pretreatment with MG132 substantially increased the amount of c-Jun, which could be co-immunoprecipitated with antibody against JNK. MG132 treatment did not affect the JNK level (Fig. 2B) but increased the amount of c-Jun measured in the whole cell extracts (Fig. 2A). These findings suggest that the c-Jun-JNK complex in vitro is a target for proteasome activity. UV irradiation of mouse fibroblast cells neither affected the JNK-c-Jun association nor led to retardation of the electrophoretic mobility of c-Jun bound to JNK (Fig. 2A). These data provide further support for the role of JNK as a targeting molecule in c-Jun ubiquitination and, therefore, in determining its stability.

JNK Does Not Target ELK-1 Ubiquitination—In addition to its ability to bind and phosphorylate c-Jun, JNK is known to associate with JunB and ATF2 and to phosphorylate Elk1 and ATF2. We therefore examined the possible involvement of JNK in the regulation of ATF2, Elk1, and JunB ubiquitination. In all cases, the His-tagged substrates were used in our in vitro ubiquitination assay. To study Elk-1 ubiquitination we used a bacterially expressed histidine-tagged Elk-1 protein, which was previously shown to be a functional sequence specific DNA binding protein (17). As is evident from the data presented in Fig. 3, Elk1 is efficiently ubiquitinated by RL, even in the absence of targeting molecules. Preincubation with either purified JNK or with whole cell lysate did not alter the extent of ubiquitination (Fig. 3). These findings suggest that Elk1, which is not capable of association with JNK (17), cannot be targeted for ubiquitination by JNK (purified or in the content of the cell lysate). The ability of RL to mediate a high degree of Elk1 ubiquitination suggests that RL provides all necessary components for Elk1 ubiquitination, including enzymes of the ubiquitination machinery and targeting molecule(s), which were not depleted by the antibodies to JNK (16). As JNK phosphorylation of c-Jun protects it from subsequent targeting for ubiquitination (16, 22), we tested whether Elk1 phosphorylation affects its degree of ubiquitination. Unlike c-Jun, extensive phosphorylation of Elk1 by JNK (data not shown) did not alter its ubiquitination (Fig. 3).

JNK Targets JunB Ubiquitination—JunB preserves a δ domain-like sequence within its NH2 terminus, thus enabling JNK binding (17, 23). Basal levels of JunBhis ubiquitination by RL were higher than those found with c-Jun (data not shown). As in the instance of c-Jun, preincubation with JNK or with whole cellular extract increased the extent of JunBhis ubiquitination (Fig. 4A, compare lanes 1–3). Whole cell extract immunodepleted of JNK mediated the decreased extent of JunBhis ubiquitination, whereas addition of a purified form of JNK to these extracts restored the original degree of ubiquitination (Fig. 4A, lanes 4 and 5).

To further confirm JNK’s role in targeting JunB ubiquitination, we performed experiments using JunBhis31–44, which lacks the first 44 amino acids. This mutant lacks the ability to associate with JNK as suggested by available data (23) and evidenced by an in vitro binding assay (Fig. 4B). Ubiquitination of JunBhis31–44 was severely impaired. Neither JNK nor cell extract could increase JunBhis31–44 ubiquitination (Fig. 4C). Although JNK binds JunB, it cannot phosphorylate this transcription factor because of the absence of phosphoacceptor sites
Indeed, preincubation of JunB with JNK and ATP in a solid phase kinase reaction followed by JNK removal (with 3% n-octyl β-D-glucopyranoside as described previously (16)) did not result in incorporation of [32P]phosphate into JunB nor did it yield any protection from subsequent ubiquitination (data not shown).

JNK Targeting of ATF2 Ubiquitination Is Phosphorylation-dependent—Transcription factor ATF2 can associate with JNK and is a substrate for JNK-mediated phosphorylation. Surprisingly, preincubation of ATF2his with JNK2 purified from 4A-3T3 cells did not target ubiquitination of this recombinant protein (Fig. 5A, compare lanes 2 and 3). Addition of cell extract as a source of targeting molecules led to a clear increase in the extent of ATF2 ubiquitination (Fig. 5, lane 4). Immunodepletion of this protein extract with antibody to JNK substantially decreased ATF2his ubiquitination. Conversely, reconstituting this protein extract with purified JNK restored the extent of ATF2 ubiquitination (Fig. 5, lanes 5 and 6).

To further support the role of JNK in ATF2 ubiquitination, we performed experiments using ATF2440–60, which cannot bind JNK (19, 24). Although ATF2440–60 exhibits some basal ubiquitination by RL, it cannot be increased by whole cell extract (Fig. A, lanes 7 and 8).

Phosphorylation of ATF2his by JNK leads to a modest yet highly reproducible increase in the extent of its basal ubiquitination (Fig. 5B, lane 1 versus 2). However, such phosphorylation prevents an increase in the degree of ATF2his ubiquitination by mouse fibroblast protein extracts (compare lanes 3 and 4). Additional studies to confirm the protective effect of ATF2 phosphorylation by JNK in ATF2 ubiquitination utilized an ATF2 mutant in which JNK phosphorylation sites (Thr69 and Thr71) were replaced with Ala residues. Phosphorylation of this mutant by JNK was not capable of preventing whole cell extract-targeted ubiquitination (Fig. 5B; compare lanes 3 and 4 versus 7 and 8).

