COP9 Signalosome-directed c-Jun Activation/Stabilization Is Independent of JNK*

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The basic region-leucine zipper transcription factor c-Jun regulates gene expression and cell function. It participates in the formation of homo- or heterodimeric complexes that specifically bind to DNA sequences called activating protein 1 (AP-1) sites. The stability and activity of c-Jun is regulated by phosphorylation within the N-terminal activation domain. Mitogen- and stress-activated c-Jun N-terminal kinases (JNKs) have been described as the only enzymes phosphorylating c-Jun on Ser-63 and Ser-73 in vivo. The JNKs are constitutively inactive. They are components of the MAP kinase signaling pathway. In response to many stimuli such as PMA, JNKs are activated by phosphorylation via MAP kinase kinases, MKK4 and MKK7, which in turn phosphorylated by numerous MAP kinase kinase kinases (for review see Ref. 5). Although there are a few reports on JNK-independent c-Jun/AP-1 activation (e.g. Ref. 6), no alternative pathway was identified. Here we show the existence of a COP9 signalosome-directed AP-1 activation pathway.

The COP9 signalosome complex, originally identified in plant cells (7), consists of 8 subunits, which are conserved between plant and human (8, 9). The significant sequence homologies between components of the COP9 signalosome and the 26 S proteasome lid (8–12) and the colocalization of the two complexes led to the speculation that COP9 signalosome and 26 S proteasome cooperate in the regulation of signaling pathways (9). Whereas the 26 S proteasome lid components are essential for the degradation of many transcriptional factors (10) (for review see Ref. 13), COP9 signalosome might stabilize those proteins. The COP9 signalosome is involved in light signaling in plants (7) and isolated human COP9 signalosome is associated with kinase activity that phosphorylates regulators of transcription (9). Recently it has been demonstrated that the purified human COP9 signalosome complex phosphorylates c-Jun at the N-terminal activation domain including Ser-63 and Ser-73. In contrast to JNK, the isolated COP9 signalosome modifies only full-length c-Jun, whereas JNK phosphorylates N-terminal c-Jun fragments such as the Δc-Jun(1–79) and Δc-Jun(1–226) (9). The COP9 signalosome kinase has not yet been identified. Because none of the COP9 signalosome subunits contain a recognizable kinase domain, we refer to it as an associated kinase activity. Data presented here demonstrate that COP9 signalosome-directed phosphorylation of c-Jun results in the stabilization of the transcription factor in vivo accompanied by an elevated AP-1 activity.

EXPERIMENTAL PROCEDURES

In Vitro Kinase Assays—COP9 signalosome was isolated from human red blood cells as described previously (9). Kinase assays were performed with His-tagged full-length c-Jun as substrate (9). His-tagged full-length c-Jun, His-tagged Δc-Jun(1–226), and His-tagged signalosome subunit 5 (Sgn5) were produced in Escherichia coli from pQE expression vectors and isolated using the Ni-nitrilotriacetic acid purification kit (Qiagen). The complete Sgn2 cDNA has been deposited in the GenBank™ data base under GenBank™ Accession number AF084260.

Transient Transfections and Reporter Assays—HeLa cells were grown in RPMI 1640 containing 4 mm glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Life Technologies, Inc.) in a humidified 5% CO₂ atmosphere. The cells were seeded in tissue culture plates for 48 h prior to infection.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF084260.

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‡ The abbreviations used are: AP-1, activating protein-1; JNK, c-Jun N-terminal kinase; PMA, phorbol 12-myristate 13-acetate; MAP, mitogen-activated protein; MKK, MAP kinase kinase; RIPA, radioimmune precipitation buffer; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; Sgn, signalosome subunit; DNMKK4, dominant inhibitory MKK4; COP9, constitutive photomorphogenesis 9.
FIG. 1. Phosphorylation of full-length c-Jun by purified COP9 signalosome is dependent on Sgn5 also known as Jun activation domain-binding protein 1 (Jab1). The autoradiographs show decreased phosphorylation of full-length c-Jun with γ32P[ATP in the presence of increasing amounts of recombinant Δc-Jun-(1–226) or recombinant His-tagged Sgn5. Equal amounts of full-length c-Jun were added as shown by the Coomassie Blue stain.

