Yes-associated Protein and p53-binding Protein-2 Interact through Their WW and SH3 Domains*

Received for publication, September 19, 2000, and in revised form, January 11, 2001
Published, JBC Papers in Press, January 31, 2001, DOI 10.1074/jbc.M008568200

Xavier Espanel‡ and Marius Sudol
From the Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029-6574

To understand the role of the Yes-associated protein (YAP), binding partners of its WW1 domain were isolated by a yeast two-hybrid screen. One of the interacting proteins was identified as p53-binding protein-2 (p53BP-2). YAP and p53BP-2 interacted in vitro and in vivo using their WW1 and SH3 domains, respectively. The YAP WW1 domain bound to the YPPPPY motif of p53BP-2, whereas the p53BP-2 SH3 domain interacted with the VPMRLR sequence of YAP, which is different from other known SH3 domain-binding motifs. By mutagenesis, we showed that this unusual SH3 domain interaction was due to the presence of three consecutive tryptophans located within the βC strand of the SH3 domain. A point mutation within this triplet, W976R, restored the binding selectivity to the general consensus sequence for SH3 domains, the PXXP motif. A constitutively active form of c-Yes was observed to decrease the binding affinity between YAP and p53BP-2 using chloramphenicol acetyltransferase/enzyme-linked immunosorbent assay, whereas the overexpression of c-Yes did not modify this interaction. Since overexpression of an activated form of c-Yes resulted in tyrosine phosphorylation of p53BP-2, we propose that the p53BP-2 phosphorylation, possibly in the WW1 domain-binding motif, might negatively regulate the YAP-p53BP-2 complex.

WW domains are small modules that mediate protein/protein interactions (1, 2). The major features of the WW domain primary structure are (i) two conserved tryptophans spaced by 20–22 amino acids within the 40-amino acid long domain, (ii) a block of two or three aromatic amino acids located centrally between the two signature tryptophans, and (iii) a conserved proline located +3 to the second conserved tryptophan (3). The three antiparallel β-strands of WW domains form a hydrophobic patch that binds proline-rich or proline-containing motifs (4). Based on the ligand-binding specificity, one can divide WW domains into five groups. Group I WW domains interact with the core sequence PXXY (2, 5). Group II WW domains interact with a long stretch of prolines interrupted by a leucine (6, 7). Group III WW domains bind to PPR-containing motifs (8). Group IV WW domains interact with phosphoserine that is followed by a proline (9). Group V WW domains interact with polyprolines interrupted by a glycine and flanked by arginine (10). A WW-like fold was identified in the platlet-derived growth factor receptor subfamily of tyrosine kinases (11).

SH3 domains are composed of 50–70 amino acids forming a structure containing multiple β sheets (12). These modules also mediate protein/protein interactions through proline-rich motifs. Based on the binding specificity, the SH3 domains are divided into three major groups. Group I SH3 domains interact with basic-X-hydrophobic-proline-X-hydrophobic-proline (+Xo)(PX)(p), whereas Group II binds to hydrophobic-proline-X-hydrophobic-proline-X-basic (PXpPX+). Group III SH3 domains represented by the Eps8 family select ligands with PXDXY consensus cores (13).

Yes-associated protein (YAP), the first protein in which a WW domain was identified, is a phosphoprotein of 65 kDa that interacts with the SH3 domain of the c-yes proto-oncogene product, a non-receptor tyrosine kinase of the Src family. YAP expression is ubiquitous, with a high expression in ovaries (14). YAP has two isoforms: a short form (YAP) that possesses only one WW domain (WW1) and a long form (LYAP) that has two WW domains (WW1 and WW2) (Fig. 1A). In addition, there is a PDZ domain-binding motif, TWL, at the carboxyl-terminal end of YAP that allows the interaction with a submembranous scaffolding protein, EBP50 (ERM-binding phosphoprotein) (15). Since the modular structure of YAP is reminiscent of adaptor-type signaling proteins, we have decided to identify cognate partners of YAP to understand its molecular function. Using the human YAP WW1 domain to screen a mouse embryonic expression library, we have previously identified two putative ligand proteins: WW domain-binding protein (WBP)-1 and WBP-2 (2). The analysis of these two ligands showed that the YAP WW1 domain binds to the PXXY core sequence (PY motif). Recently, it has been shown that YAP can also interact with polyomavirus enhancer binding protein-2α (PEBP-2α), a transcription factor (16).

