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The Subunit 1 of the COP9 Signalosome Suppresses Gene Expression Through its N-terminal Domain and Incorporates into the Complex Through the PCI Domain

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The COP9 signalosome is a conserved protein complex composed of eight subunits. Individual subunits of the complex have been linked to various signal transduction pathways leading to gene expression and cell cycle control. However, it is not understood how each subunit executes these activities as part of a large protein complex. In this study, we dissected structure and function of the subunit 1 (CSN1 or GPS1) of the COP9 signalosome relative to the complex. We demonstrated that the C-terminal half of CSN1 encompassing the PCI domain is responsible for interaction with CSN2, CSN3, and CSN4 subunits and is required for incorporation of the subunit into the complex. The N-terminal fragment of CSN1 cannot stably associate with the complex but can translocate to the nucleus on its own. We further show that CSN1 or the N-terminal fragment of CSN1 (CSN1-N) can inhibit *c-fos* expression from either a transfected template or a chromosomal transgene (*fos-lacZ*). Moreover, CSN1 as well as CSN1-N can potently suppress signal activation of a AP-1 promoter and moderately suppress serum activation of a SRE promoter, but is unable to inhibit PKA-induced CRE promoter activity. We conclude that the N-terminal half of CSN1 harbors the activity domain that confers most of the repression functions of CSN1 while the C-terminal half allows integration of the protein into the COP9 signalosome.

Keywords: COP9 signalosome; c-fos; PCI domain; protein complex; GPS1

The COP9 signalosome is a multi-component protein complex conserved among most eukaryotes including vertebrate animals, flies, plants, and fission yeast *Schizosaccharomyces pombe*, but not budding yeast *Saccharomyces cereviciae* (Wei & Deng, 1999; Freilich *et al*., 1999; Mundt *et al*., 1999; Deng *et al*., 2000). The overall organization and the amino acid sequences of the COP9 signalosome subunits resemble the lid subcomplex of the 19 S regulatory particle for the 26 S proteasome (Glickman *et al*., 1998; Seeger *et al*., 1998; Wei *et al*., 1998). Both complexes are composed of eight subunits: six of them containing the PCI domain (Proteasome-COP9 complex-Initiation factor 3) and two of them containing the MPN domain (Mpr1-Pad1-N-term.) (Aravind & Ponting, 1998; Glickman *et al*., 1998; Hofmann & Bucher, 1998; Wei & Deng, 1999). The architectures deduced from electron microscopy data of the purified COP9 signalosome and the proteasome lid appear non-identical, but common structural features can be found (Kapelari *et al*., 2000).

In Arabidopsis, the COP9 signalosome is genetically defined as a suppressor of light-dependent development. An important function of the complex is to prevent the expression of the genes involved in photomorphogenesis in the absence of light signals (Deng & Quail, 1999; Osterlunt *et al*., 2000). Characterization of the mutants in Arabidopsis subunit 1 (atCSN1), also known as *fus6/cop11* mutants (Castle & Meinke, 1994; Wei *et al*., 1994b), indicates that atCSN1 is an essential subunit of the complex with regard to both structural integrity and functionality (Wei *et al*., 1994a; Chamovitz *et al*., 1996; Staub *et al*., 1996). Biochemically, both plant and animal CSN1 (FUS6/GPS1) and CSN8 (COP9) subunits are found exclusively

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associated with the complex (Wei *et al*., 1994a; Staub *et al*., 1996; Wei & Deng, 1998). Correspondingly, atCSN1 (COP11/FUS6) and atCSN8 (COP9) proteins are nearly undetectable in the Arabidopsis mutants missing the other subunit of the COP9 signalosome (Wei *et al*., 1994a; Staub *et al*., 1996; Kwok *et al*., 1998), suggesting that accumulation of CSN1 and CSN8 proteins require the assembly of an intact complex.

The physiological role of the COP9 signalosome in animals has not yet been clearly defined at present. The purified COP9 signalosome was found to associate with a novel kinase activity that can phosphorylate Iκb and c-Jun *in vitro* (Seeger *et al*., 1998). Studies on individual subunits suggest that the complex is involved in multiple cellular processes including the stress-activated MAP kinase pathway (Claret *et al*., 1996; Spain *et al*., 1996; Naumann *et al*., 1999), nuclear hormone receptor (Lee *et al*., 1995; Dressel *et al*., 1999; Chauchereau *et al*., 2000), integrin-mediated gene expression (Bianchi *et al*., 2000), and in cell cycle control (Mahalinggam *et al*., 1998; Mundt *et al*., 1999; Tomoda *et al*., 1999).

