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The DEXD/H-box RNA helicase RHII/Gu is a co-factor for c-Jun-activated transcription

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Tandem affinity purification (TAP) and mass spectrometric peptide sequencing showed that the DEAD-box RNA helicase RHII/Gu is a functional interaction partner of c-Jun in human cells. The N-terminal transcription activation region of, c-Jun interacts with a C-terminal domain of RHII/Gu. This interaction is stimulated by anisomycin treatment in a manner that is concurrent with, but independent of, c-Jun phosphorylation. A possible explanation for this effect is provided by the observation that RHII/Gu translocates from nucleolus to nucleoplasm upon anisomycin or UV treatment or when JNK signaling is activated by overexpression of a constitutively active form of MEKK1 kinase. Several experiments show that the RNA helicase activity of RHII/Gu supports c-Jun-mediated target gene activation: dominant-negative forms of RHII/Gu, as well as a neutralizing antibody against the enzyme, significantly interfered with c-Jun target gene activity but not with transcription in general. These findings clarify the mechanism of c-Jun-mediated transcriptional regulation, and provide evidence for an involvement of RHII/Gu in stress response and in RNA polymerase II-catalyzed transcription in mammalian cells.

Keywords: c-Jun/helicase/RHII/Gu/TAP

Introduction

The AP-1 family transcription factor c-Jun plays a pivotal role in the genetic responses of cells to a number of extracellular stimuli, including stress insults, apoptotic and differentiation signals. Many of these signals are transduced to c-Jun by signaling pathways that culminate in the activation of Jun-N-terminal kinase (JNK), causing the phosphorylation and activation of c-Jun (Leppä and Bohmann, 1999; Davis, 2000).

In spite of a large body of literature on the topic, the molecular mechanism by which the transcriptionally active form of c-Jun stimulates mRNA synthesis is still incompletely understood, but it presumably involves the recruitment of specific co-factors. Several c-Jun-interacting proteins have been described (for a review see Chinenov and Kerppola, 2001). Examples are the co-activators CBP and p300, which bind to the N-terminal transactivation domain of c-Jun *in vitro* and enhance AP-1-mediated promoter activation (Chinenov and Kerppola, 2001). Remarkably, none of the proteins reported to bind to the transactivation domain of c-Jun and to facilitate c-Jun-mediated transcription has been shown to interact with c-Jun in *in vivo* binding assays. In that regard, a further development of methods for the identification of proteins that participate in c-Jun-mediated gene activation *in vivo* is required.

Recent progress in mass spectrometric protein sequencing technology as well as the rapid growth of protein and genome databases have made direct approaches to map protein–protein interactions feasible. Because the identification of interacting proteins by a biochemical route is not based on a transcriptional read-out, as is the case in yeast two-hybrid assays, it is also amenable to the study of transcription factor complexes.

Results

Identification of RHII/Gu RNA helicase as an interaction partner of c-Jun by tandem affinity purification

In order to isolate proteins that interact with the transactivation domain of c-Jun in human cells, we employed the tandem affinity purification (TAP) method (Rigaut *et al*., 1999). Briefly, the protein of interest is fused to two ligand-binding domains, one derived from a calmodulin-binding peptide, the other from protein A (Figure 1A). This construction endows the bait protein with high affinity for both calmodulin and IgG affinity purification resins. The calmodulin-binding peptide and protein A affinity tags are separated by the recognition sequence for tobacco etch virus (TEV) protease, permitting proteolytic elution of the fusion protein from the IgG affinity resin (Rigaut *et al*., 1999; Puig *et al*., 2001). The tagged protein, potentially in a complex with interacting factors, can be purified from crude extracts by two consecutive affinity chromatographic steps.

We developed a mammalian expression vector that codes for a fusion protein consisting of amino acids 1–223 of c-Jun linked to the TAP tandem affinity purification domain (c-Jun$^{1–223}$TAP, Figure 1A). For a typical experiment such as the one shown in Figure 1B, 10^8 293 cells