In this report, using yeast two-hybrid screening, we identified another YAP WW1 domain partner, p53-binding protein-2 (p53BP-2) (17). Originally, p53BP-2 was isolated as one of two proteins that interact with the wild-type p53 tumor suppressor protein, but not with a mutant form, in a yeast two-hybrid screen (17). In fact, the most common point mutations of p53 found in cancers prevent p53/p53BP-2 interaction if they are located in the DNA-binding domain (18). Human p53BP-2, composed of 1005 amino acids, possesses a PY motif, four

* This work was supported by Grants CA45757 and AR45626 from the National Institutes of Health and by Grant RG0234 from the Geneva, Switzerland. Tel.: 41-22-706-9788; Fax: 41-22-794-6965; E-mail: xavier.espanel@serono.com.

† To whom correspondence should be addressed: Serono Pharmaceutical Research Inst., 14, chemin des Aulx, Plan-Les-Ouates, 1228 Geneva, Switzerland. Tel.: 41-22-706-9788; Fax: 41-22-794-6965; E-mail: xavier.espanel@serono.com.

‡ This work was supported by Grants CA45757 and AR45626 from the National Institutes of Health and by Grant RG0234 from the Hu-

1 The abbreviations used are: YAP, Yes-associated protein; WBP, WW domain-binding protein; p53BP-2, p53-binding protein-2; CAT/ELISA, chlamydomenial acetyltransferase/enzyme-linked immunosorbent assay; GST, glutathione S-transferase; PCR, polymerase chain reaction; HEC, human embryonic kidney; MAPK, mitogen-activated protein kinase.
ankyrin repeats, and one SH3 domain at the carboxyl-terminal end (Fig. 1A). Using the two-hybrid system and x-ray diffraction, it has been shown that the fourth ankyrin repeat and the SH3 domain of p53BP-2 interact with the DNA-binding domain of p53, thus preventing the interaction between DNA and p53 (17, 18). The p53BP-2 SH3 domain does not interact with p53 via a PXXP or PXXDY consensus motif. Like p53, p53BP-2 is part of a protein network since it has been shown that p53BP-2 can also interact with Bcl-2 (an anti-apoptotic protein), protein phosphatase-1, NF-κB subunit p65 (a transcription factor), and APCL (adenomatous polyposis coli-like) protein (a tumor suppressor-like protein) (19–22). These interactions are mutually exclusive and can be competed by p53.

We show here that the interaction between p53BP-2 and YAP is dependent on the presence of the YAP WW1 and p53BP-2 SH3 domains. Using the SPOT technique, the binding motifs of these two domains were mapped. The p53BP-2 WW domain-binding motif seemed to be due to the presence of three consecutive tryptophans within the βC sheet of the SH3 domain. By CAT/ELISA, we confirmed that YAP was a putative transcription factor and that the interaction between YAP and p53BP-2 occurred in vivo. In addition, overexpression of a constitutively active form of c-Yes phosphorylated, directly or indirectly, p53BP-2. We also provided evidence that this phosphorylation might decrease or abolish the binding between YAP and p53BP-2.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The screening was performed with a Matchmaker kit from CLONTECH. We used the human brain library from CLONTECH, in which cDNAs were cloned into the pGAD-10 vector (GenBankTM/EBI accession number U13188) at the EcoRI site using an EcoRI linker (CCGGAACTCGG). Baits were cloned into the pGBT-BSE vector, which corresponds to a modified pGBT9 vector (GenBankTM/EBI accession number U07646): the sequence between EcoRI and BamHI was replaced by GAATTGGGATCCCGGTGAATTCC (the old EcoRI and BamHI sites are in italic and the new BamHI and EcoRI sites are underlined). Confirmation of interactions was performed in the pGAD-BSE vector, which corresponds to the pGAD-424 vector (GenBankTM/EBI accession number U07647) modified the same way as the pGBT9 vector.