The human CSN1 (GPS1) was isolated by its ability to suppress the lethality of a constitutively activated MAP kinase pathway involved in the pheromone response in *S. cerevisiae* (GPS1, G-protein pathway suppressor 1, Spain *et al*., 1996). Transient expression of CSN1 in mammalian cells was shown to inhibit JNK1 activity and repress c-Jun-dependent promoter activity (Spain *et al*., 1996). On the other hand, the subunit CSN5, or JAB1 (Jun activation domain binding protein 1) was reported to interact with c-Jun and to act as a co-activator (Claret *et al*., 1996). Additionally, CSN2 (Trip15/SGN2) overexpression in mammalian cells was also found to cause an increase in AP-1-dependent transcription (Naumann *et al*., 1999). In the case of CSN2, the apparent AP-1 activation was speculated to be mediated by a putative COP9 signalosome-associated kinase (Naumann *et al*., 1999). However in these studies, it was not shown whether the observed effects were caused by the complex or by the overexpressed free subunits. To comprehend how COP9 signalosome works as a large protein complex, we reasoned that it is necessary to understand not only the activity of each subunit but also the structural relationship between a given subunit with the complex. In this study, we have focused on the largest subunit of the COP9 signalosome, CSN1, for a systemic structure and function analysis.

CSN1 interacts with CSN2, CSN3, and CSN4 through the carboxyl-half of the protein

We first examined pair-wise interactions between CSN1 and other subunits of the complex in yeast two-hybrid assays. As shown in Figure 1(a), CSN1 interacts strongly with CSN2, CSN3, and CSN4, three PCI domain-containing

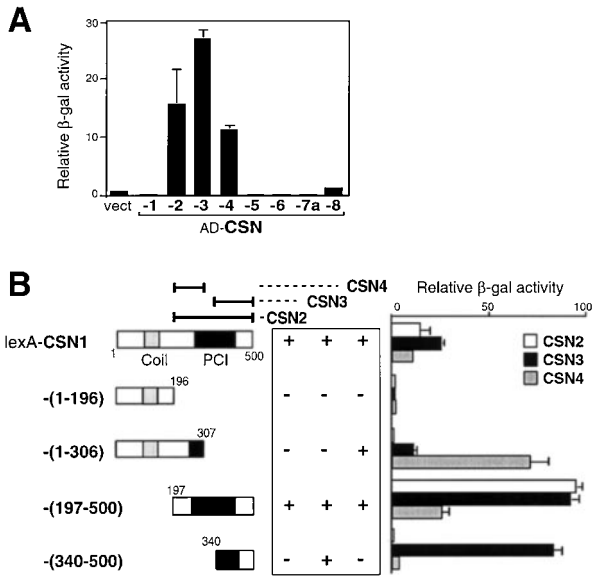

Figure 1. The C-terminal half of CSN1 mediates interactions with CSN2, CSN3, and CSN4 in yeast-two-hybrid assays. (a) Full-length human CSN1 was expressed as a LexA DNA binding domain (LexA-CSN1) fusion protein, and other subunits of the COP9 signalosome were expressed as transcription activation domain (AD-CSNs) fusion proteins (Golemis *et al*., 1994). The pair-wise interactions were indicated by relative β-galactosidase activities of the reporter plasmid (pSH18-34). The values were relative to the positive control, pSH17-4 (at 100) (LexA-AD fusion). Six independently transformed samples were used to calculate the averages and the standard deviations (error bars). (b) Deletions of LexA-CSN1, as depicted on the left, were used in the yeast two-hybrid assay with full-length AD-CSN2, AD-CSN3, and AD-CSN4. The quantitative data of pair-wise interactions are shown on the right and summarised in the middle section of the Figure. The data are expressed as folds of induction over the corresponding deletion construct with empty AD-fusion prey vector. The regions on CSN1 sufficient for interaction with the three subunits studied are indicated on the top. In all samples, protein expression for the respective construct was confirmed by immunoblotting using anti-LexA (Clontech) and anti-HA antibodies (Santa Cruz).

subunits. These results are consistent with the CSN1-CSN2 interaction found with the *S. pombe* subunits (Mundt *et al*., 1999), CSN1-CSN4 interaction with the Arabidopsis subunits (Serino *et al*., 1999), and CSN1-CSN3 interaction found by a filter binding assay (Kapelari *et al*., 2000). However, we did not detect the CSN1-CSN5 and CSN1-CSN7 interactions as reported with the Arabidopsis subunits for reasons currently not understood (Kwok *et al*., 1998; Karniol *et al*., 1999). We next proceeded to delineate the domains on CSN1 that mediate the interactions.

Deletion mutants of LexA-CSN1, as diagramed in Figure 1(b) (left panel), were tested for their abilities to interact with AD-CSN2, AD-CSN3, and

AD-CSN4 in yeast. The amino-terminal fragment 1-196 amino acid residues (aa) fail to interact with any of the subunits, demonstrating that the C-terminal half of CSN1 is essential for interacting with all three subunits. Correspondingly, the C-terminal half, aa 197-500, is sufficient for interaction with all three subunits. Further deletion analysis within the C-terminal region suggest that aa 340-500 is sufficient to interact with CSN3 and that aa 197-307 likely contains the CSN4 binding module. The CSN2 binding region overlaps with those of CSN4 and CSN3 (Figure 1(b)). Together, these results suggest that the middle and the C-terminal portion of CSN1 containing the PCI domain is necessary and sufficient to mediate interactions with the CSN2, CSN3, and CSN4 subunits. A similar observation, in regard to the role of the PCI domain, has been reported in an interaction study between *Drosophila* dmCSN2 and dmCSN7 (Freilich *et al.*, 1999). Our data further suggest that regions responsible for interaction with individual subunits may be separable.