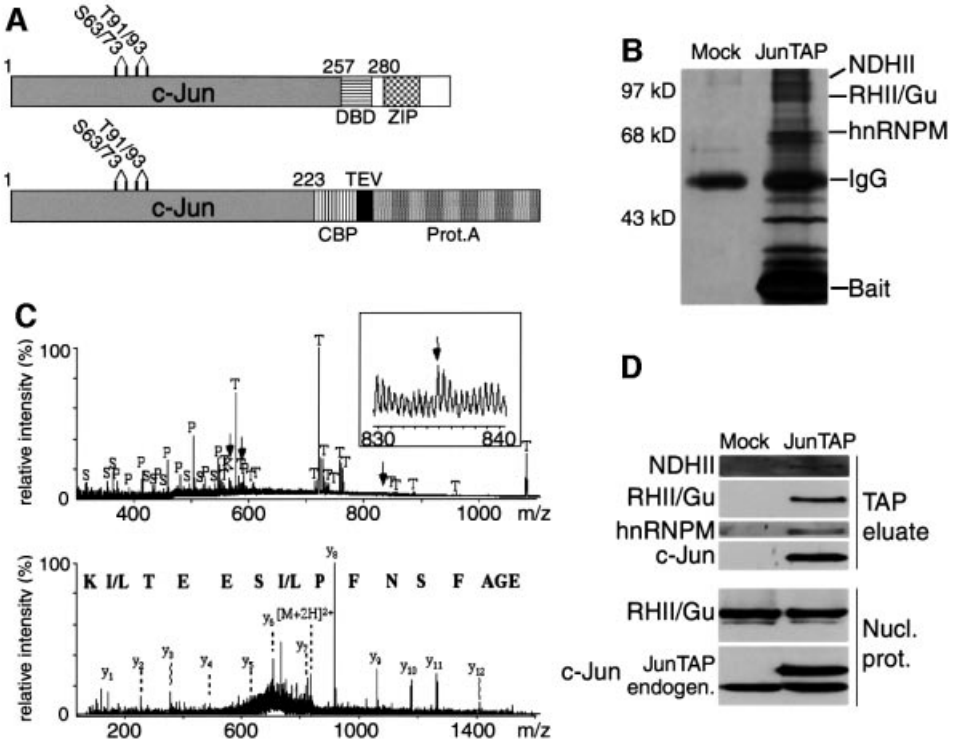

Fig. 1. Identification of the c-Jun-binding protein complex by TAP purification. (**A**) Schematic representation of c-Jun (top) and c-JunTAP structures (bottom). The region of the DNA-binding domain (DBD) and the leucine zipper dimerization domain (ZIP) in c-Jun has been replaced with the TAP tag, consisting of a calmodulin-binding peptide (CBP), a consensus TEV cleavage site (TEV) and the IgG-binding domain of protein A (Prot. A). The position of regulatory JNK MAP kinase phosphorylation sites is indicated. (**B**) Analysis of the c-JunTAP-interacting proteins. Proteins of the TAP eluate from untransfected 293 mock-treated cells or cells transfected with the c-JunTAP expression vector were resolved on an SDS–polyacrylamide gel and silver stained. The protein bands from which NDHII, RHII/Gu and hnRNP M peptide sequences were identified are indicated. In both lanes, a prominent band at ~50 kDa derived from human IgG leeched off the first column of the TAP purification. In the c-JunTAP lane, a prominent band at ~30 kDa represents the bait after enzymatic removal of the protein A affinity tag by TEV. (**C**) Identification of the 84 kDa protein band as RHII/Gu by tandem mass spectrometry. The mass spectrum of the extracted peptides after tryptic digestion is shown in the top panel. Ions from various sources were observed and evaluated based upon the following criteria. Known autoproteolysis products of trypsin (T) and common keratin peptides (K) were identified by comparison of the spectrum with a blank based on their mass-to-charge values and charge states, whereas singly charged ions due to polymer contamination of the solvents used were determined by their characteristic 44 Da repeats (P), all of which were excluded from tandem mass spectrometric analysis. Finally, singly charged peptide ions (S) known to provide limited sequencing information due to poor fragmentation were not considered, while three doubly charged peptide ions marked by arrows were identified and fragmented individually. From all three peptides, tandem mass spectra were acquired that allowed RHII/Gu to be identified independently. An example of a tandem mass spectrum acquired from a doubly charged ion at *m*/*z* 834.8 (see enlarged part of the mass spectrum as inset in top panel) is shown in the bottom panel. The peptide sequence was derived from the mass difference between adjacent fragments of the *y*-ion series. The peptide sequences thus determined and their corresponding location in the RHII/Gu protein sequence are: EGAFSNFPISEETIK (203–217), APQVLVLAPTR (277–287) and IGVPSATEIIK (581–591). (**D**) Verification of the interaction between c-Jun and interacting proteins by western blot analysis. Nuclear proteins from HT-1080 cells stably transfected with the c-Jun$^{1-223}$TAP expression construct or from control cells bearing empty expression vector were subjected to TAP purification. Aliquots of nuclear proteins and TEV eluates were analyzed by western blot using specific antibodies against human c-Jun, NDHII, RHII/Gu and hnRNP M. Note that the TAP-tagged c-Jun protein derivatives in the starting material migrate at slightly higher apparent molecular mass than the endogenous c-Jun.

were transiently transfected with the c-Jun$^{1-223}$TAP expression construct. After 36 h, nuclear extracts were prepared and applied to dual affinity chromatography according to the TAP protocol. The purified eluates contained a number of polypeptide species that co-purified specifically with the TAP-tagged c-Jun bait, but were absent in the fraction purified from control cells (Figure 1B). These products were therefore candidates for c-Jun-interacting proteins.

Bands representing putative c-Jun-binding proteins were purified by SDS–PAGE and analyzed by mass spectrometric peptide sequencing. Comparison of the obtained peptide sequences with protein databases identified several proteins that previously had been linked to mRNA metabolism and transcription. Here we report the identification of three proteins, the DNA/RNA helicase NDHII, hnRNP M and the RNA helicase RHII/Gu, as novel interaction partners for c-Jun. The characterization of other interacting proteins will be reported elsewhere. The identification of RHII/Gu (Valdez *et al*., 1996) was based on sequences obtained from three peptides showing complete identity to the human RHII/Gu protein sequence (see Figure 1C and figure legend). The other interaction partners were identified in a similar manner.

To verify the identity of NDHII, RHII/Gu and hnRNP M as c-Jun-binding partners, we performed a small-scale TAP experiment using an HT-1080 human fibrosarcoma line that carries the c-JunTAP expression vector integrated into

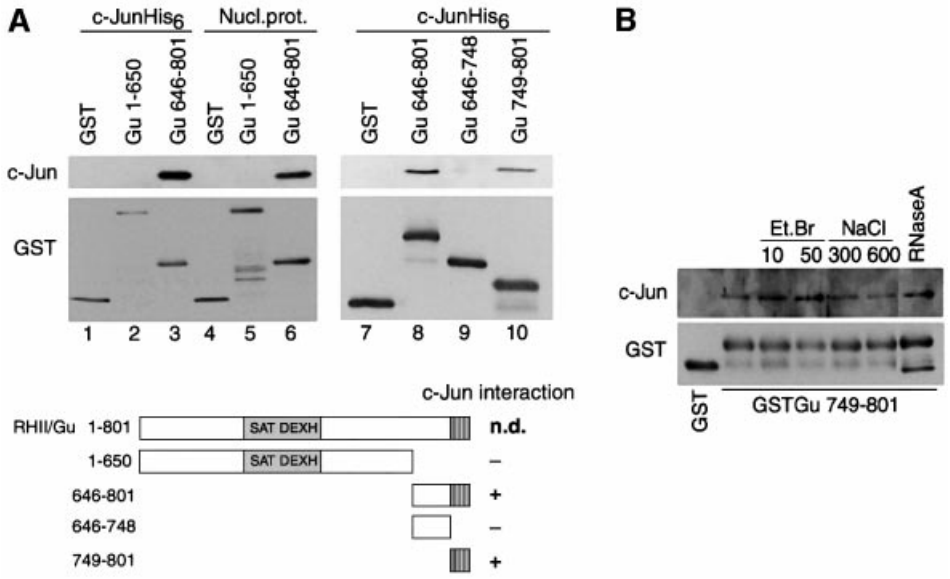

Fig. 2. Direct protein–protein interaction between RHII/Gu and c-Jun. (**A**) Mapping of the c-Jun-binding domain of RHII/Gu. Bacterially expressed GST–RHII/Gu proteins were immobilized on glutathione–beads and incubated with either bacterially expressed full-length c-JunHis$_6$ protein or with nuclear proteins from unstimulated 293 cells. After extensive washing, proteins bound to the beads were eluted in protein sample buffer and analyzed by western blotting with c-Jun (top) or GST antibodies (bottom). A schematic representation of the structure of RHII/Gu and summary of the binding data are shown below. (**B**) The interaction between RHII/Gu and c-Jun is not mediated by DNA or RNA. GST pull-down experiments between GST–RHII/Gu 749–801 and c-JunHis$_6$ were performed in the absence or presence of ethidium bromide (10 or 50 μg/ml), 40 μg/ml RNase A or NaCl (300 or 600 mM).

its genome. Immunoblot analyses with NDHII-, RHII/Gu- and hnRNP M-specific antibodies revealed cross-reactive protein species of the expected molecular masses in the relevant eluate fraction (Figure 1D, upper panel). We conclude that NDHII, RHII/Gu and hnRNP M were retained on the column by means of their interaction with c-JunTAP. These results validate the mass spectrometric identification of NDHII, RHII/Gu and hnRNP M as c-Jun-binding proteins. Furthermore, the experiment confirms that the interaction of c-Jun with the analyzed proteins is not restricted to 293 cells and occurs when the c-JunTAP fusion protein is expressed at levels comparable with endogenous c-Jun as they are present in the stably transfected HT-1080 cells.

