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Heterogeneous Nuclear Ribonuclear Protein U Associates with YAP and Regulates Its Co-activation of Bax Transcription*

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Although initially described as a cytosolic scaffolding protein, YAP (Yes-associated protein of 65 kDa) is known to associate with multiple transcription factors in the nucleus. Using affinity chromatography and mass spectrometry, we show that YAP interacts with heterogeneous nuclear ribonuclear protein U (hnRNP U), an RNA- and DNA-binding protein enriched in the nuclear matrix that also plays a role in the regulation of gene expression. hnRNP U interacts specifically with the proline-rich amino terminus of YAP, a region of YAP that is not found in the related protein TAZ. Although hnRNP U and YAP localize to both the nucleus and the cytoplasm, YAP does not translocate to the nucleus in an hnRNP U-dependent manner. Furthermore, hnRNP U and YAP only interact in the nucleus, suggesting that the association between the two proteins is regulated. Co-expression of hnRNP U attenuates the ability of YAP to increase the activity of a p73-driven Bax-luciferase reporter plasmid. In contrast, hnRNP U has no effect when co-expressed with a truncated YAP protein lacking the hnRNP U-binding site. Because YAP is distinguished from the homologue TAZ by its proline-rich amino terminus, the YAP-hnRNP U interaction may uniquely regulate the nuclear function(s) of YAP. The YAP-hnRNP U interaction provides another mechanism of YAP transcriptional regulation.

Gene expression is regulated through a variety of mechanisms including the modulation of transcription factor localization, post-translational processing, and turnover (1–3). In addition, transcription factors may associate with a large number of protein co-factors that either enhance or repress transcriptional activity in a cell type-specific context (4, 5). These associations function in part to localize transcription factors to specific protein complexes and can recruit proteins of the basal transcription machinery. In addition, specific protein interactions can modulate the localization of transcription factors in the nucleus or cytoplasm and play an important role in the integration of signal transduction cues.

One protein recently shown to function as a modulator of multiple transcription factors in a variety of cell types is YAP (Yes-associated protein of 65 kDa). YAP is a modular adaptor protein with multiple protein interaction domains that was originally isolated based on its ability to bind the SH3 domain of the Src family kinase c-Yes (6). In addition to its SH3-binding motif, YAP contains a proline-rich amino terminus, a 14-3-3-binding site, one (YAP1) or two (YAP2) WW domains (hereafter, YAP will refer to YAP1), a coiled-coil, and a PDZ interaction motif at the extreme COOH terminus (7–9). Although initially thought to play a role in the anchoring and or targeting of c-Yes to specific subcellular compartments, many studies suggest that YAP plays a role in the regulation of gene expression through its ability to associate with a broad array of transcription factors (7, 10–14). These interactions typically involve either of the two YAP WW domains that associate directly with PPxY motifs within the transcription factors, but other mechanisms of interaction also occur (7, 11–14). In a two-hybrid screen, YAP was found to associate with PEBP2α (polyomavirus enhancer-binding protein 2), a transcription factor important for hematopoiesis and osteogenesis (14, 15). *In vivo*, YAP enhanced the transcriptional activity of PEBP2α, and this enhancement required an association of the YAP WW domain with the PPxY motif of the transcription factor. Likewise, overexpression of YAP increased the transcriptional activity of the TEAD/TEF family of transcription factors and endogenous YAP co-purified with overexpressed TEAD-2 in a cell culture model system (12). The long forms of p73, homologues of the p53 tumor-suppressor gene, also interact with YAP via its WW domain (10, 13). Like p53, p73 proteins can induce several anti-proliferative effects, such as cell cycle arrest, apoptosis, and cellular differentiation (16–19). However, the mechanisms underlying p73 protein stability are distinct from p53; the two proteins differentially regulate gene expression (20, 21) and have distinct binding partners (22, 23). Co-expression of YAP enhanced transcription of Bax and mdm2 by p73α and p73β but had no effect on p53-mediated gene transcription (13).

Although YAP can modulate the activity of multiple transcription factors, several studies suggest that it localizes predominantly in the cytoplasm. For example, in airway epithelial cells, YAP associates via its PDZ interaction motif with EBP50 (ezrin/radixin/moesin-binding phosphoprotein of 50 kDa), a scaffolding protein localized predominantly to the apical membrane (8). YAP also associates with cytoplasmic 14-3-3 proteins (10) involved in multiple cellular processes such as signal transduction, cell cycle control, and apoptosis (for reviews see Refs. 24 and 25). 14-3-3 binds phosphorylated serine or threonine residues on target proteins, often sequestering the protein or maintaining it in an inactive state (26, 27). In the case of YAP, binding by 14-3-3 sequesters YAP in the cytoplasm and prevents it from entering the nucleus (10). The 14-3-3-binding site on YAP is also a phosphorylation site for Akt; phosphorylation by Akt attenuates p73-mediated transcription by YAP in U2OS cells (10).