DNA Constructs—pDBGAL4-BSE was obtained by inserting the

![Fig. 1. Schematic representations of protagonists. A, modular organization of YAP and p53BP-2 proteins. SB, SH3 domain-binding motif; PB, PDZ domain-binding motif; CC, coiled-coil region; PY, PPXY motif. B, schematic representation of the major constructs used in this study. Mutant constructs are symbolized by m within the concerned drawings. For PY mutations, partial sequences with the underlined point mutation are shown. GBT, DNA-binding domain of GAL4 (yeast expression vector); GAD, activation domain of GAL4; DB, DNA-binding domain of GAL4 (mammalian expression vector).](image-url)
**Regulation of the YAP-p53BP-2 Complex**

HindIII-EcoRI fragment corresponding to the DNA-binding domain of GAL4 coming from the pG-BEST vector into the pcDNA3.1(+)-vector (Invitrogen).

**pG-BEST-BSE (pGST), pGAD-BSE (pGAD), pGEX-2TK (pGEX),** and **pDB-**

**GAL4-BSE (pDB) Constructs—**pG-BEST-YAP (where YAP is the GAL4 DNA-binding domain (mammalian expression vector)) possesses the BamH1-EcoRI fragment of human YAP, which corresponds to the full-length cDNA, except that for the streptomycin, and 2.5 μg of the protein extract used for CAT/ELISA in the presence of 100 μM of o-nitrophenyl β-D-galactosidase buffer (60 mM NaHPO4, 40 mM NaCl, 100 mM β-mercaptoethanol) and 100 μM of o-nitrophenyl β-D-galactosidase. CAT/LacZ corresponds to the ratio of CAT absorbance to LacZ absorbance (27).

**GST Purification—**Bacteria were transfected with different DNA constructs cloned into the pGEX-2TK vector. The GST fusion proteins were induced by 1 mM isopropyl-β-D-thiogalactosidase for 2 h at 30 °C. GST fusion proteins were extracted and purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described previously (24).

**Full-drown Experiments—**Chicken brains were lysed in radioimmune precipitation assay buffer (10 mM Tris, pH 7.5, 300 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, and 1% sodium deoxycholate) in the presence of the protease inhibitor mixture Complete (Roche Molecular Biochemicals). After clarification by centrifugation, lysates were diluted with 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Tween 20, and 1% bovine serum albumin) with protease inhibitors (Complete) and incubated at 4 °C for 14 h under agitation with GST fusion protein (50 μg) bound to glutathione beads. The beads were washed three times with Tween buffer without bovine serum albumin.

**Anti-MBP-2 Antibody—**We expressed human p53BP2 from amino acids 480 to 626 as a GST fusion protein (GST-Ab). This construct was obtained by cloning GST into the pG-Ab vector (25). The GST fusion proteins were purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described previously (28).

**Western Blots—**Protein samples in loading buffer (25% glycerol, 62.5 mM Tris, pH 6.8, 5 μg/ml bromophen blue, 2.5% β-mercaptoethanol, 0.1% SDS, 0.1% v/v SDS) were run on SDS-polyacrylamide gels and then electro-transferred to nitrocellulose membranes. Blots were blocked for 1 h at room temperature in 20% milk Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 with either 5% low-fat dried milk or 3% bovine serum albumin (for the 4G10 antibody). For Western immunoblotting, anti-YAP (29), anti-MBP-2, and 4G10 (anti-phosphatase from Upstate Biotechnology, Inc.) antibodies were diluted 1:2000. After 1 h, membranes were washed with the same buffer. The blots were incubated for 1 h at room temperature with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:8000 dilution; Amersham Pharmacia Biotech). For visualization of the signal, we used the enhanced chemiluminescence kit from PerkinElmer Life Sciences.