DEAD-box RNA helicases have been identified previously as auxiliary factors in transcription and translation processes (Eisen and Lucchesi, 1998; Luking *et al*., 1998; Linder and Daugeron, 2000). NDHII, for example, binds CREB, a leucine zipper transcription factor and a distant relative of c-Jun. NDHII mediates an interaction between RNA polymerase II (pol II) and CREB-binding protein (CBP) and supports CREB-mediated transcription activation in reporter gene assays, an effect that requires the ATPase function of the enzyme (Nakajima *et al*., 1997). It is thus not surprising that NDHII also interacts with c-Jun. It is conceivable that this enzyme supports c-Jun transcription in a manner comparable with its role in CREB target gene activation.

Interestingly, one other newly identified c-Jun interaction partner, RHII/Gu, is a human DEAD-box RNA helicase (Figure 1B and D). Unlike NDHII, however, RHII/Gu has not been implicated previously in RNA pol II-mediated transcription. The presence of RHII/Gu in a complex with c-Jun thus prompted further studies on the role of this enzyme in AP-1-regulated gene expression.

RHII/Gu binds c-Jun directly

To study whether the interaction between RHII/Gu and c-Jun is mediated by direct protein–protein contact, *in vitro* pull-down assays were performed. Different bacterially expressed GST–RHII/Gu fusion proteins were immobilized on glutathione–beads and subsequently incubated with various sources of c-Jun. Immunoblot analyses revealed that both bacterially expressed c-Jun (c-JunHis$_6$) and c-Jun present in 293 cell nuclear extracts were retained specifically on the immobilized C-terminal domain of RHII/Gu (amino acids 646–801; Figure 2A, lanes 3 and 6). However, no interaction of c-Jun with the N-terminal part of RHII/Gu or with GST alone could be detected (Figure 2A, lanes 1, 2, 4 and 5). This result shows that the interaction observed by the TAP method can also be reconstituted *in vitro* and, importantly, that it is direct and does not rely on any intermediary proteins that might have been present in 293 or HT-1080 cells. Furthermore, these results show that RHII/Gu protein may interact with the non-fused wild-type form of c-Jun present in nuclear extracts.

Analysis of deletion mutants of RHII/Gu in the pull-down assay revealed that the c-Jun interaction domain on RHII/Gu resides in the most C-terminal part of the protein between amino acids 749 and 801 (Figure 2A). Importantly, the interaction between c-Jun and GST–Gu 749–801 was not affected by addition of high concentrations of ethidium bromide or RNase A, or by 600 mM NaCl (Figure 2B), demonstrating that the interaction

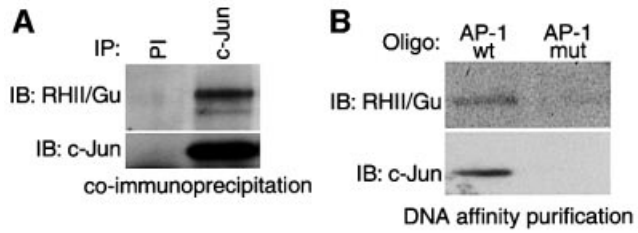

Fig. 3. Interaction between endogenous RHII/Gu and c-Jun proteins in 293 and HT-1080 cells. (**A**) RHII/Gu specifically co-immunoprecipitates with c-Jun. Nuclear extracts from non-transfected subconfluent 293 cells were subjected to immunoprecipitation (IP) with either specific rabbit anti-c-Jun antiserum or rabbit pre-immune serum (PI). Immunoprecipitated proteins were analyzed by western blotting (IB) using anti-RHII/Gu or anti-c-Jun antibodies. Similar amounts of starting material were used in each lane. (**B**) RHII/Gu and c-Jun form a protein complex on AP-1-binding sites. Whole-cell extracts from non-transfected subconfluent HT-1080 cells were incubated with 5′-biotinylated double-stranded 25mer oligonucleotides carrying either a consensus or a mutated AP-1-binding site. The oligonucleotides were recovered using streptavidin-conjugated magnetic beads. Bound proteins were visualized by western blot using anti-RHII/Gu or anti-c-Jun antibodies. Similar amounts of starting materials were used in each lane. Both panels show representative examples of two or three experiments with similar results.

between the recombinant proteins is not mediated by contaminating DNA or RNA, and is stable at moderately high ionic strength.

Interaction between endogenous c-Jun and RHII/Gu

After showing that RHII/Gu and c-Jun interact *in vitro* or after overexpression of c-Jun in transfected cells, it was important to show that the interaction between the proteins at their endogenous expression levels also occurs in cells. To this end, we used two independent approaches. First, we performed co-immunoprecipitation studies on nuclear extracts from non-transfected 293 and HT-1080 cells. Immunoblotting confirmed that c-Jun antiserum efficiently co-immunoprecipitated endogenous RHII/Gu and c-Jun from both cell lines, whereas no precipitation of either of the proteins was observed with control pre-immune serum (PI) (Figures 3A and 4B). This experiment demonstrates that c-Jun and RHII/Gu proteins interact in human cells at their endogenous level of expression.

In the next experiment, we used a DNA affinity purification assay to examine whether RHII/Gu interacts with DNA-bound c-Jun. A biotinylated double-stranded DNA oligonucleotide probe, containing a consensus AP-1-binding site, retrieved both c-Jun and RHII/Gu from extracts of non-transfected HT-1080 and 293 cells (Figure 3B and data not shown). In contrast, no significant binding of either c-Jun or RHII/Gu proteins was observed with a control oligonucleotide carrying a mutated AP-1-binding site (Figure 3B). This finding indicates that the binding of c-Jun to DNA and to RHII/Gu is not mutually exclusive, and c-Jun could conceivably deliver RHII/Gu to a transcription unit.