YAP interacts with multiple proteins in the nucleus and in

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the cytoplasm and is an adaptor protein with many potential functions *in vivo*. Many of these functions are likely shared by a paralogue of YAP called TAZ (transcriptional co-activator with a PDZ-binding motif). The two human proteins are ~45% identical and share many functional sequence domains and motifs (28). TAZ also binds 14-3-3 and was originally identified in a screen for novel 14-3-3-binding proteins (28). In addition, TAZ and YAP both bind E3KARP (NHE3 kinase A regulatory protein), a closely related member of the EBP50 family (29). Furthermore, the predicted ligand-binding sequence of the TAZ WW domain is similar to that of the YAP WW domain, and TAZ also functions to enhance the transcriptional activity of PEBP2α *in vivo* (28). Thus the two proteins likely share many overlapping functions. Although the two proteins differ in their ability to bind c-Yes (28), another difference between YAP and TAZ is that YAP contains a proline-rich amino terminus that is not found in TAZ. To identify protein interactions in this region, we used fusion proteins made to the first 57 amino acid sequence of human YAP in biochemical screens to identify novel YAP-associated proteins. We find that the proline-rich amino terminus of YAP associates with heterogeneous nuclear ribonucleoprotein U (hnRNP U),[1] a protein that is enriched in the nuclear matrix and has been shown to regulate gene transcription (30–33). hnRNP U binds conserved AT-rich regions within the genome called scaffold attachment regions, which allow chromatin to attach to the nuclear matrix and form ~5–200-kb loops (34). Indeed, hnRNP U was identified independently as a protein that was capable of binding these scaffold attachment region elements and named scaffold attachment factor A (35). The interaction of YAP and hnRNP U expands the association of YAP with nuclear proteins beyond those that function as transcription factors and provides an alternate mechanism whereby YAP may modulate gene expression in cells.

MATERIALS AND METHODS

Cell Culture and Immunofluorescence—The cells were maintained as described previously (8, 36). Immunofluorescence and confocal microscopy were performed as described previously (8, 36) using the antibodies and dilutions described below.

Antibodies and Reagents—YAP antisera were generated in rabbits using GST-tagged human YAP(274–454) as immunogen. Recombinant fusion protein was purified from the soluble fraction of bacterial lysates on glutathione agarose beads, and the purified protein was injected into two New Zealand White rabbits (Covance, Princeton, NJ). Serum (NC252) was further purified on DEAE ion exchange and GST-YAP(274–454) affinity columns and used for Western blotting (1:1000), immunofluorescence (1:500), and immunoprecipitation (2 μg). The anti-hnRNP U monoclonal antibody 3G6 was a gift from Dr. Gideon Dreyfuss (University of Pennsylvania) and used at 1:20000 dilution. All other antisera were obtained from commercial sources.

The pGL3-Bax luciferase construct was a gift from Dr. Giovanni Blandino from the Regina Elena Cancer Institute (Rome, Italy). A β-actin-LacZ plasmid expressing β-galactosidase under control of human β-actin promoter was a gift from Dr. Keith Yamamoto (University of San Francisco), and the triple-tandem GFP (3XGFP) vector was a gift from Dr. Yuri Lazebnik (Cold Spring Harbor Laboratory).

cDNAs encoding full-length human YAP and full-length human hnRNP U were generated by PCR and cloned into the SalI and NotI sites of pCMV.HA (Clontech, Palo Alto, CA). Plasmids containing the amino terminus (amino acids 1–57) and WW1 domain (amino acids 162–217) of human YAP fused to GST were generated by PCR and cloned into the SalI and NotI sites of pGEX.5X2 (Amersham Biosciences). cDNAs encoding fragments of YAP for expression in mammalian cells were generated by PCR and cloned into the BamHI site of the 3XGFP plasmid. The sequences of all plasmids were verified at the University of North Carolina sequencing facility.

Affinity Purification of YAP-interacting Proteins and Immunoprecipitation Analysis—The expression of GST, GST-YAP(1–57), and GST-YAP(162–217) fusion proteins was induced for 3 h with 1 mM isopropyl-β-D-thiogalactopyranoside at 25 °C in terrific broth. The proteins were harvested by sonication in phosphate-buffered saline containing 0.5% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg of leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml benzamidine. The soluble fusion protein was purified on glutathione-Sepharose beads, and initial pull-down assays were performed using 20 μg of purified fusion protein. The cell lysates were prepared from a single 100-mm dish of 16HBE14o− cells labeled with [^{32}P]orthophosphate for 3 h in serum-free and phosphate-free Dulbecco's modified Eagle's medium (Sigma). The cells were lysed in RIPA buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), and the lysates were diluted 1:5 in binding buffer (50 mM Tris, pH 7.0, 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA). The assays were performed as described previously (8, 37), and the proteins bound to GST, GST-YAP(1–57), and GST-YAP(162–217) were compared by SDS-PAGE and visualized by silver staining (Amersham Biosciences) or PhosphorImager analysis. For mass spectrometric analysis, 100 μg of GST and GST-YAP(1–57) were used in pull-down assays as described above except that the cell lysate was prepared from 30 × 100-mm dishes of 16HBE14o− cells. After extensive washing, the bound fraction was analyzed by SDS-PAGE, and the proteins were visualized with 0.01% colloidal Coomassie in 10% acetic acid. The visible bands were excised, and the samples were analyzed by MALDI-TOF-MS (Bruker Instruments Co., Bremen, Germany) and Nano-ESI-MS/MS on an API QSTAR-Pulsar (QSTAR, Applied Biosystems Div., Perkin-Elmer Corp., Foster City, CA) as described (38).