The carboxyl-half of CSN1 is responsible for incorporation into the COP9 signalosome

To verify in mammalian cells that the PCI region indeed serves a structural role for CSN1 as suggested by the yeast two-hybrid studies, we transiently expressed the Flag-epitope tagged derivatives of full-length CSN1 ($Csn1^{flag}$), the N-terminal fragment encompassing aa 1-196 ($Csn1N^{flag}$), or the C-terminal fragment aa 220-500 ($Csn1C^{flag}$). These gene products were then immunoprecipitated with anti-Flag antibodies and immunoblotted with antibodies against Flag and various CSNs (Figure 2(a)). Consistent with the interaction results in yeast, $Csn1^{flag}$ and $Csn1C^{flag}$ can pull down all of the endogenous COP9 signalosome subunits tested, whereas $Csn1N^{flag}$ cannot (Figure 2(a)). It is probable that the entire complex of eight subunits was pulled down, regardless whether the individual subunits directly contact CSN1 or not. This result demonstrates that the full-length and the C-terminal fragment (aa 220-500), but not the N-terminal fragment (aa 1-196), are capable of incorporation into the COP9 signalosome. The lower intensity of the $Csn1C^{flag}$ band (Figure 2(a)) is due to its insolubility (see below) rather than the expression level.

The co-immunoprecipitation results suggest that $Csn1C^{flag}$ would be in the complex form whereas $Csn1N^{flag}$ would not. To confirm this, we used gel filtration chromatography which can easily separate the free subunits of COP9 signalosome (below 60 kDa) from the complex (about 500 kDa) (Kwok *et al.*, 1998). The cell extracts were fractionated through a Superose 6 column and the fractions were probed with anti-Flag to identify the transfected gene products, and with anti-CSN1 and anti-CSN8 to identify the endogenous COP9 signalosome subunits (Figure 2(b)). As shown previously (Wei & Deng, 1998), the endogenous CSN1 and CSN8 co-fractionate in a peak around 500 kDa without detectable free subunits. In contrast, most of the transiently overexpressed $Csn1^{flag}$ protein is in free forms (Figure 2(b), panel $Csn1^{flag}$). In addition, $Csn1^{flag}$ is found in essentially all fractions larger than its monomeric size including the fractions containing the COP9 signalosome. Importantly, the gel filtration pattern of the endogenous CSN8 is essentially identical to that of the untransfected control (Figure 2(b), panel Untransfected). This result indicates that the structural integrity of the endogenous COP9 signalosome is more or less unaffected by overexpression of $Csn1^{flag}$, because intermediate complexes or CSN8 free subunits would have been detected otherwise (Kang *et al.*, 2000).

As predicted, the $Csn1N^{flag}$ is found only in fractions corresponding to low molecular weight proteins, indicating that it exists in a free form apart from the complex (Figure 2(b), panel $Csn1N^{flag}$). This fractionation pattern is consistent with its inability to co-immunoprecipitate with the complex (Figure 2(a)). In contrast, $Csn1C^{flag}$ is not detected in the free subunit fractions, but is found to co-fractionate with the COP9 signalosome (500 kDa) or in fractions of very large molecules around the excluded volume of the column (6 MDa) (Figure 2(b), panel $Csn1C^{flag}$). In fact, most of $Csn1C^{flag}$ proteins were found in the insoluble fraction (not shown), possibly as a result of self-aggregation or membrane association. Taken together, we have shown that $Csn1N^{flag}$ is present exclusively in free forms whereas $Csn1C^{flag}$ is present only in the complex forms, a small portion of which is in the COP9 signalosome. The full-length subunit, $Csn1^{flag}$, accumulates in both free and complex forms.

The COP9 signalosome has previously been shown to localize predominantly in the nucleus (Chamovitz *et al.*, 1996; Wei & Deng, 1998; Dressel *et al.*, 1999; Schaefer *et al.*, 1999). Here, we examined the subcellular localization pattern of transiently expressed CSN1 proteins by immunofluorescence staining of the Flag-epitope (Figure 2(c)). $Csn1^{flag}$ is found in both nuclear and cytoplasmic compartments, and is frequently found enriched in the nucleus and around the nuclear periphery (Figure 2(c), also see Figure 4(a)). $Csn1N^{flag}$ localization pattern is similar to $Csn1^{flag}$ (Figure 2(c)). Since $Csn1N^{flag}$ cannot stably associate with the complex, its nuclear localization indicates that this truncated protein can translocate to the nucleus on its own. In contrast, $Csn1C^{flag}$ exhibits a grainy-looking staining pattern that is mostly excluded from the nucleus. We suspect that either self-aggregation of $Csn1C^{flag}$ or association with Golgi membranes may account for this staining pattern and for its gel filtration profile where it is predominantly found in high molecular weight complexes (Figure 2(b)). Some weakly stained cells, presumably expressing low level of $Csn1C^{flag}$ proteins, showed diffused nuclear staining (Figure 2(c), indicated by an arrowhead). These