Anisomycin stimulates c-Jun–RHII/Gu interaction

The part of c-Jun that is present in c-Jun$^{1-223}$TAP carries regulatory phosphorylation sites for the MAP kinase JNK. To examine whether the interaction between RHII/Gu and c-Jun might be influenced by the phosphorylation state of the latter, the binding of RHII/Gu to different phosphorylation site mutants of c-Jun$^{1-223}$TAP was examined (Figure 4A). In c-Jun$^{1-223}$AspTAP, the JNK phosphorylation sites were replaced with aspartic acid to create a mimic of phosphorylated c-Jun (Treier *et al*., 1995). A c-Jun$^{1-223}$AlaTAP construct, with alanine substitutions of the phosphorylation sites, was generated in order to create an uninduced, inactive form of c-Jun. 293 cells were transiently transfected with c-Jun$^{1-223}$AlaTAP, c-Jun$^{1-223}$TAP or c-Jun$^{1-223}$AspTAP. In addition, these cells received an expression vector for ΔMEKK1, a constitutively active JNKKK that induces high JNK activity and phosphorylation of both endogenous c-Jun and wild-type c-Jun$^{1-223}$TAP protein (Figure 4A). Probing of the eluate fractions with anti-RHII/Gu serum showed that all three c-Jun$^{1-223}$TAP proteins were expressed and recovered at comparable levels and co-purified with approximately the same amounts of RHII/Gu (Figure 4A, lanes 2–4). This finding indicates that c-Jun phosphorylation does not influence binding to RHII/Gu. However, it does not exclude the possibility that the interaction between the proteins might be regulated by signals that increase the transcriptional activity of c-Jun, in a manner that is not mediated by the c-Jun phosphorylation sites. To test this idea, non-transfected HT-1080 cells were treated with anisomycin and the amount of RHII/Gu in c-Jun co-immunoprecipitates was measured by western blotting. Anisomycin treatment induces potent activation of the JNK pathway and prominent phosphorylation of the immunoprecipitated c-Jun protein (Figure 4B). Interestingly, more RHII/Gu was co-immunoprecipitated with c-Jun from anisomycin-treated cells, whereas the amount of c-Jun in the immunoprecipitate was identical in treated and control cells. These results suggest that anisomycin-induced signaling regulates the interaction between c-Jun and RHII/Gu. To confirm that this effect is independent of c-Jun phosphorylation, as suggested by the experiment shown in Figure 4A, we tested whether anisomycin treatment would also increase RHII/Gu binding to a c-Jun derivative that lacks the relevant phosphorylation sites. For this purpose, we used a stable HT-1080 cell line expressing the c-Jun$^{1-223}$AspTAP fusion protein, and performed TAP purification from untreated or anisomycin-treated cells. As shown in Figure 4C, anisomycin treatment significantly increased binding of endogenous RHII/Gu to c-Jun$^{1-223}$AspTAP, but did not cause increased expression of RHII/Gu protein or an elevated recovery of c-Jun$^{1-223}$AspTAP in the eluate of the affinity column (Figure 4C).

JNK signaling regulates subnuclear localization of RHII/Gu

The results above show that the stoichiometry of interaction between endogenous c-Jun and RHII/Gu proteins can be increased by cellular signaling that induces JNK activity. As this effect does not depend on c-Jun phosphorylation on the known JNK substrate sites, it is possible that it is mediated by a JNK-induced change in the properties of RHII/Gu. RHII/Gu protein has been reported previously to be a predominantly nucleolar protein. Interestingly, treatment of cells with cytotoxic drugs results in translocation of RHII/Gu from nucleolus to

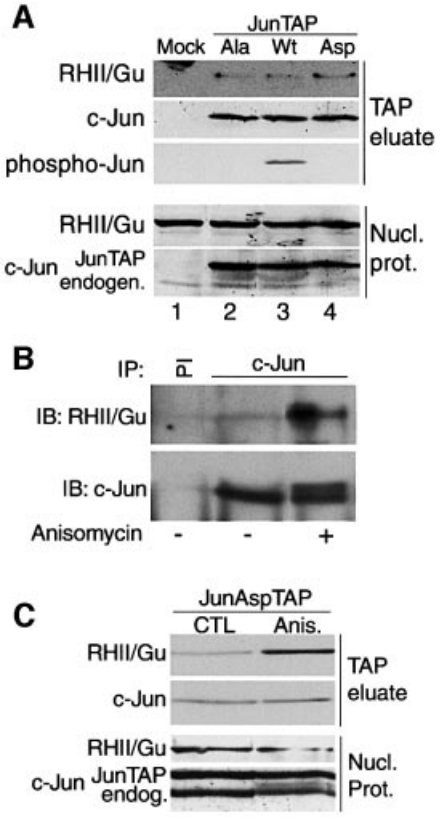

Fig. 4. The interaction between c-Jun and RHII/Gu is increased upon stress signaling. (**A**) Phosphorylation of c-Jun in JNK phosphorylation sites is not required for interaction between c-Jun and RHII/Gu. Subconfluent 293 cells were transiently co-transfected with vectors directing the expression of ΔMEKK1 and different c-JunTAP constructs, containing either wild-type c-Jun sequences (Wt), or derivatives thereof in which the MAP kinase phosphorylation sites at position 63, 73, 91 and 93 were mutated to alanine (Ala) or aspartic acid (Asp) residues. Nuclear proteins were prepared and subjected to IgG affinity purification and TEV cleavage. Aliquots of nuclear proteins and TEV eluates were analyzed by western blot using specific antibodies against human c-Jun or RHII/Gu. (**B**) The interaction between endogenous c-Jun and RHII/Gu proteins is increased upon stress signaling. Nuclear extracts from untreated or anisomycin-treated (5 μg/ml, 1 h) subconfluent HT-1080 cells were subjected to immunoprecipitation with either c-Jun antiserum or rabbit pre-immune serum (PI). The amounts of c-Jun and RHII/Gu in the immunoprecipitates were analyzed by western blotting with RHII/Gu and c-Jun antibodies. Phosphorylation of c-Jun in the anisomycin-treated samples is apparent by the mobility shift of the protein. Similar amounts of starting material were used for each lane. (**C**) Anisomycin-induced increase of RHII/Gu binding is independent of c-Jun phosphorylation state. Subconfluent HT-1080 cells stably expressing c-Jun1–223AspTAP were treated for 1 h with anisomycin (5 μg/ml), or left untreated, and nuclear proteins were subjected to IgG affinity purification and TEV cleavage. The amounts of c-Jun and RHII/Gu proteins in the TEV eluate and nuclear proteins used as a starting material were examined by western blot using RHII/Gu and c-Jun antibodies. Phosphorylation of endogenous c-Jun in the anisomycin-treated samples is discernable by mobility shift of the protein. All panels show representative examples of two or three experiments with similar results.

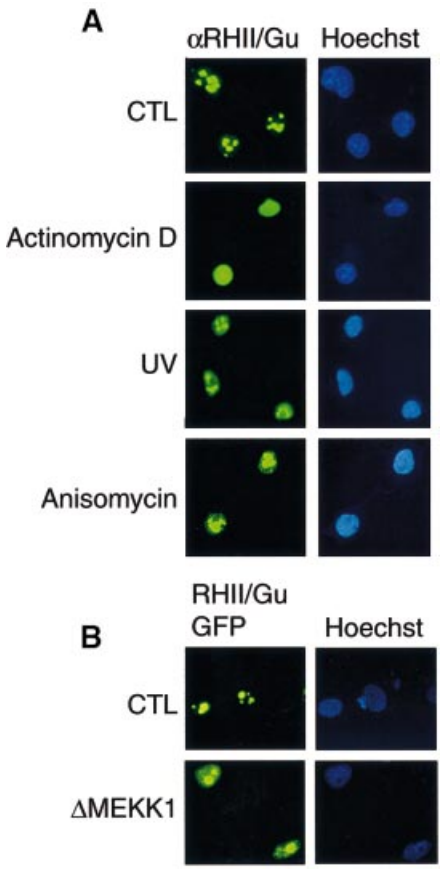

Fig. 5. JNK activity regulates subnuclear localization of RHII/Gu. (**A**) Chemical activators of JNK signaling cause nucleoplasmic retention of RHII/Gu in HT-1080 cells. Serum-starved HT-1080 were left untreated (CTL), treated with anisomycin (5 μg/ml), UVC (20 J/m^2) or actinomycin D (0.2 μM) for 1 h, cells were fixed, immunostained with RHII/Gu antisera and analyzed by fluorescence microscopy. (**B**) Overexpression of constitutively active MEKK1 causes nucleoplasmic retention of RHII/Gu in HT-1080 cells. HT-1080 cells were transiently co-transfected with an expression construct coding for ΔMEKK1 together with RHII/Gu–GFP, and after 24 h cells were serum starved for 12 h, after which subcellular localization of RHII/Gu–GFP fusion protein was studied by fluorescence microscopy. Hoechst staining was used to visualize the morphology of nuclei. Shown are representative images of four experiments showing similar results.