16 h before infection, the medium was replaced by fresh RPMI 1640 medium supplemented with 5% fetal calf serum. Transactivating activity of AP-1 was measured at 50–70% confluence by cotransfection of a luciferase expression plasmid containing three repeats of the AP-1 binding site and other expression constructs using cationic liposomes (DAC-30, Eurogentec, Sart Tilman, Belgium). Expression constructs Sgn5, Sgn2, and Δc-Jun-(1–226) were cloned into the pCDNA3 vector (Invitrogen) expressing a N-terminal Flag-tagged sequence. 16 h after transfection, cells were either treated with 40 nM PMA (Sigma) or left untreated. Luciferase assays were performed 3–4 h after treatment as recommended by the manufacturer’s instructions (Promega). The results were recorded on a Wallac 409 counter (Berthold-Wallac). Representative results from more than three independent experiments are shown as fold induction or percentage induction compared with the control. Activities varied <10% between transfection experiments.

Immunoprecipitation and Protein Kinase Assays—To analyze the kinase activities of JNK and p38, cells were lysed in RIPA buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS, 5% glycerol, 1 mM EGTA, 10 mM NaF, 10 mM K2HPO4, 1 mM Na3VO4, 100 μM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 4 μM aprotinin. For immunoprecipitation, RIPA buffer-lysed cells were disrupted and incubated with anti-JNK1 (sc-474, Santa Cruz Biotechnology) or anti-p38 (sc-535, Santa Cruz Biotechnology) antibodies. Immunocomplexes were recovered and washed, and immunoprecipitates were used for in vitro kinase reactions using 1 μg of GST-c-Jun (Santa Cruz Biotechnology) or GST-ATF2 (Santa Cruz Biotechnology) as substrate for JNK and 1 μg of GST-ATF2 (Santa Cruz Biotechnology) for p38. The samples were separated in SDS-PAGE and dried, and substrate phosphorylation was visualized by autoradiography. Equal amounts of each sample were used for immunoblot analysis to indicate equivalent protein amounts in all lanes as described previously (14).

Density Gradient Centrifugation—For separation in 10–30% glycerol gradients 16 h after transfection, cells were lysed with RIPA buffer as described above. Lysate from approximately 1 × 107 cells was subjected to density gradient centrifugation (9). Trichloroacetic acid-precipitated proteins from fractions 6–15 were separated by 12.5% SDS-PAGE and dried, and substrate phosphorylation was visualized by autoradiography. Equal amounts of each sample were used for immunoblot analysis to indicate equivalent protein amounts in all lanes as described previously (14).

RESULTS AND DISCUSSION

It has been shown that c-Jun interacts via amino acids 31–57 with Jun activation domain-binding protein 1 (Jab1) (15), recently identified as Sgn5 of the COP9 signalosome (9). To test whether COP9 signalosome-directed c-Jun activation is dependent on Sgn5-c-Jun interaction, we performed in vitro competition assays with Δc-Jun-(1–226). As mentioned above, Δc-Jun-(1–226) is not phosphorylated by COP9 signalosome but should bind to Sgn5. Fig. 1 shows that increasing amounts of Δc-Jun-(1–226) added to constant amounts of full-length c-Jun inhibit c-Jun phosphorylation by purified COP9 signalosome. 2.4 μg of Δc-Jun-(1–226) corre-

FIG. 2. Stimulation of AP-1 transactivation activity by Sgn2. Relative luciferase activity was measured in HeLa cells transfected with an AP-1 reporter plasmid in the presence of sgn2 or sgn5 expression vectors or PMA (40 nM), as indicated. Data are expressed as fold induction.
incorporation into the COP9 signalosome was observed (see Fig. 3). To see whether the transfections had an impact on c-Jun stabilization, the same glycerol gradient fractions were analyzed for c-Jun amounts using an anti-c-Jun antibody. Whereas endogeneous c-Jun can barely be detected in Sgn5-transfected cells and is very low in the controls, increased cellular c-Jun amounts were found in Sgn2-transfected HeLa cells (Fig. 3). This increase of c-Jun concentration is most likely because of stabilization of the protein and is responsible for the increase of AP-1 activity.