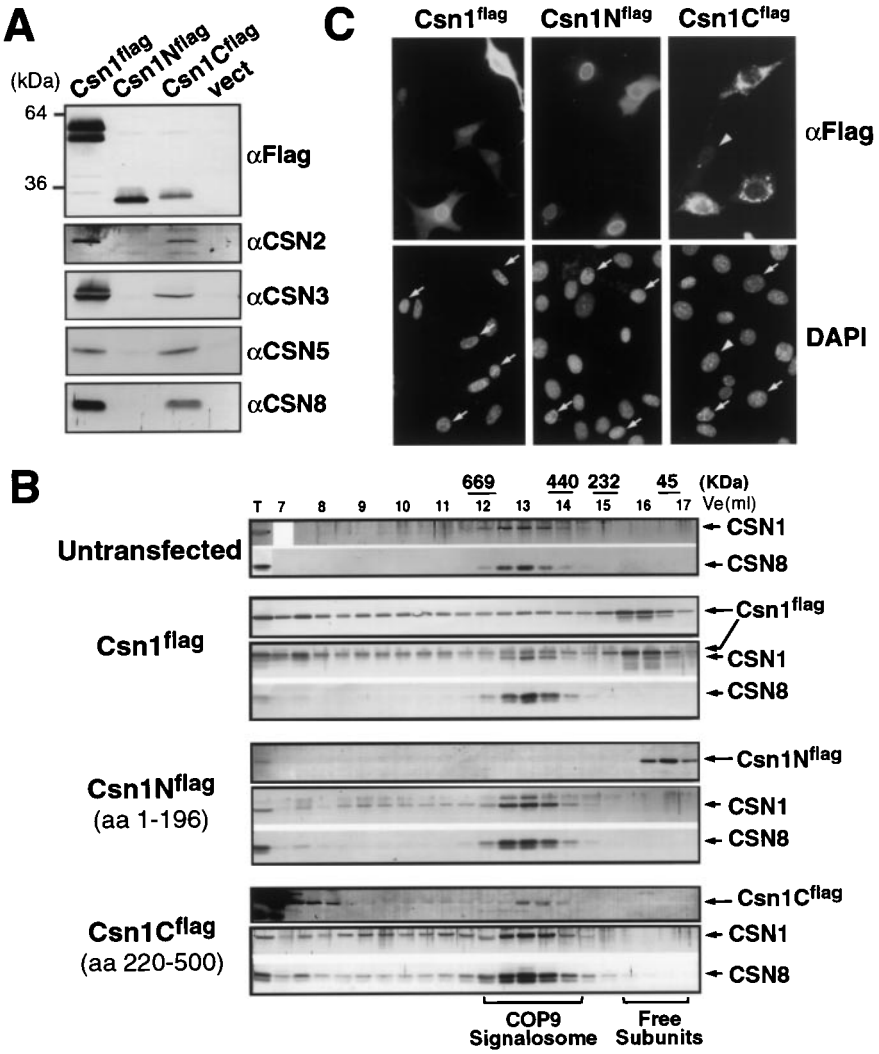

Figure 2. The C-terminal half of CSN1 is responsible for the subunit to assemble into the COP9 signalosome. NIH3T3 cells were transfected with 10 μg per 10 cm dish of CSN1 expression plasmids including full-length ($Csn1^{flag}$), the N-terminal fragment (aa 1-196) ($Csn1N^{flag}$), and the C-terminal fragment (aa 220-500) ($Csn1C^{flag}$) using lipofectamine reagent (GIBCO/BRL). (a) Co-immunoprecipitation. Anti-Flag (M2) beads (Sigma) were used to immunoprecipitate the Flag fusion proteins from the cell extracts 40 hours post-transfection in the buffer containing 10 mM Hepes (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 200 mM NaCl, 0.4 % Tween-20, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail (Roche). After rocking at 4 °C for four hours, the beads were washed for five times with PBS containing 0.2 % Tween-20. Proteins co-precipitated with the beads were subject to immunoblot analysis with antibodies against the Flag tag (Sigma), CSN2, CSN3, CSN8 (hCOP9) (Affiniti Research Products), or CSN5/Jab1 (kindly provided by Dr E. Bianchi) (Bianchi *et al*., 2000). (b) Crude cell extracts derived from untransfected cells or 48 hours post-tranfected cells were fractionated through a Superose 6 column (Pharmacia) at a flow rate of 0.3 ml/minute in a buffer containing 20 mM Tris-HCl (pH 7.2), 200 mM NaCl, and 10 % (v/v) glycerol. Fractions of 0.5 ml each were collected starting from the on-set of the void volume (7 ml) and were concentrated by 20 μl/fraction Strataclean™ beads (Stratagene). Fractions were subjected to immunoblot analysis with anti-Flag antibody to detect the flag-tagged full-length or mutant Csn1 gene products, or with anti-CSN1 and anti-CSN8 antibodies to detect the endogenous CSN1 and CSN8 subunits. Fractions corresponding to the peak position of the COP9 signalosome (500 kDa) and to the elution positions of free subunits are labeled at the bottom. In panel $Csn1^{flag}$, the Flag-tagged $Csn1^{flag}$ migrated slightly slower than the endogenous CSN1 on the SDS-PAGE. (c) Immunofluorescence staining using the anti-Flag antibodies was carried out 24 hours post-transfection (see Figure 4 legend for detail). Cells were double stained with DAPI to identify the nuclei in the corresponding field.

proteins are likely to represent the small portion that is properly incorporated into the complex as indicated from gel filtration and co-IP experiments.