nucleoplasm (Perlaky *et al*., 1997). This suggests that RHII/Gu has both nucleolar and nucleoplasmic functions. Thus, we investigated whether JNK activation might also cause accumulation of RHII/Gu in the nucleoplasm, which would provide an explanation for the JNK-dependent stimulation of c-Jun–RHII/Gu interaction. We found, in addition to the prevalent nucleolar stores, a significant fraction of RHII/Gu in the nucleoplasm of both 293 and HT-1080 cells. Next, we examined if activation of the JNK pathway might cause an enhanced translocation of RHII/Gu into the nucleoplasm. To this end, HT-1080 cells were seeded on glass coverslips and, after treatment with anisomycin (5 μg/ml), UVC (20 J/m^2) or actinomycin D (0.2 μM) for 1 h, cells were fixed and immunostained with RHII/Gu antisera. As reported earlier, actinomycin D treatment induced significant translocation of RHII/Gu from nucleolus to nucleoplasm, resulting in uniform nucleoplasmic staining (Figure 5A). Interestingly, treatment of cells with anisomycin and UVC also increased nucleoplasmic levels of RHII/Gu (Figure 5A).

Even though anisomycin and UVC treatments have been used as prototypical activators of JNK signaling, they influence multiple cellular processes. In order to study specifically the role of JNK signaling in RHII/Gu translocation, HT-1080 cells were transfected with RHII/Gu–green fluorescent protein (GFP) alone or together

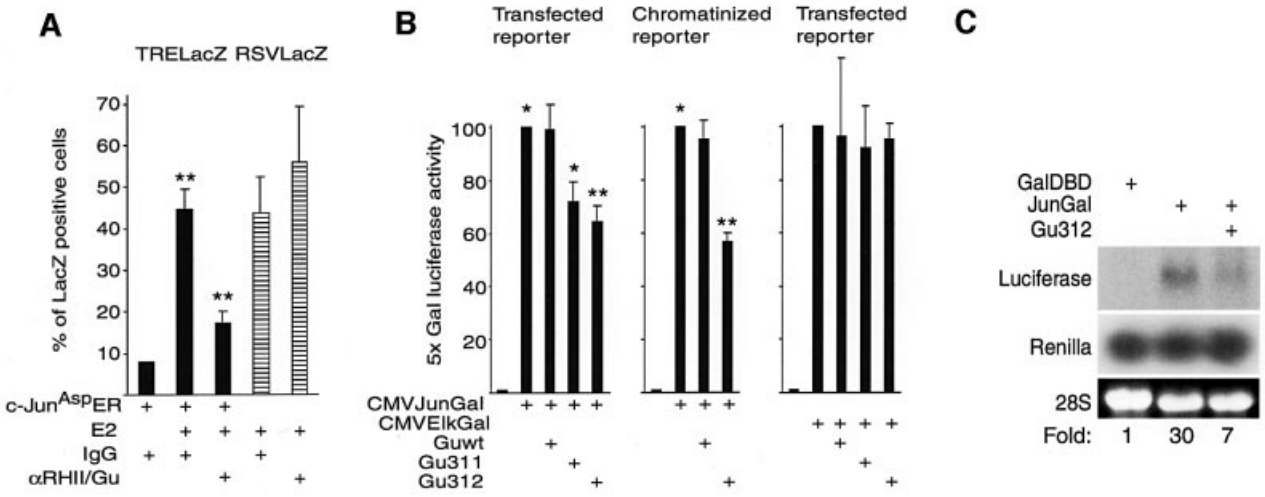

Fig. 6. RNA helicase and ATPase activities of RHII/Gu contribute to transcription regulation by c-Jun. (**A**) A RHII/Gu neutralizing antibody inhibits c-Jun-induced activation of an AP-1 reporter in HT-1080 cells. Subconfluent HT-1080 cells were microinjected with a c-Jun^{Asp}ER expression construct along with either an AP-1 reporter plasmid (TRELacZ) or a non-AP-1 responsive control reporter (RSVLacZ), and either an affinity-purified human RHII/Gu neutralizing antibody (αRHII/Gu) or control human IgG (IgG). At 12 h after injection, cells were treated with 1 μM estradiol (E2) and incubation was continued for 18 h. Thereafter, cells were fixed and stained for β-galactosidase activity. The bar graph represents the percentage of LacZ-positive cells among the injected cells (average ± SD of three independent experiments), $^{**}P < 0.02$ (Student's *t*-test). (**B**) The helicase activity of RHII/Gu is required for stimulation of c-Jun activity. 293 cells were transiently transfected with c-JunGal4 or ElkGal4 expression constructs together with either wild-type (Guwt), helicase mutant (Gu311) or ATPase mutant (Gu312) forms of RHII/Gu (left and right panels). Subconfluent 293 cells carrying a stably integrated 5×Gal4 reporter construct were transiently transfected with c-JunGal4 expression construct alone or in combination with the either wild-type (Guwt) or ATPase mutant (Gu312) form of RHII/Gu (middle panel). Luciferase activity in cell lysates was measured 24 h after transfection. Transfection efficiency was monitored by co-transfecting the cells with a Ubi–*Renilla* luciferase construct. Shown are mean values ± SD of three or four independent experiments, each in duplicate, $^{*}P < 0.05$; $^{**}P < 0.02$ (Student's *t*-test). (**C**) Inhibition of RHII/Gu activity impairs c-Jun-mediated induction of mRNA expression. 293 cells were transiently transfected with the 5×Gal4 reporter construct, the Ubi–*Renilla* expression vector and c-JunGal4 expression construct alone or together with Gu312. After 24 h, cell lysates from parallel samples were prepared and luciferase activity assays were performed along with northern blots to quantitate the abundance of luciferase and *Renilla* luciferase mRNAs. Equal loading of mRNA was confirmed by ethidium bromide staining of the rRNA.

with an expression vector for ΔMEKK1. Activation of JNK signaling by ΔMEKK1 resulted in markedly increased nucleoplasmic staining of RHII/Gu–GFP (Figure 5B). Taken together, these findings provide evidence that in addition to the well-established function of activating c-Jun by direct phosphorylation, JNK also promotes the translocation of RHII/Gu from the nucleolus to the nucleoplasm. This mechanism provides a plausible explanation for the observed stimulation of the c-Jun and RHII/Gu interaction in response to anisomycin-induced JNK signaling.