For immunoprecipitations, HeLa cells were transfected for 18 h with plasmid vectors encoding HA-YAP. The cells were washed in phosphate-buffered saline, scraped into 1 ml of phosphate-buffered saline and centrifuged 1000 × *g* for 4 min. Cytosol was extracted with cytoplasmic extraction buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.2% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 75 s. The nuclei were collected by centrifugation at 1000 × *g* for 5 min and washed three times in cytoplasmic extraction buffer without detergent. Nuclear proteins were extracted for immunoprecipitation using nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) for 10 min. Total nuclear proteins were extracted by resuspending nuclei in RIPA buffer for 10 min. Then nuclear and cytoplasmic fractions were diluted 1:5 in binding buffer, and 2 μg of affinity-purified YAP antisera was added to each fraction and tumbled 1 h at 4 °C. The samples were collected on protein A beads, washed, and solubilized in Laemmli sample buffer for SDS-PAGE and Western blot analysis.

Luciferase Assays—The cells were transfected with pGL3-Bax luciferase and Lac-Z reporter plasmids either alone or with plasmids encoding HA-YAP and/or HA-hnRNP U as noted in the figure legends. Empty pCMV-HA vector was transfected to normalize the amount of DNA used in each sample, and β-galactosidase enzyme assays (Promega, Madison, WI) were performed to normalize transfection efficiencies across samples. The cells were harvested in passive lysis buffer (Promega, Madison, WI) for 18 h post-transfection, and the lysates were analyzed using a Molecular Devices luminometer (Molecular Devices, Sunnyvale, CA). Statistical significance was determined by Student's *t* test.

RESULTS

YAP Associates with hnRNPU in Vitro—At the amino acid level human YAP shares 45% sequence identity and 57% homology with its paralogue TAZ. One obvious difference at the level of primary structure is a proline-rich amino terminus found only in YAP (Fig. 1*A*). To identify proteins that associate with YAP via this unique region, we immobilized 20 μg of GST, GST-YAP(1–57), or GST YAP(162–217) on glutathione-agarose beads and incubated the fusion proteins with cell lysates prepared from 16HBE14o− cells labeled with [^{32}P]orthophosphate. A unique 120-kDa phosphoprotein bound specifically to GST-YAP(1–57) but not to GST alone or GST-YAP(162–217) (Fig. 1*B*). We also analyzed proteins bound to GST or GST-YAP(1–57) by silver staining (Fig. 1*C*). The 120-kDa protein bound to GST-YAP(1–57) but not to GST alone, and the interaction was abolished when the affinity matrix was washed in

[1] The abbreviations used are: hnRNP U, heterogeneous nuclear ribonuclear protein U; Pol II, RNA polymerase II; CTD, carboxyl-terminal domain; CTF, COOH-terminal fragment; GST, glutathione *S*-transferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; HA, hemagglutinin; GFP, green fluorescent protein.

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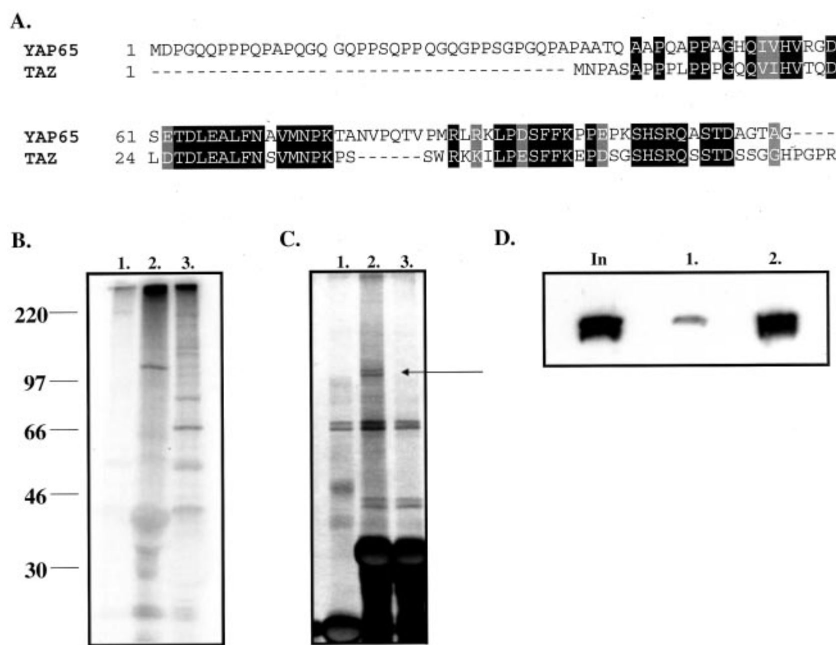

FIG. 1. **hnRNP U binds the amino terminus of YAP.** *A*, sequence analysis of the amino-terminal domains of human YAP and TAZ. Identical amino acids are *shaded black*, whereas conservative substitutions are *shaded gray*. *B*, cells were labeled for 3 h with [^{32}P]orthophosphate, lysed in RIPA buffer, and then diluted 1:5 in binding buffer as described under "Materials and Methods." The lysates were incubated with GST alone (*lane 1*), GST-YAP(1–57) (*lane 2*), or GST-YAP(162–217) (*lane 3*) for 1 h and then washed in binding buffer. The samples were resolved on 9% SDS-PAGE gels and applied to PhosphorImager screens. *C*, cell lysates were prepared as described in *B* and incubated with GST (*lane 1*), GST-YAP(1–57) (*lane 2*), or GST-YAP(1–57) with 500 mM NaCl wash (*lane 3*). The bound proteins were analyzed on 9% SDS-PAGE gels and visualized with silver staining. The *arrow* indicates proteins identified by mass spectrometry. *D*, cell lysates were prepared as described in *B* and incubated with GST (*lane 1*) or GST-YAP(1–57) (*lane 2*). The bound proteins were fractionated on 9% SDS-PAGE gels, transferred to Immobilon-P, and blotted with a monoclonal antibody against hnRNP U. A shorter exposure indicated that the monoclonal antibody recognized both bands of the doublet visualized in *B*. *In*, input, representing 20% of the total cell lysate in each assay. All of the gels are representative of two to four replicates with identical results.