Collectively, we have demonstrated that the C-terminal portion of CSN1 encompassing the PCI domain is responsible for incoporation of the subunit into the COP9 signalosome. Additionally, our data indicate that the regions within the CSN1 PCI domain sufficient for interaction with different subunits, such as CSN3 and CSN4, are separable

(Figure 1(b)). The PCI domain is predicted to consist of multiple α-helices extending over aa 180 residues, and is found in subunits of the proteasome lid and eIF3 besides the COP9 signalosome (Aravind & Ponting, 1998; Glickman *et al*., 1998; Hofmann & Bucher, 1998; Wei *et al*., 1998). The notion that the PCI domain serves as a structural scaffold for the assembly of the COP9 signalosome may potentially extend to the other multisubunit complexes.

CSN1 can repress the *c-fos* promoter

Since the N-terminal portion of CSN1 has no structural role, we reasoned that it could potentially carry biological activities. To establish a functional assay and to define the functional domain(s) of CSN1, we first examined its ability to regulate gene expression by using the *c-fos* promoter, a prototype immediate-early promoter, in transient assays. Expression from the *c-fos* promoter can be rapidly and transiently induced by a broad range of stimuli, such as serum, phorbol ester, and UV irradiation, in a variety of cell types (Curran & Morgan, 1995; Hill & Treisman, 1995; Treisman, 1995; Whitmarsh *et al*., 1997). For example, UV irradiation or MEKK1, the upstream kinase of the SAPK/JNK pathway (Yan *et al*., 1994), has been shown to activate *c-fos* expression predominantly by stimulating TCF/Elk1 activity at SRE (serum response element) through JNK (Cavigelli *et al*., 1995; Gille *et al*., 1995; Whitmarsh *et al*., 1995) as well as the p38 and the ERK pathways (Price *et al*., 1996; reviewed by Schlesinger *et al*., 1998). Studies with *fos-lacZ* transgenic mice showed that multiple regulatory elements are required in concert for tissue- and stimulus-specific regulation of *c-fos* promoter (Robertson *et al*., 1995).

We first tested possible non-specific effects of CSN1 overexpression in transcription-reporter assays by examining DBD-VP16-mediated transactivation from a Gal4-DNA binding site containing promoter-reporter, *Gal4-luc*. We found that $Csn1^{flag}$ and $Csn1N^{flag}$ showed little effect on VP16-mediated transactivation, while $Csn1C^{flag}$ overexpression appeared to moderately reduce the activity (Figure 3(a)). The non-specific effect of $Csn1C^{flag}$ on transcription assays and its altered subcellular distribution pattern (Figure 2(c)) suggest that this construct is unsuitable for transcription assays. Therefore we used $Csn1^{flag}$ and $Csn1N^{flag}$ to investigate the role of CSN1 in c-fos expression in a similar assay. We constructed a *fos-luc* reporter plasmid containing a human *c-fos* promoter (−711 to +45) fragment which has all of the known upstream regulatory elements to drive the expression of a luciferase reporter gene. In NIH3T3 cells, expression of $Csn1^{flag}$ or $Csn1N^{flag}$ lead to reduced *fos-luc* activity (Figure 3(b)), indicating that the full-length and the N-terminal region of CSN1 can repress the basal activity of *fos-luc*. We next co-expressed a constitutively active MEKK1 kinase domain (MEKK1Δ) (Fanger *et al*., 1997) to induce *c-fos* expression and asked whether the *fos-luc* induction by MEKK1 is affected by CSN1. We found that both $Csn1^{flag}$ and $Csn1N^{flag}$ can efficiently repress the MEKK1-induced *fos-luc* activity to a level equal to that without MEKK1 stimulation, suggesting a role of CSN1 in signal suppression.

Since the COP9 signalosome is related to the lid subcomplex of the 26 S proteasome, we asked whether CSN1-mediated repression of *fos-luc* involves the activity of the 26 S proteasome. Similar experiments were carried out in the presence of a proteasome inhibitor MG132. For the basal activity of *fos-luc*, a six-hour incubation with MG132 resulted in no dramatic alterrations with regard to the extent of repression by $Csn1^{flag}$ or $Csn1N^{flag}$ (Figure 3(c), upper panel). In the presence of MEKK1 activation, however, MG132 treatment resulted in an additional three to fourfold reduction in *fos-luc* activity (Figure 3(c), lower panel), suggesting a synergistic effect between inactivation of the 26 S proteasome and overexpression of CSN1. This result directly opposes the idea that CSN1 might facilitate the proteasome-mediated degradation of transactivators of *c-fos* expression. However, our result cannot rule out a possibility that CSN1-mediated repression of gene expression might involve inactivation of the proteasome.