RHII/Gu contributes to transcription activation by c-Jun

The observation that RHII/Gu preferentially interacts with c-Jun *in vivo* in conditions that coincide with target gene activation raises the possibility that it supports this process. To test this idea, we performed experiments in which HT-1080 cells were microinjected with an expression construct for a c-Jun^{Asp}–estrogen receptor fusion protein (c-Jun^{Asp}ER) and an AP-1-responsive LacZ reporter construct. In addition, the injected cells received an anti-RHII/Gu antibody, which blocks helicase activity, or a control antibody. The c-Jun^{Asp}ER was used for these experiments because it allows a rapid induction of a transcriptionally active c-Jun protein without engaging other signaling pathways. Treatment of c-Jun^{Asp}ER-expressing cells with 1 μM estradiol, 12 h after injection, significantly increased the fraction of β-galactosidase-positive cells (Figure 6A), indicating that activation of the c-Jun protein stimulated the TRELacZ reporter. Estradiol treatment had no effect on TRELacZ reporter activity in cells that did not express c-Jun^{Asp}ER (data not shown). Significantly, when the RHII/Gu neutralizing antibody was co-injected, c-Jun^{Asp}ER-dependent TRELacZ promoter activation was essentially abolished. The suppressive effect of the RHII/Gu neutralizing antibody was not due to a general suppression of transcription, as it did not interfere with the activity of an RSVLacZ reporter, which is insensitive to changes in AP-1 activity (Figure 6A). This is strong evidence that the endogenous RHII/Gu in the injected cells supports transcriptional activation by c-Jun.

The neutralizing RHII/Gu antibody that interfered with c-Jun-dependent transcription specifically inhibits the RNA helicase activity of RHII/Gu (Valdez *et al*., 1997). This suggests that the helicase activity of RHII/Gu is required for c-Jun-mediated transcriptional activation. To address the role of helicase function of the RHII/Gu in c-Jun-mediated transcriptional activation more specifically, we generated mammalian expression vectors coding for full-length RHII/Gu in which either the helicase or the ATPase domain of RHII/Gu was inactivated by point mutations. We have shown previously that both mutations completely abolish the RNA helicase activity of RHII/Gu *in vitro* (Valdez *et al*., 1997). To study the effects of the mutants on c-Jun-mediated promoter activation, we used

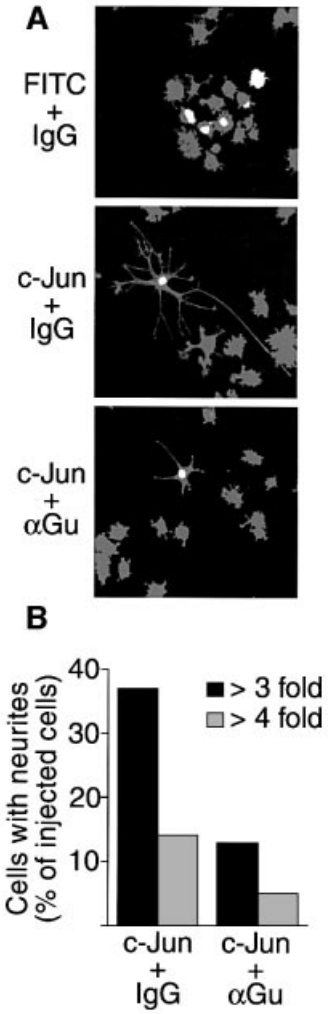

Fig. 7. RHII/Gu activity supports c-Jun-mediated PC12 cell differentiation. (**A**) Neurite outgrowth in microinjected PC12 cells. PC12 cells were microinjected with FITC-labeled dextran or HA-c-Jun^{Asp} expression construct together with control IgG or RHII/Gu blocking antibodies (αGu) as indicated. After 48 h, cells were fixed and cells expressing HA-c-Jun^{Asp} were identified by staining with anti-HA antibody and FITC-labeled secondary antibody (bright nuclei). The morphology of the cells was visualized by actin staining. (**B**) Quantification of neurite outgrowth. The percentage of HA-c-Jun^{Asp}-injected cells with neurites 3- or 4-fold the cell length among microinjected (FITC-positive) cells is shown. The graph is a representative example out of three experiments with similar results.

constructs in which the N-terminal transactivation domain of c-Jun was fused to the Gal4 DNA-binding domain. The effects of the overexpressed proteins were determined by measurement of 5×UAS luciferase reporter activity. Overexpression of wild-type RHII/Gu had no effect on c-JunGal4-mediated promoter activation (Figure 6B), probably due to a high abundance and non-limiting concentration of the endogenous RHII/Gu in these cells. In accordance with the results of the microinjection experiment, both the helicase mutant (Gu311) and the ATPase mutant (Gu312) forms of RHII/Gu, however, significantly suppressed the c-JunGal4-induced promoter activation (P-values <0.05 and <0.02, Figure 6B). Importantly, overexpression of the wild-type and mutant forms of RHII/Gu had no significant effect on the basal activity of the 5×UAS luciferase reporter (data not shown) or when the luciferase reporter was activated by Gal4-Elk1, a derivative of a transcription factor that is unrelated to c-Jun (Figure 6B). These results suggest that the helicase activity of RHII/Gu specifically facilitates c-Jun-mediated transcription.

To investigate whether the results of the transfection experiments could be repeated in a chromatin context and were not influenced by the episomal state of the reporter constructs, we expressed c-JunGal4 alone or together with wild-type and the Gu312 mutant of RHII/Gu, in a 293 cell line bearing a genomically integrated 5×UAS luciferase reporter. In these cells, a similar significant suppressive effect of Gu312 on c-JunGal4-induced reporter activation was observed (P-value <0.02, Figure 6B).

In addition to the proposed role of DEAD-box RNA helicases in transcription, some of these enzymes promote mRNA translation (Chuang *et al*., 1997; Luking *et al*., 1998; Linder and Daugeron, 2000). We wanted to determine whether RHII/Gu might affect reporter expression at the translational rather than the transcriptional level. 293 cells were transfected with the 5×UAS luciferase reporter, along with c-JunGal4 in the absence or presence of the RHII/Gu mutant Gu312, and both luciferase activity and luciferase mRNA expression were determined from parallel samples. As before, Gu312 inhibited c-JunGal4-dependent induction of luciferase activity (Figure 6B). Furthermore, Gu312 expression significantly (by 75%) decreased the abundance of luciferase mRNA as measured by northern blots (Figure 6C), indicating that helicase activity of RHII/Gu is involved in the transcriptional regulation of gene expression. In accordance with previous experiments, inhibition of helicase activity of RHII/Gu did not have any non-specific inhibitory effect on the expression of ubiquitin promoter-driven *Renilla* luciferase mRNA, which was transcribed from an internal control vector (Figure 6C).