buffers containing 500 mM NaCl to disrupt ionic interactions (Fig. 1*C*, *arrow*). Using MALDI-TOF and electrospray tandem MS/MS, we identified the interacting protein as hnRNP U (also known as scaffold attachment factor A). To confirm the mass spectrometry analysis, we performed identical pull-down assays using GST or GST-YAP(1–57) and analyzed the bound fractions using a monoclonal antibody specific for hnRNP U (33). As predicted by the mass spectrometry analysis, hnRNP U specifically bound the affinity resin containing the GST-YAP(1–57) fusion protein but did not associate with GST alone (Fig. 1*D*). To determine whether an indirect association of YAP and hnRNP U is mediated by nucleic acids, we repeated the same experiments in the presence of RNase and DNase but found that GST-YAP(1–57) still bound hnRNP U under these conditions (not shown). Thus we conclude that nucleic acids do not indirectly mediate the association of YAP and hnRNP U.

YAP and hnRNP U Co-localize in the Nucleus—For YAP and hnRNP U to interact *in vivo*, they must co-localize, at least in part, to the same subcellular compartment. Although YAP has been described as a transcriptional co-activator based on its ability to associate directly with multiple transcription factors *in vitro* (11–14), the localization of endogenous YAP has not been extensively studied. In polarized epithelial cells, YAP localizes primarily to the apical membrane, and no protein was detected in the nucleus (8). In fibroblasts, the localization of YAP was studied by overexpression of the epitope-tagged protein with conflicting results (12, 14), and the localization of the endogenous protein has never been studied. Therefore we tested whether endogenous YAP localized to the nucleus, where it could potentially interact with hnRNP U. HeLa cells were stained with antibodies directed against YAP and hnRNP U and the distribution of each protein studied by confocal microscopy. A significant fraction of the endogenous YAP protein was found in the cytoplasm and on the plasma membrane; however, we also detected endogenous YAP in the nucleus of HeLa cells (Fig. 2*A*). As expected we also found significant amounts of hnRNP U in the nucleus of HeLa cells; both YAP and hnRNP U were distributed throughout the nucleoplasm, as well as concentrated in speckled foci (Fig. 2*A*). Although far less hnRNP U was detected in the cytoplasm of the cells, we consistently observed a pool of the protein diffusely distributed throughout the cytoplasm (Fig. 2*A*). We used subcellular fractionation and Western blot analysis to confirm these results and to determine the ratio of cytoplasmic and nuclear YAP. Equal ratios of cytoplasmic and nuclear fractions were separated by SDS-PAGE, and the purity of the fractions was assessed by Western blot analysis of the cytoplasmic protein IκB (39, 40) or the nuclear histone deacetylase Sin3 (41) (Fig. 2*B*). Consistent with our localization studies, YAP was found in both nuclear and cytoplasmic fractions with ~5- 20% of the endogenous protein present in the nucleus based on densitometric analysis of multiple preparations. We were also able to confirm our finding that some hnRNP U resides in the cytoplasm, although the majority of the protein clearly fractionated with the nuclear compartment (Fig. 2*C*).

YAP Interacts with hnRNP U in Vivo—Because we detected an interaction between YAP and hnRNP U *in vitro* and the two proteins co-localize in cells, we next asked whether the two proteins interacted *in vivo*. Furthermore, because both proteins were found in nuclear and cytoplasmic compartments, we asked whether the interaction of YAP and hnRNP U occurred in the nucleus, in the cytoplasm, or in both subcellular compartments. Cells expressing HA-tagged full-length YAP were separated into cytoplasmic and nuclear fractions, and the soluble protein in each fraction was incubated with affinity-purified YAP antibodies or a nonimmune IgG control. After exten-

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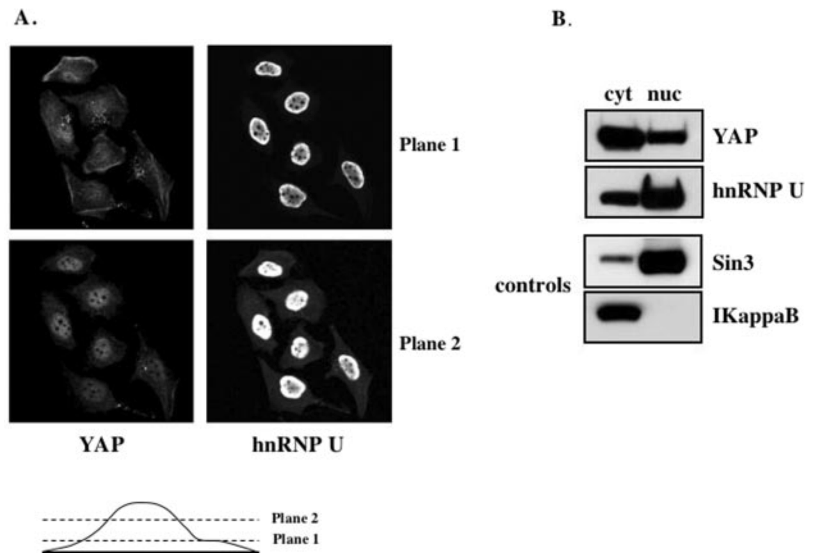