The *c-fos* promoter contains a complex array of regulatory elements including AP-1, SRE, and CRE (cAMP response element), which allow it to respond to a wide range of stimuli. To dissect the involvement of CSN1 in different upstream signaling pathways regulating *c-fos* expression, we examined the effect of CSN1 overexpression on transcription activation from independent responsive element (Figure 3(d)). The activities of *AP1-luc* or *CRE-luc* promoter-reporters were induced by co-expressing MEKK1Δ or the catalytic subunit of PKA (protein kinase A), respectively, whereas *SRE-luc* was induced by 20 % serum. Similar to *fos-luc* as described above (Figure 3(b)), expression of $Csn1^{flag}$ and $Csn1N^{flag}$ effectively inhibited activation of *AP1-luc* and *SRE-luc* (Figure 3(d) upper two panels). In addition, $Csn1^{flag}$ and $Csn1N^{flag}$ can also inhibit MEKK1Δ activation of *SRE-luc* to a similar degree (not shown). By contrast, neither $Csn1^{flag}$ nor the truncated forms are able to repress the PKA induction of the CRE-driven promoter, *CRE-luc*, in NIH3T3 or Hela cells (Figure 3(d) lower panel). These results indicate that CSN1 predominantly acts to suppress the growth factor and, in particular, the stress-activated MAP kinase pathways.

CSN1 represses the signal induction of a *fos-lacZ* transgene

We next asked whether CSN1 could also repress a chromosomally resided *c-fos* promoter. We approached this by utilizing the fibroblast cell line derived from a *fos-lacZ* transgenic mouse (Smeyne

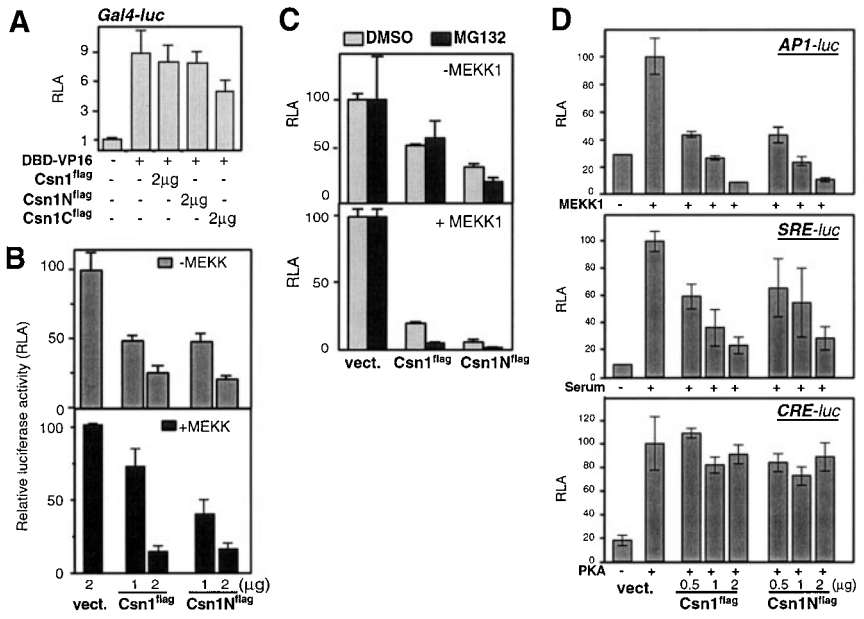

Figure 3. CSN1 (GPS1) represses *c-fos* promoter through its N-terminal domain. (a) The *Gal4-luc* reporter, which contains luciferase reporter gene controlled by five copies of the Gal4 binding sequences, was activated by co-transfection of a Gal4 DNA-binding domain-VP16 fusion plasmid (DBD-VP16, 1 μg). The Flag-epitope tagged derivatives of CSN1 ($Csn1^{flag}$), the N-terminal (aa 1-196) ($Csn1N^{flag}$), and the C- terminal (aa 220-500) ($Csn1C^{flag}$) fragments were also expressed. The expression of $Csn1^{flag}$ or $Csn1N^{flag}$ did not significantly affect the VP16-mediated transcription, while $Csn1C^{flag}$ caused moderate reduction of the reporter. (b) $Csn1^{flag}$ and $Csn1N^{flag}$ were transiently expressed in NIH3T3 cells with *fos-luc* (1 μg) for 24 hours (upper panel). In the lower panel, *fos-luc* activity was induced by co-expressing the active MEKK1Δ (1 μg). The β-galactosidase and luciferase assays (Promega) were performed 24-36 hours after the transfection was initiated. The relative luciferase activities (RLA) were compared to the vector control (set to 100). (c) Similar experiment as described in (b) was carried out with or without a six-hour incubation with proteasome inhibitor MG132 (10 μM, Sigma) or its vehicle (DMSO). The DNA expressing $Csn1^{flag}$ and $Csn1N^{flag}$ was used at 2 μg for each reaction. The relative luciferase activities (RLA) were adjusted relative to the respective vector controls (set to 100) in both MG132+ and MG132− (DMSO) series. (d) NIH3T3 cells were transfected with plasmids of *AP1-luc* promoter-reporter (1 μg) and MEKK1Δ (50 ng) where indicated. The plasmids expressing $Csn1^{flag}$ or $Csn1N^{flag}$ in the amounts as indicated or the empty vector (2 μg) were co-transfected. Luciferase activities were measured 24 hours post-transfection. Similar results were obtained with Hela cells. In the *SRE-luc* promoter-reporter experiments, NIH3T3 cells were serum-starved for 30 hours. Six hours after addition of 20 % serum, cells were collected for luciferase assay. Hela cells were tranfected with *CRE-luc* (1 μg), pFc-PKA (0.5 μg) where indicated, and the luciferase activity was assayed 30 hours post-transfection. In all panels, relative luciferase activities to the values of the induced vector control sample (set to 100) for respective reporters are plotted. In all cases, the averages and the standard deviations for each reaction were calculated from three independent samples.