RHII/Gu promotes c-Jun-mediated neuronal differentiation of PC12 cells

We wanted to study whether RHII/Gu is required for regulation of complex cellular programs induced by c-Jun. c-Jun potently induces neuronal differentiation of PC12 cells (Leppä *et al*., 2001). To study whether RHII/Gu activity is involved in this differentiation function, we performed an experiment in which PC12 cells were microinjected with an expression vector for hemagglutinin (HA) epitope-tagged c-Jun together with either a control antibody or a neutralizing antibody against RHII/Gu. At 48 h after injection, the effect of the RHII/Gu antibody on c-Jun-mediated neuronal differentiation of the PC12 cells was measured. Injection of PC12 cells with control antibody alone did not have any effect on the cell phenotype, whereas prominent neurite outgrowth was observed in cells injected with c-Jun expression construct together with control antibody (Figure 7A). Remarkably, injection of the RHII/Gu neutralizing antibody strongly decreased c-Jun-induced neurite outgrowth, showing that RHII/Gu activity is required for induction of the neuronal differentiation program initiated by c-Jun in these cells (Figure 7A and B). Importantly, comparable numbers of HA epitope-positive cells were observed in both groups of cells (data not shown), indicating that the RHII/Gu

antibody did not have adverse effects on the survival of injected PC12 cells. These results provide further evidence in support of a contribution of endogenous RHII/Gu to c-Jun-mediated cellular processes and imply that RHII/Gu supports the activation of endogenous c-Jun target genes, in addition to synthetic reporter genes.

Discussion

Many fundamental functions of the eukaryotic cell such as transcription initiation, splicing, replication and DNA repair are catalyzed by multiprotein complexes consisting of many dozens of subunits with enzymatic as well as regulatory functions (Hirose and Manley, 2000; Lemon and Tjian, 2000). To learn more about the biology of these processes and the multiprotein complexes involved, the identification of their subunits and the characterization of their interactions is essential. The data presented here document how the TAP technology that was developed initially to characterize protein interactions in yeast can be adapted successfully for transiently and stably transfected mammalian tissue culture systems. Based on its generic nature (Rigaut *et al*., 1999; Puig *et al*., 2001), this method should be widely applicable to the identification and characterization of known or suspected protein–protein interactions in higher eukaryotic cells.

We have identified a group of proteins that bind to c-Jun and provide evidence that the DEAD-box RNA helicase RHII/Gu is a novel transcriptional co-activator of c-Jun. Several of the other proteins identified as c-Jun interaction partners have established or supposed roles in the transcriptional regulation of gene expression, making it plausible that we have identified a set of proteins that facilitate c-Jun-mediated promoter activation and mRNA synthesis.

The data presented in this study indicate that the RNA helicase activity of RHII/Gu is involved specifically in the stimulation of c-Jun-mediated reporter activation and for activation of endogenous c-Jun targets during PC12 cell neuronal differentiation. The fact that the substrate specificity of RHII/Gu is limited to RNA–RNA and RNA–DNA duplexes suggests that it acts at a step of transcription different from DNA helicases such as NDHII (Nakajima *et al*., 1997; Eisen and Lucchesi, 1998). Based on our reporter gene assays, the RNA helicase activity of RHII/Gu facilitates efficient mRNA synthesis but, in contrast to other RNA helicases such as eIF-4A and the yeast RNA helicase Ded1p (Chuang *et al*., 1997; Linder and Daugeron, 2000), it appears not to be involved in translational regulation of gene expression. In addition, since the reporter genes used in this study do not contain introns, it is evident that the stimulatory effect of RHII/Gu on their expression cannot be explained by effects at the level of pre-mRNA splicing as has been shown for yeast DEAD-box PRP proteins (Luking *et al*., 1998). Furthermore, the finding that overexpression of mutant forms of RHII/Gu equally potently suppressed c-Jun-mediated transcriptional activation in both an episomal and a chromatin context suggests that RHII/Gu is not involved in chromatin decondensation.

The mechanism by which RHII/Gu participates in c-Jun-stimulated mRNA synthesis remains a matter of speculation at this point. Interestingly, it was shown recently that the vaccinia virus DEXD/H protein NPH-II can displace protein from RNA (Jankowsky *et al*., 2001). This might suggest an additional mechanism to explain how these proteins could influence gene transcription, for example by facilitating promoter clearance. Further conceivable possibilities for a role of RHII/Gu in support of c-Jun include assisting in the packaging of the RNA into hnRNPs or even a function in transcription-coupled DNA repair or the removal of a stalled polymerase from damaged templates.

Our data show that the binding of RHII/Gu to c-Jun increases in response to anisomycin treatment by a c-Jun phosphorylation-independent mechanism. This observation could be explained by the JNK-regulated translocation of RHII/Gu from nucleoli to nucleoplasm. Such a model would not be without precedent. In addition to being a site for rRNA synthesis and assembly, the nucleolus is now recognized as a store for proteins with latent nucleoplasmic functions (e.g. Mdm2; Visintin and Amon, 2000). It has been established that nucleolar proteins may shuttle continuously between nucleolus and nucleoplasm (Phair and Misteli, 2000). Interestingly, the C-terminal domain of RHII/Gu that binds to c-Jun also serves as a nucleolar localization signal for RHII/Gu (Ou *et al*., 1999), indicating that the interaction between RHII/Gu and c-Jun might prevent relocalization of RHII/Gu to the nucleolus.

A final point of note is that both c-Jun and RHII/Gu have been shown to be necessary for cell growth and/or survival (Ou *et al*., 1999; Davis, 2000). These findings indicate that the interaction between RHII/Gu and c-Jun might be required for the regulation of c-Jun target genes important for cell cycle progression. Further studies will be required to elucidate a potential function of interaction between c-Jun and RHII/Gu in cell growth and transformation.

Materials and methods

Plasmids and construction of expression vectors

Bacterial expression plasmids for His_6-tagged c-Jun (Treier *et al*., 1994), mammalian HA-tagged c-Jun^{Asp} (Treier *et al*., 1995), constitutively active ΔMEKK1 (Whitmarsh *et al*., 1995), GST–RHII/Gu fusion proteins (Valdez *et al*., 1996) and reporter plasmids RSVLacZ and TRELacZ (Yeung *et al*., 1999) have been described. Information about cloning of the mammalian expression constructs for RHII/Gu mutants Jun^{Asp}ER and c-$Jun^{1–223}$TAP is available on request.

TAP purification

For TAP purification experiments, subconfluent 293 cells were transiently transfected by the calcium phosphate method with 5 μg of CMVJunTAP constructs, 2 μg of ΔMEKK1 or 7 μg of empty cytomegalovirus (CMV)-driven expression construct per 10 cm plate. c-JunTAP complexes were purified using a published procedure (Rigaut *et al*., 1999; Puig *et al*., 2001) with minor modifications. High salt nuclear extractions from mock- or $Jun^{1–223}$TAP-transfected cells were prepared as described (Andrews and Faller, 1991) and subsequently adjusted to IgG-binding conditions: 180 mM NaCl, 10 mM Tris–HCl pH 8.0, 0.2% NP-40, 0.5 mM dithiothreitol (DTT), complete protein inhibitors (Roche), 10 mM β-glycerolphosphate and 20 mM NaF. Diluted extracts were rotated overnight at 4°C with 100 μl of IgG matrix (Amersham Biotech), after which the beads were washed extensively in binding buffer. Washed beads were resuspended in TEV cleavage buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.3% NP-40, 0.5 mM EDTA, 0.5 mM DTT), and 5–15 μl of recombinant TEV enzyme (150 U; Life Technologies) was added to the mixture. After rotation for 2 h at 16°C, the TEV eluate from the IgG column was recovered and adjusted to calmodulin-binding conditions: 45 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.7 mM Mg-acetate, 0.7 mM imidazole, 2.5 mM $CaCl_2$, 0.2 % NP-40, 10 mM

β-mercaptoethanol, and rotated for 2 h at 4°C with 50 µl of calmodulin affinity resin (Stratagene). After binding, sedimented beads were washed extensively with calmodulin-binding buffer. Bound proteins were recovered by boiling the calmodulin beads for 3 min in protein sample buffer and loaded onto a preparative 12% SDS–polyacrylamide gel. Proteins were detected by silver staining (Shevchenko *et al*., 1996).