As mentioned above, c-Jun is stabilized by phosphorylation including serines 63 and 73, and JNKs have been described to be the responsible kinases (2). To study the possibility whether Sgn2 overexpression leads to JNK activation resulting in elevated AP-1 activity, JNK was immunoprecipitated from Sgn2, transfected Sgn5, or PMA-stimulated cells. The precipitate was assayed for immunocomplex kinase activity with full-length c-Jun as a substrate. As shown in Fig. 4A, PMA treatment led to JNK activation as expected. In contrast, there was no JNK-dependent c-Jun phosphorylation as a consequence of Sgn2 or Sgn5 overexpression. Similar data were obtained in experiments in which another regulator of AP-1 activity, p38 MAP kinase (5), was analyzed (data not shown). These data demonstrate that increased AP-1 activity induced by Sgn2 overexpression is independent of JNK or p38 kinase activities. Thus, AP-1 activity can be stimulated via c-Jun phosphorylation by two different pathways COP9 signalosome-dependent and JNK-dependent signaling. To further discriminate between the two signaling pathways dominant inhibitory MKK4(K116R) (DNMKK4) was transfected into HeLa cells. MKK4 is a physiological activator of JNK at the MAP kinase kinase level and also functions as an activator of p38 MAP kinase (5). As illustrated in Fig. 4B, transfection of DNMKK4 into PMA-stimulated cells led to a dose-dependent inhibition of AP-1 activity. This c-Jun activation is dependent on JNK activation by MKK4. On the other hand, the COP9 signalosome-directed c-Jun activation is not affected by DNMKK4, again demonstrating JNK-independent signaling.

Δc-Jun-(1–226) and recombinant Sgn5 inhibit the phosphorylation of full-length c-Jun by the COP9 signalosome-associated kinase in vitro (see Fig. 1). Whether similar effects could be obtained under cellular conditions was tested in HeLa cells cotransfected with reporter plasmids and with Sgn5 or Δc-Jun-(1–226) expression vectors as indicated, are shown. Data are expressed as percentage induction.
signalosome-directed phosphorylation of full-length c-Jun. Similar data were obtained with PMA-stimulated cells using Δc-Jun-(1-226) as competitor for JNK.

The presented data demonstrate that ectopically expressed Sgn2 incorporates into the cellular COP9 signalosome complex accompanied by a significant de novo complex formation. Increased amounts of COP9 signalosome lead to a stabilization of endogenous c-Jun and increased AP-1 transactivation activity. Thus, transcriptional regulation of Sgn2 might represent a mechanism for controlling COP9 signalosome amounts and cellular activity, e.g. c-Jun activation/stabilization. The resulting stimulation of AP-1 activity is independent of JNK and MKK4 activities but depends on COP9 signalosome. Therefore, stabilization of c-Jun is because of phosphorylation of the transcription factor at its N-terminal activation domain by the COP9 signalosome-associated kinase as demonstrated with the isolated complex (9). Additional evidence for the existence of a JNK-independent COP9 signalosome-directed c-Jun signaling comes from the fact that the activity of the purified complex is inhibited by curcumin (11), a known inhibitor of AP-1 activity (17). Interestingly, there seems to be a cross-talk between the COP9 signalosome-directed c-Jun activation and the JNK pathway. The G-protein suppressor 1 (Gps1), identical to signalosome subunit 1, has been shown to act as a suppressor of JNK (18). It is perhaps advantageous for the cell to block the stress-activated protein kinases, although the COP9 signalosome is active.

In addition to c-Jun stabilization, COP9 signalosome-dependent phosphorylation might also affect the transport of the transcription factor into the nucleus. It has been shown in Arabidopsis that a functional COP9 signalosome complex is essential for the nuclear accumulation of COP1, a transcriptional regulator, in dark adapted plants (19). In addition, the relocation into the cytoplasm of another protein which binds Sgn5, p27Kip1, might be regulated by COP9 signalosome (16). However, because the p27Kip1 relocation was induced by Sgn5 overexpression, one should be cautious with the interpretation in light of the effects of Sgn5 overexpression presented in this paper. If COP9 signalosome is involved in the regulation of p27Kip1, large amounts of free Sgn5 might trap the cell cycle regulator and prevent its interaction with the COP9 signalosome.

The high homologies of COP9 signalosome subunits with components of the 26 S proteasome lid (8–12) could be because of a common ancestor and perhaps a functional divergence of the two complexes during evolution. In the case of c-Jun, interaction with the COP9 signalosome leads to stabilization of the transcription factor, whereas the 26 S proteasome lid is involved in its ubiquitin-dependent degradation. The balance of the two processes, stabilization and degradation of c-Jun, is crucial for the decision whether cells proliferate, differentiate, or go into apoptosis.

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