FIG. 2. **The localization of endogenous YAP and hnRNP U in HeLa cells.** *A*, HeLa cells were fixed and stained as described previously (36) using a polyclonal antibody to YAP and a monoclonal antibody to hnRNP U followed by the fluorescently labeled secondary antibodies. The cells were visualized in *xy* sections by confocal microscopy (*scale bar*, 20 μm). Several different sections of the cells were visualized, and the data presented are representative of two different planes of focus to highlight nuclear and cytoplasmic images. *B*, HeLa cells were separated in cytoplasmic (*cyt*) and nuclear (*nuc*) fractions as described under "Materials and Methods." Equal ratios of each fraction was resolved on 4–20% SDS-PAGE gels and analyzed by Western blotting as noted in the figure. Western blots are representative of fractionation experiments performed at least five times.

sive washing the bound fractions were separated by SDS-PAGE and analyzed by Western blot analysis with HA antibodies and with antibodies directed against hnRNP U. Importantly, the distribution of HA-YAP was indistinguishable from the distribution of endogenous YAP (not shown), and the overexpressed protein was observed in both the nuclear and cytoplasmic compartments. We found that YAP interacted with hnRNP U selectively in the nuclear compartment (Fig. 3), even though a pool of hnRNP U was available in the cytoplasmic fraction for interaction (Fig. 3). Furthermore cytoplasmic YAP was capable of protein-protein interaction because we reliably detected the interaction of cytoplasmic HA-YAP and 14-3-3 (Fig. 3), a protein involved in sequestering YAP in the cytoplasm (10). Thus we conclude that YAP and hnRNP U interact *in vivo* and that the interaction occurs selectively in the nuclei of HeLa cells.

Interaction of YAP with hnRNP U Is Not Responsible for YAP Translocation to the Nucleus—Multiple labs have proposed that YAP cycles between the cytoplasm and nucleus to integrate specific signal transduction events, but YAP contains no classical nuclear localization sequence, and the mechanism by which it enters the nucleus is unknown. Although hnRNP U stably interacts with YAP only in the nuclear fraction, we considered the possibility that one function of the hnRNP U-YAP interaction was to shuttle YAP into the nucleus where the two proteins remained stably associated. Therefore we asked whether amino acids 1–57 of YAP, which mediate the interaction with hnRNP U (Fig. 1), are necessary and sufficient for targeting YAP to the nucleus in HeLa cells. Because the truncated YAP protein was able to passively diffuse into the nucleus, we fused full-length and truncated YAP proteins to a triple-tandem green fluorescent protein tag (3XGFP) and compared the distributions of 3XGFP and various 3XGFP-YAP proteins, including YAP(1–57), which is capable of binding hnRNP U, and YAP(58–474), which lacks the site of interaction with hnRNP U.

HeLa cells were transiently transfected with 3XGFP alone, 3XGFP-YAP, 3XGFP-YAP(1–57), or 3XGFP-YAP(58–474). The 3XGFP protein accumulated exclusively in the cytoplasm (Fig. 4*A*). In contrast, 3XGFP-YAP localized to both the nucleus and the cytoplasm in a distribution similar to the endogenous YAP protein (Fig. 4*B*; see Fig. 2). The overexpressed 3XGFP-YAP(1–57) protein localized predominantly to cytoplasm, whereas 3XGFP-YAP(58–474) localized both to the nucleus and the cytoplasm similar to full-length YAP (Fig. 4, *C* and *D*). Because the majority of overexpressed 3XGFP-YAP(1–57) protein resides in the cytoplasm, whereas YAP(58–474) resides in both the nucleus and the cytoplasm, the interaction of YAP with hnRNP U mediated by this region is unlikely to play a role in the translocation of YAP into the nucleus. Furthermore, amino acids 1–57 of YAP are neither necessary nor sufficient to target YAP to the nucleus.

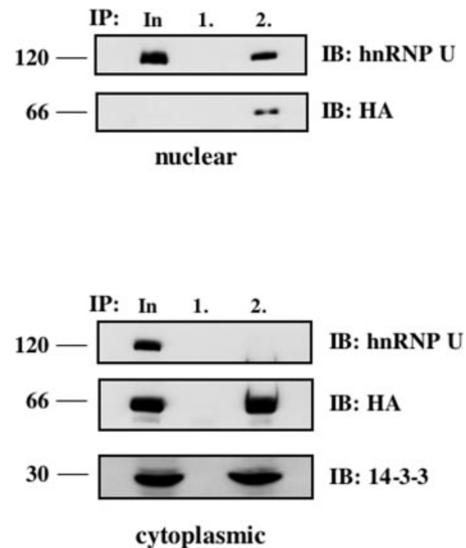

FIG. 3. **YAP and hnRNP U selectively interact in the nuclear fraction.** HeLa cells were transiently transfected with plasmid encoding HA-YAP, harvested 18 h later, and separated into nuclear and cytoplasmic fractions as indicated. The cytoplasmic and nuclear fractions were subjected to immunoprecipitation with preimmune IgG (*lane 1*) or anti-YAP IgG (*lane 2*) for 1 h and collected on protein A beads for 45 min. The samples were electrophoresed on 4- 20% gradient SDS-PAGE gels, transferred to Immobilon-P, and analyzed by Western blotting with HA, hnRNP U, or pan-14-3-3 antibodies. The results are representative of three experiments. *IP*, immunoprecipitation; *IB*, immunoblot.