Peptide sequences of c-Jun-specific interaction partners were identified as described previously (Bouveret *et al*., 2000) except that a Q-Tof mass spectrometer (Micromass) was used for peptide sequencing by tandem mass spectrometry. Protein bands were excised from the silver-stained gel, reduced, alkylated and digested overnight with trypsin, and peptide sequences were obtained by nano-electrospray tandem mass spectrometry and by comparison with a comprehensive non-redundant protein database using the program PeptideSearch.

For western blot analysis, aliquots of nuclear proteins or TEV eluates after IgG affinity purification and TEV cleavage were loaded onto 12% SDS–polyacrylamide gels and transferred to nitrocellulose membrane.

GST pull-down experiments

Pull-down assays with purified $JunHis_6$, a histidine-tagged recombinant full-length c-Jun, were performed by incubating 1 µg of soluble $JunHis_6$ protein with molar equivalent amounts (1–3 µg) of GST–RHII/Gu deletions immobilized on glutathione–Sepharose beads in 0.5 ml of buffer containing 20 mM Tris–HCl pH 7.4, 0.2 mM EDTA, 0.1 M NaCl and 1 mM DTT. In experiments in which nuclear proteins containing c-Jun were used, nuclear proteins were prepared from 293 cells (Andrews and Faller, 1991), adjusted to the binding conditions described above. In experiments in which ethidium bromide, RNase A or higher concentrations of NaCl were used, these reagents were added to the binding reaction prior to addition of the recombinant proteins. Bound proteins were boiled in sample buffer, resolved by SDS–PAGE and immunoblotted with anti-GST and anti-c-Jun antibodies.

Co-immunoprecipitations and DNA affinity purification assay

Protein G–Sepharose (Pharmacia) beads were agitated on a tumbler for 2 h at 4°C with c-Jun antiserum or with rabbit pre-immune serum in 20 mM HEPES–KOH pH 7.5, 400 mM NaCl, 0.25 mM EGTA, 1.5 mM $MgCl_2$, 0.25% NP-40, protease inhibitors (Roche), 10 mM β-glycerolphosphate, 20 mM NaF and 0.5 mM DTT. Before co-immunoprecipitation, 293 and HT-1080 nuclear extracts were sonicated and pre-cleared for 2 h at 4°C with rabbit pre-immune serum immobilized to protein G–Sepharose (Pharmacia) beads, and supernatants from the pre-cleared samples were incubated with immobilized c-Jun antiserum or control rabbit pre-immune serum overnight at 4°C. Thereafter, sedimented beads were washed four times with 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.3% NP-40 and 0.5 mM DTT, and bound proteins were analyzed by western blotting using mouse anti-c-Jun or human anti-RHII/Gu antibodies. For the DNA affinity purification assay, HT-1080 cells were lysed in RIPA buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors (Roche), 10 mM β-glycerolphosphate, 20 mM NaF, 0.5 mM DTT]. The cell lysates were then sonicated and incubated with 3 µg of biotinylated double-stranded oligonucleotides at 4°C for 3 h. Oligonucleotide–protein complexes were recovered using streptavidin-conjugated magnetic beads (Dynal) and washed extensively. The following double-stranded oligomers containing either wild-type or mutated AP-1-binding sites (underlined) were used: (AP-1wt) 5′-biotinTAAAGCATGAGTCAGACACCTCTG-3′ or (AP-1mut) 5′-biotinTAAAGCAGGGCCTCAGACACCTCTG-3′.

Proteins bound to DNA were resolved by SDS–PAGE and analyzed by western blotting using c-Jun and RHII/Gu antibodies.

Reporter gene assays

293 cells were transfected at 50–70% confluency by the calcium phosphate method with 1 µg of reporter plasmid pf2Luc (Stratagene) together with CMVGal4DBD, CMVJunGal4 (Stratagene), CMVElk1Gal4 (pFA2-Elk1, Stratagene) (10 ng each), pHook-Flag-Gu, pHook-FLAG-Gu311 or pHook-FLAG-Gu312 (150 ng each) expression constructs. Control cultures were co-transfected in parallel with equivalent amounts of the corresponding empty expression vector. Transfection efficiency was controlled by co-transfection with 10 ng of ubiquitin promoter-driven *Renilla* luciferase reporter construct. At 24 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and lysed with passive lysis buffer (Promega). Firefly luciferase and *Renilla* luciferase activities were measured separately.

Microinjection reporter assays

HT-1080 cells were seeded on CELLocate coverslips (Eppendorf) to facilitate identification of control IgG and RHII/Gu neutralizing antibody-injected cells after staining. A total of 100–150 cells were co-injected with the $Jun^{Asp}ER$ expression construct (100 µg/ml) and TRELacZ reporter construct (200 µg/ml) or with RSVLacZ control vector (5 µg/ml) together with 90 ng/µl of either purified human IgG or affinity-purified human RHII/Gu neutralizing antibody (Valdez *et al*., 1996, 1997). At 12 h after microinjection, cells were treated with 1 µM estradiol and incubation was continued for 18 h. Cells were washed thereafter with PBS, fixed with 2% paraformaldehyde (PFA) and LacZ stained as described previously (Rose *et al*., 1999). Cells were inspected by light microscopy and the injected cells that exhibit blue staining were counted as positive.

PC12 cell differentiation assay

PC12 cells were microinjected and stained as described previously (Leppä *et al*., 2001). Briefly, 100–150 PC12 cells were injected with CMV HA $c\text{-}Jun^{Asp}$ expression construct (Treier *et al*., 1995) together with 90 ng/µl of purified human IgG or affinity-purified human RHII/Gu neutralizing antibody (Valdez *et al*., 1997). Control cells were injected with purified human IgG together with fluorescein isothiocyanate (FITC)-labeled dextran (Molecular Probes). At 48 h after injection, cells were fixed with 2% PFA, and nuclear expression of $HAc\text{-}Jun^{Asp}$ was visualized by staining of cells with a monoclonal anti-HA antibody (Roche) and FITC-conjugated secondary antibody (Dianova). The morphology of cells was visualized using tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma). For visualization of the cells and quantification of neurite outgrowth, mounted samples were examined using a Zeiss LSM410 confocal imaging system.

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