ATF2 and JunB Proteins Associated with JNK Are Substrates for Degradation by Proteasomes—Treatment of mouse fibroblasts with proteasome inhibitor MG132 (but not with calpain inhibitor LLM) increased the amount of JunB co-immunoprecipitated with anti-JNK antibody (Fig. 6A). Blocking the proteasome pathway did not alter the total amount of JunB, suggesting that only a small portion of JunB is bound to JNK and susceptible for JNK-targeted ubiquitination and proteasome-mediated degradation. We cannot rule out the possibility that calcium-dependent proteases play a role in a JunB degradation in the JNK-independent manner.

Immunoblotting analysis of the same experiment with antibodies to ATF2 revealed multiple splicing variants of ATF2 and the cross-reactive ATFa (Fig. 6B) which is also known to bind JNK (25) (Fig. 6B). While the amount of full-length ATF2 (molecular mass, 68 kDa) slightly increased after MG132 treat-
ment, its association with JNK was noticed only in the immunoprecipitates from MG132-treated cells when the blot was overexposed (not shown). The 41.5-kDa splicing version of mouse ATF2, which is a constitutively active transcription factor (26), was sensitive to proteasome inhibition, especially when complexed with JNK (Fig. 6B). These data demonstrate that JNK-associated JunB and ATF2 are substrates for a proteasome pathway.

**DISCUSSION**

Post-translational modifications of preexisting transcription factors play a central role in the immediate cellular response to damage and stress. Members of the AP1 and ATF families are among the stress-activated transcription factors that we studied in the present investigation. Phosphorylation of c-Jun by JNK correlates with its transactivation (27, 28), providing the mechanistic link between JNK signaling and the induction of de novo expression of stress-responsive genes. Since the transactivating potential of v-Jun, which lacks the δ domain, seems to be independent of phosphorylation (29, 30), it may be attributed to the increased stability that is due to its lack of JNK association and targeted ubiquitination (15, 16). That association of JNK with c-Jun via the δ domain impairs this protein’s ability to undergo transactivation in nonstressed cells, which has been previously proposed and is consistent with our model (31).

The data presented here, together with our previous results, suggest that JNK actively participates in the regulation of c-Jun’s stability and, therefore, availability. In nonstressed cells, JNK functions as a targeting molecule for ubiquitination, an activity which depends not on the fact that it is a kinase, but on its ability to associate tightly with c-Jun. This association attracts the enzymes of ubiquitination machinery to c-Jun, thereby marking it for a proteasome-dependent degradation. The necessity of JNK for ubiquitination (and degradation) is directly supported by the data presented here.

JNK regulates c-Jun ubiquitination depending on (i) JNK association with c-Jun and (ii) c-Jun phosphorylation status. The fact that c-Jun is protected from ubiquitination upon being phosphorylated by JNK (16, 22) prompted us to test the role of JNK in ubiquitination of its associated substrate ATF2 (19), its nonassociated substrate Elk1 (17) and associated yet non-substrate JunB (17, 23). JNK was incapable of targeting the ubiquitination of Elk1 (Fig. 3). Moreover, phosphorylation of Elk1 by JNK did not protect this transcription factor from ubiquitination. These observations suggest that JNK requires a physical association to regulate ubiquitination.

JNK participates in the targeting of JunB for ubiquitination through its association with this protein (Fig. 4), albeit in a non-phosphorylation-dependent manner since JunB is not phosphorylated by JNK (17, 23). The JunB mutant, which lacks
the JNK binding domain (Fig. 4B) (23), is poorly ubiquitinated (Fig. 4C). Interestingly, the inability to bind JNK was also documented for another member of the Jun family, JunD (17, 23). JunD, which does not have a JNK binding domain, is poorly ubiquitinated compared with c-Jun (32). Together, these data strengthen JNK's role in ubiquitination of its associated proteins.

ATF2 is another member of the bZIP transcription factor superfamily which participates in the cellular response to stress/damage (19, 24, 33). The ATF2-c-Jun heterodimers were shown to mediate transactivation from the UV-responsive element (35) the motif which regulates various stress-responsive genes (i.e. c-Jun, ERCC3) (36). JNK-mediated phosphorylation of ATF2 at Thr\(^{69}\) and Thr\(^{71}\) has been shown to induce its transactivation potential (19, 24, 33). As for c-Jun, the flanking phosphorylation, which would result in weaker association with cellular adapters and/or ubiquitin machinery enzymes.

Change in conformation would also coincide with greater stability of these proteins, as shown for their \(\delta\) domain-deleted counterparts. The activity of various proteins is often limited by their availability, as shown for another JNK-associated protein, p53 (41). We suggest that stabilization of c-Jun and ATF2 through their protection from ubiquitination by JNK-mediated phosphorylation provides an important mechanism for their activation.

In all, the emerging model from our studies suggests that JNK targeting for ubiquitination requires tight interaction with its associated protein. Phosphorylation of the associated protein decreases JNK targeting capacity, as shown for c-Jun and ATF2, probably due to altered conformation of the associated protein, which is likely to affect this binding affinity. Ubiquitination of c-Jun, JunB, and ATF2 targeted by JNK is expected to play a central role in rendering transcription factors such as these inactive through their rapid degradation in nondamaged cells. We hypothesize that JNK targeting ubiquitination of ATF2, which regulates the expression of c-Jun, as of c-Jun itself, is a functionally important mechanism that maintains a balanced expression of key regulatory proteins in normally proliferating cells. The activation of these proteins via phosphorylation and gained stability, in response to stress, has been implicated as an early signal in apoptosis (10).

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