hnRNP U Negatively Regulates YAP Co-activation of Bax Transcription—Because YAP and hnRNP U interact in the nucleus, we next asked whether hnRNP U had an effect on known YAP functions in the nucleus. YAP was shown to robustly co-activate p73-mediated gene expression to regulate the transcription of the pro-apoptotic gene Bax (10, 13). Because HeLa cells express significant amounts of p73 (42), we tested whether co-expression of hnRNP U had an effect on the ability of YAP to stimulate the expression of Bax. To do this we used a reporter plasmid with a p73-responsive Bax promoter upstream of the firefly luciferase gene. HeLa cells were tran-

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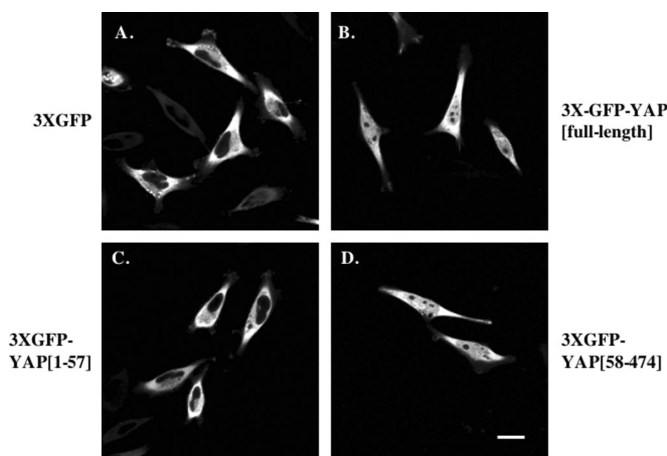

FIG. 4. **hnRNP U is not responsible for translocating YAP to the nucleus.** HeLa cells were transiently transfected with cDNAs encoding 3XGFP (*panel A*), 3XGFP-YAP (*panel B*), 3XGFP-YAP(1-57) (*panel C*), or 3XGFP-YAP(58-474) (*panel D*). After 18 h the cells were fixed and analyzed by confocal microscopy (*scale bar*, 20 μm). The figures are representative of two separate transfection experiments and the visual examination of at least 100 GFP-positive cells.

siently transfected with the reporter construct plus empty vector, full-length YAP and/or hnRNP U. As reported previously (10, 13), overexpression of YAP induced Bax-luciferase activity 8–12-fold above basal levels (Fig. 5). Overexpression of hnRNP U alone had no effect on activation of the reporter gene, but when co-expressed with full-length YAP, hnRNP U significantly attenuated the ability of YAP to increase Bax-luciferase activity (Fig. 5). Overexpressed YAP(58–474), lacking the amino-terminal domain that mediates the interaction with hnRNP U, robustly increased Bax-luciferase activity and did not differ significantly from the effect observed when full-length YAP was co-expressed (Fig. 5). However, in contrast to what we observed with the full-length YAP protein, hnRNP U was unable to block the YAP(58–474)-mediated increase of Bax-luciferase activity (Fig. 5). Therefore we conclude that the association of hnRNP U and YAP is required for modulation of p73-mediated Bax reporter gene activity in HeLa cells. Furthermore we conclude that YAP and hnRNP U functionally interact in the nucleus and that one outcome of the interaction is to block the ability of YAP to function as a transcriptional co-activator in some cellular contexts.

DISCUSSION

Here we show that YAP physically (Figs. 1 and 3) and functionally (Fig. 5) interacts with hnRNP U, a multifunctional nuclear protein and a constituent of the nuclear matrix and scaffold (31, 32, 43–47). YAP associates with hnRNP U via a proline-rich amino terminus lacking in the related protein TAZ (Fig. 1). Thus the ability to associate with hnRNP U is another distinguishing feature of these two related proteins and may help explain differences in their cellular functions.

Our subcellular fractionation and localization studies clearly show that YAP and hnRNP U are localized in both the cytoplasm and nucleus (Fig. 2); however, we were only able to co-immunoprecipitate the two proteins from the nuclear fraction (Fig. 3). Further biochemical studies will be required to determine whether the association of YAP and hnRNP U occurs in subcompartments of the nucleus including the matrix, a site where hnRNP U is known to significantly accumulate (32, 43). It is not clear why the YAP-hnRNP U association occurs preferentially in the nucleus. One possibility is that YAP and hnRNP U do indeed interact in the cytosol but with a lower affinity than the interaction observed in the nucleus. It is also possible that YAP and/or hnRNP U interact with other proteins in the cytoplasm and that these protein interactions may prevent the interaction between YAP and hnRNP U. Alternatively, a nuclear-specific protein may mediate an indirect interaction between YAP and hnRNP U. Because we identified hnRNP U in pull-down assays from cell lysates, we cannot rule out this possibility. However, sensitive silver staining approaches failed to visualize additional proteins eluting from the GST-YAP(1–57) affinity matrix. Finally it is also possible that the interaction of YAP and hnRNP U is regulated by compartment-specific phosphorylation or dephosphorylation of one or both proteins. Both YAP (6) and hnRNP U (Fig. 1*B*) are constitutively phosphorylated, and Basu *et al.* (10) showed that YAP is differentially phosphorylated in the cytoplasm and nucleus. Furthermore, phosphorylation of YAP has already been shown to promote specific protein-protein interactions in the cytoplasm, where Akt-mediated phosphorylation at serine 127 leads to 14-3-3 binding (10). Regardless of the mechanism involved in this compartment-specific interaction, it will be important to compare the full complement of YAP-interacting proteins in the nucleus and cytoplasm to gain a full appreciation of its cellular functions.