et al., 1992). The *fos-lacZ* transgene contains all of the known *c-fos* regulatory elements including the promoter, 315 N-terminal amino acid residues, and the 3′ untranslated region of the *c-fos* gene (Schilling *et al*., 1991). Importantly, the *fos-lacZ* transgene recapitulates both tissue- and stimulus-specific regulation of *c-fos* expression *in vivo* (Smeyne *et al*., 1992; Curran & Morgan, 1995; Robertson *et al*., 1995).

We transiently expressed $Csn1^{flag}$ in the *fos-lacZ* cells and then stimulated *fos-lacZ* expression with serum, UV irradiation, or TPA. The expression of *fos-lacZ* can be monitored by β-galactosidase staining, and the transfected cells *versus* untransfected cells can be distinguished by immunofluorescence staining of the Flag epitope (Figure 4(a)). As with the native *c-fos* gene, *fos-lacZ* transgene was efficiently induced by serum, UV and TPA treatment. Under these conditions, most of the cells expressing $Csn1^{flag}$ exhibited reduced (cells marked with arrowheads) or undetectable (cells marked with arrows) LacZ staining comparing to the untransfected cells in the same dish (Figure 4(a)), although a small portion of transfected cells still showed *fos-lacZ* expression. To gain a statistical picture of the effect of $Csn1^{flag}$, we counted the number of cells with or without detectable LacZ staining among transfected or untransfected cells. The data revealed that the numbers of *fos-lacZ* expressing cells are reduced in the transfected cell populations to below half (UV), about half (serum), or about two/third (TPA) of the untransfected populations (Figure 4(b)). Similar results were obtained with $Csn1N^{flag}$ (not shown). These results demonstrate that CSN1 can repress signal activation of a chromosomal *c-fos* promoter.

Notably, the 196 aa N-terminal fragment of CSN1 ($Csn1N^{flag}$) is sufficient to confer the repression activities we have observed with the full-length CSN1. Considering that $Csn1N^{flag}$ does not stably associate with the complex but can accumulate in the nucleus, we propose that the 196 aa N-terminal region of CSN1 (CSN1-N) contains a repression domain (Figure 4(c)) that can act autonomously. CSN1 has been shown to inhibit JNK1 kinase activity (Spain *et al*., 1996). Therefore, it seems more likely that CSN1, through its N-terminal domain, functions to suppress the