Epidermal growth factor receptor activation and subsequent phosphorylation by Akt stimulates the nuclear exit of YAP (10), but we know relatively little regarding how YAP enters the nucleus where it functions to modulate transcription of a variety of genes (7, 10–14). Furthermore, it is unclear whether there is a stable pool of nuclear YAP or whether specific extracellular signals promote the nuclear accumulation of YAP. YAP lacks any known nuclear localization signal; thus the association with cytoplasmic proteins may play a key role in the nuclear entry of YAP. For example, binding of heregulin to ErbB-4 results in two proteolytic cleavages that release the ErbB-4 intracellular COOH-terminal fragment (CTF) from the membrane (48, 49). The CTF translocates from the cytoplasm to the nucleus where it may affect the transcription of target genes (7, 50). Because YAP associates with the plasma membrane-associated ErbB-4 receptor and the CTF requires YAP for stimulation of transcription via the Gal4 transactivation system (7), YAP may translocate from the cytoplasm to the nucleus in association with the ErbB-4 CTF. Because we found hnRNP U in the cytoplasm (Fig. 2), and hnRNP U contains an amino-terminal nuclear localization signal (30), we first considered the possibility that the association with hnRNP U played a role in the translocation of YAP from the cytoplasm to the nucleus. However, using triple GFP-tagged YAP fusion proteins, we found YAP(1–57) excluded from the nucleus, whereas full-length YAP and YAP(58–474) was found in both the cytoplasm and in the nucleus (Fig. 4, *B* and *D*). Because amino acids 1–57 of YAP mediate its interaction with hnRNP U, we conclude that the YAP-hnRNP U interaction plays no role in targeting YAP to the nucleus. Furthermore, the distribution of GFP-YAP(58–474), which can not bind hnRNP U, resembles that of full-length YAP (Fig. 4), implying that this interaction does not play a major role in retaining YAP in the nucleus.

Although the hnRNP U- YAP interaction does not play a role in regulating the subcellular localization of YAP, our functional studies show that co-expression of hnRNP U significantly attenuates the ability of YAP to increase p73-driven Bax-luciferase activity in HeLa cells (Fig. 5). We do not yet understand how hnRNP U modulates the ability of YAP to function as a transcriptional co-activator. Interaction with hnRNP U may directly block a binding site needed for YAP co-activation. This is unlikely, because the proline-rich amino terminus that binds hnRNP U is not the region of YAP implicated in transcriptional co-activation. Alternatively, hnRNP U may shuttle YAP away

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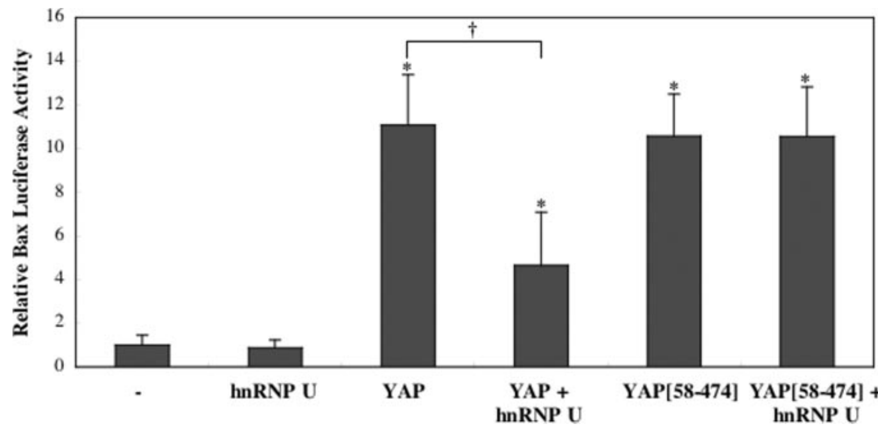

FIG. 5. **Functional analysis of the YAP-hnRNP U interaction.** HeLa cells were transiently transfected 150 ng of the indicated plasmids encoding HA-YAP or HA-YAP(58–474) with or without HA-hnRNP U, together with Bax-luciferase and Lac-Z reporter plasmids. The total amount of transfected DNA was maintained constant by the addition of empty pCMV-HA plasmid when necessary. After 18 h the cell extracts were prepared in passive lysis buffer, luciferase activity was measured, and the data were normalized to β-galactosidase activity. The samples were analyzed in triplicate, and the data are expressed as the fold change relative to the negative control and analyzed by Student's t test. All of the conditions were performed at least three times with similar results, and protein expression was verified by Western blot analysis with HA antibodies in parallel dishes. *, $p < 0.001$; †, $p < 0.001$.

from sites of active gene transcription (Fig. 6). Evidence supporting this model comes from studies demonstrating that overexpression of hnRNP U represses glucocorticoid-mediated transcription by recruiting the activated receptor to the nuclear matrix (31, 51). The nuclear matrix may be involved in RNA transcription and processing and is a repository for many transcription factors, splicing factors (52), and proteins involved in chromatin remodeling (53–55). Association of transcriptional regulators with the nuclear matrix could serve multiple purposes, including inhibition of transcription by sequestration away from the sites of active transcription. Sequestration to the nuclear matrix may also provide a local storage pool capable of rapid dynamic regulation without the need to recruit proteins from the cytoplasm upon activation (56). Additionally, because the sites of chromatin attachment to the nuclear matrix serve as local gene expression loops, matrix-associated regions could be specifically enriched for the factors needed to enhance or repress the genes within these local active regions (57).

One intriguing possibility is that instead of directly acting on YAP, hnRNP U may affect the function of YAP by acting on RNA polymerase II (Pol II). Pol II contains a noncatalytic COOH-terminal domain (CTD) that can be phosphorylated, a prerequisite for transcript elongation (58, 59). Although hnRNP U binds directly to the phosphorylated CTD of Pol II (60), hnRNP U does not always localize to sites of active transcription (61) and can be found in the nuclear matrix (62). Therefore hnRNP U may sequester activated Pol II in the nuclear matrix until it is needed. Alternatively, hnRNP U may affect YAP co-activation by directly repressing transcriptional elongation by activated Pol II. hnRNP U associates with transcription factor IIH, a kinase complex known to phosphorylate the Pol II CTD to enable transcript elongation (58, 63). Binding of hnRNP U to transcription factor IIH inhibits the phosphorylation of the CTD (46). Thus transcriptional co-activation by YAP may be inhibited indirectly if hnRNP U prevents the phosphorylation (and activation) of Pol II. Although this model is intriguing, hnRNP U fails to affect the function of YAP proteins lacking the hnRNP U-binding site (Fig. 5). However, if a ternary complex including YAP, Pol II, and hnRNP U was formed because of other protein-protein interactions, this type of indirect regulation could still occur. Further studies will be needed to elucidate the mechanisms involved in YAP co-activation and to determine how hnRNPU U affects the nuclear functions of YAP.

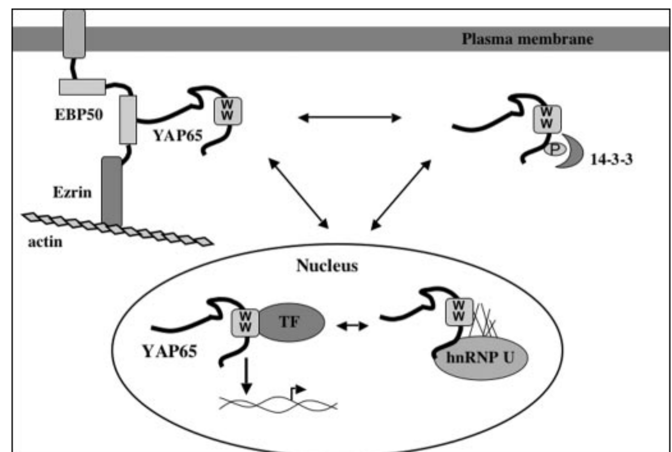

FIG. 6. **A model of compartment-specific interactions of YAP.** In the cytoplasm of epithelial cells YAP interacts with EBP50 at the plasma membrane (8). Because EBP50 is known to interact with receptors, ion channels, and signaling intermediates, this interaction may recruit YAP (and it associated nonreceptor tyrosine kinase) into cell surface-associated signaling complexes (64–66). In some cell types YAP may associate with other PDZ proteins, including MUPP1 (12). In addition YAP may interact with 14-3-3 proteins, and this interaction may function to sequester YAP in the cytoplasm and away from its nuclear targets. Currently, it is unknown whether 14-3-3 associates with YAP in the plasma membrane pool or only in the cytoplasm, and the relationship between these pools of YAP is poorly understood. In the nucleus, YAP interacts with multiple transcription factors as well as hnRNP U, a component of the nuclear matrix. Although the model shows two separate pools of nuclear YAP, one at sites of active transcription and one in the nuclear matrix in association with hnRNP U, the existence of these pools and their relationship to each other has not been explored. *Tf*, transcription factor.

Another important question is whether hnRNP U affects other nuclear functions of YAP. hnRNP U interacts with the amino terminus of YAP and not with the YAP WW domains, the site of binding for several transcription factors (13, 14). Thus hnRNP U may modulate the ability of YAP to regulate the activity of multiple transcription factors. Interestingly, the TEAD/TEF transcription factors bind a region of YAP that overlaps with the hnRNP U-binding site (12). Further studies will be needed to determine whether hnRNP U and TEAD binding is competitive or cooperative and whether the association of YAP and hnRNP U attenuates TEAD transcriptional activity.

Together with work in a variety of laboratories, our data

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suggest that YAP exists in the nucleus and in the cytoplasm in multiple distinct pools (Fig. 6). For example, in the cytoplasm YAP may target the plasma membrane by association with PDZ proteins (8) or may exist in the cytoplasm in association with 14-3-3 (10). Likewise, in the nucleus YAP may exist in association with hnRNP U in the nuclear matrix or at sites of active transcription in association with a variety of transcription factors. Further work will be needed to clearly demonstrate the existence of each of these protein complexes and to clarify the relationship between the different pools of YAP protein. Furthermore, it will be critical to define extracellular signals that drive the accumulation of YAP preferentially in one compartment or subcompartment of the cell. Given that YAP has multiple protein interaction domains and is ubiquitously expressed, tissue- and cell type-specific interactions are also likely to help explain the functions of YAP as an adaptor protein and as a regulator of gene transcription.

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