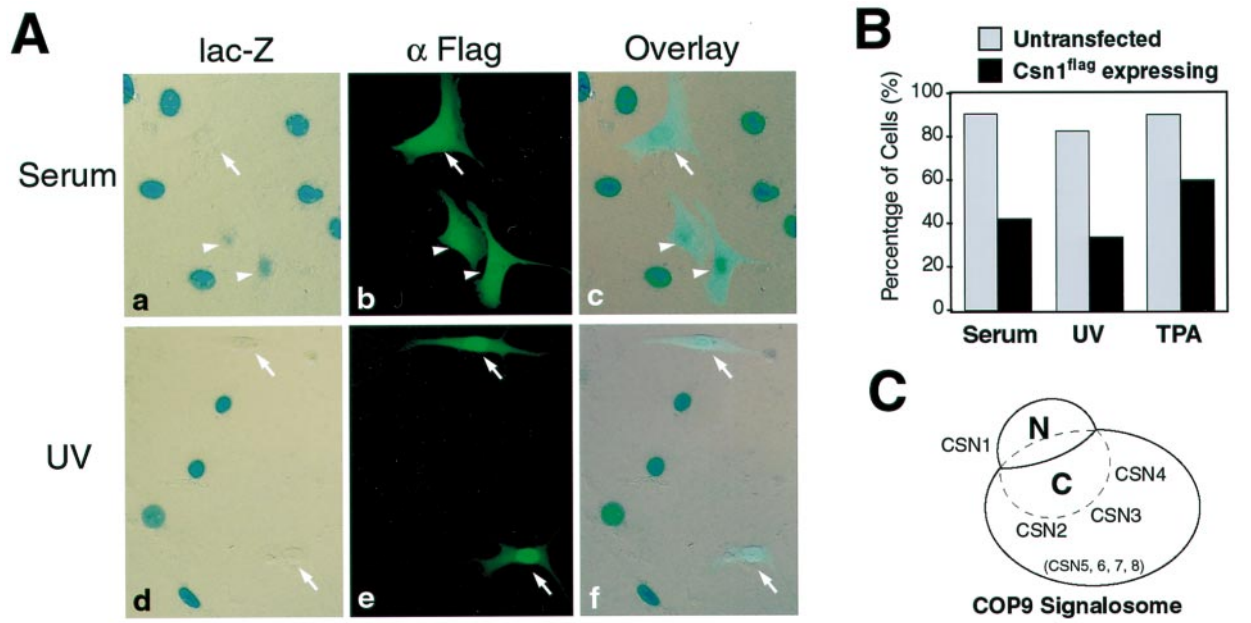

Figure 4. Expression of Csn1^{flag} inhibits chromosomal *fos-lacZ* expression. The fibroblast cells derived from *fos-lacZ* transgenic mice (kindly provided by Dr T. Curran) were seeded on cover slips and were transfected with 2 μg of plasmid expressing Csn1^{flag}. (a) Cells were serum-starved for 40 hours followed by induction with 20 % serum ((a), (b) and (c)) or with UV (50 J/m^2) ((d), (e) and (f)). After three hours, cells were fixed with 2 % paraformaldehyde for 20 minutes and permeabilized with 0.2 % Triton X-100 in PBS containing 1 mM $MgCl_2$ and 0.2 mM EGTA for ten minutes. Transfected cells expressing Csn1^{flag} were detected by indirect immuno-florescence staining with anti-Flag antibody ((b) and (e)). After the immuno reactions, cells were stained for β-galactosidase to detect the expression of *fos-lacZ* fusion protein by incubation in 1 mg/ml X-gal solution containing PBS and 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02 % (v/v) Nonidet P-40, and 0.1 mg/ml deoxycholate acid. Note that cells expressing Csn1^{flag} showed no (cells marked by arrows) or reduced (cells marked by triangles) LacZ staining ((a) and (d), (c) and (f)). (c) and (f) Pictures superimposing (a) to (b), or (d) to (e), respectively. (b) After staining, the number of transfected (expressing Csn1^{flag}) cells displaying detectable LacZ staining was counted. On the same field, the number of untransfected cells displaying LacZ staining was counted separately. These numbers were calculated as percentage of the total transfected or untransfected cell populations. For each treatment, a total of 200 to 500 cells were counted. (c) A model for the COP9 signalosome depicting the structural relationship of CSN1 to the complex is shown. The amino (N)-terminal region of CSN1, which harbors the biological activity, likely resides toward the exterior of the complex. The carboxyl (C)-terminal half of CSN1 functions to integrate the subunit into the complex, probably through interaction with other subunits including CSN2, CSN3, and CSN4.

signal activation instead of acting as a DNA-specific transcription repressor.

Combining the structural and functional data, we conclude that the C-terminal half of CSN1 serves a structural role allowing the subunit to incorporate into the complex while the N-terminal half of CSN1 harbors the activity module (Figure 4(c)). It is conceivable that excess amount of such CSN1-N activity can account for the repression effect caused by expression of Csn1^{flag} or Csn1N^{flag}. In principle, Csn1C^{flag}, which lacks the putative activity domain and can integrate into the complex, could have acted as a dominant-negative molecule of COP9 signalosome. However, most of the expressed Csn1C^{flag} proteins displayed reduced solubility and altered localization pattern with respect to the endogenous COP9 signalosome (Figure 2(b) and (c)). These may be caused, in part, by its tendency to aggregate as a consequence of transient over-production in the absence of stoichiometric balance of other interacting subunits.

To address whether the observed repression reflects a constitutive activity or a dominant negative activity of the COP9 signalosome complex, two alternative proposals are considered. In one model, the CSN1-mediated repression results from dominant negative interference with the function of the COP9 signalosome, because the free CSN1 or its N-terminal fragment blocks the action of the complex by, for example, sequestering its substrates/effectors. This model would imply that the COP9 signalosome acts as an activator of *c-fos* expression and JNK/AP-1 pathway. In the second model, the CSN1-mediated repression would represent an aspect of overall activities of the COP9 signalosome. This model would predict that the COP9 signalosome functions to repress *c-fos* expression and signal induction of AP1, in part, through the CSN1-N repression domain. By association with the complex through the C-terminal half of CSN1, the activity carried by CSN1-N could be stabilized and regulated through the functions and the signal responsiveness of the other subunits of

the complex. The genetic studies in plants seem to support the second model, because the Arabidopsis mutants deficient in the COP9 signalosome exhibit constitutive activation of stress- and light-induced genes (Wei *et al*., 1994b; Kwok *et al*., 1996; Mayer *et al*., 1996). Furthermore, overexpression of a functional CSN1 homolog in stable transgenic plants confers a gain-of-function phenotype that is opposite to the mutant phenotype (Kang *et al*., 2000). As CSN1 provides an essential functionality to the complex, elucidating the nature of the activity located at CSN1-N in the future will certainly further our understanding about the function of the COP9 signalosome.

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