**Immunoprecipitation of p53BP2—**HEK 293 cells were lysed in radioimmunoprecipitation assay buffer. About 200 μg of protein were incubated with anti-MBP-2 antibody (diluted 1:20) for 1 h at 4 °C under agitation, and then protein A-agarose beads were added and incubated for 1 h. The beads were washed once with radioimmunoprecipitation assay buffer and then twice with phosphate-buffered saline.

**GST Radiolabeling—**The pGEX-2TK vector codes for GST fusion proteins harboring a protein kinase A site. GST fusion proteins (50 μg) were radiolabeled with [γ-32P]ATP by protein kinase A from bovine heart (Sigma) according to the protocol previously described (24).

**SPOT Techniques—**Peptides were synthesized on a derivatized cellulose membrane provided by Genosys Biotechnologies, Inc. (30, 31). The spots were blocked in Western wash solution (10 mM Tris, pH 7.4, 0.1% Triton X-100, and 150 mM NaCl) plus 1% blocking buffer (Genosys Biotechnologies, Inc.) for 2 h at 4 °C under agitation. Radiolabeled GST fusion proteins were added (50 μg) to the membranes for 14 h at 4 °C under constant shaking. Washes were performed with Western wash solution. Before autoradiography, filters were dried at room temperature.
To identify binding partners of the YAP WW1 domain, pull-down experiments on cell lysates containing YAP were performed. As controls, GST alone and the GST-YAPWW1-P202A mutant, which is known to render the domain inactive for binding to PPXY-containing WBP-1 (23), were used. After separation on SDS-polyacrylamide gel, the precipitated proteins were transferred to nitrocellulose membranes and probed with radioactively labeled GST-YAPWW1. As shown in Fig. 2, there were four major specific proteins of 120, 100, 90, and 40 kDa that were pulled-down from chicken brain extracts by the human YAP WW1 domain. Proteins of the same molecular masses were pulled-down by GST-YAPWW1 from mouse brain and HeLa cell protein extracts (data not shown).

The YAP WW1 Domain Selects p53BP-2 in the Yeast Two-hybrid Screen—To identify binding partners of the YAP WW1 domain that were visualized in the pull-down assays shown in Fig. 2, a yeast two-hybrid screen was used. A human brain cDNA library was probed with the YAP WW1 domain engineered as bait (GBT-YAPWW1). After screening $1.5 \times 10^6$ clones, 16 clones that were positive on selection medium and in a β-galactosidase colorimetric assay were obtained. The following clones were then isolated: the NADH-ubiquinone oxidoreductase (amino acids 41–174), kinesin-5 (amino acids 27–176), the KIAA0870 protein with a shifted reading frame (one time), an unknown sequence (three times), an empty pGAD vector (four times), and p53BP-2 (amino acids 517–1005; which was isolated seven times). With the exception of p53BP-2, none of the isolated clones contained the expected PPXY motif for binding to the WW1 domain of YAP. In addition, the alignment of inserts for all the remaining clones revealed no apparent consensus sequence that could be considered as a core for an alternative motif for binding to the WW1 domain of YAP. With the exception of p53BP-2, we considered all the remaining clones as false positives. In addition, isolation of p53BP-2 with its PPPPYP confirmed the following: (i) the results of Firozzi et al. (32), who showed, after a data bank screen, that the peptide of p53BP-2 containing the PY motif can interact in vitro with the YAP WW1 domain, and (ii) our previous in vitro results aimed at definition of the optimal core of the YAP WW1 binders through screening peptide repertoires displayed on phage (33). Indeed, we have shown that the peptides containing PPPPY cores display the highest affinity for the WW1 domain of human and mouse YAP (33).

To further confirm the complex formation between YAP and p53BP-2, the interaction between GBT-YAPWW1 and the p53BP-2 proline-rich motif (PY5) fused to the GAL4 transactivation domain (GAD-PY5) was assayed. In parallel, we did the reverse experiment, in which PY5 was fused to the DNA-binding domain of GAL4 (GBT-PY5), whereas the YAP WW1 domain was fused to the GAL4 transactivation domain (GAD-YAPWW1). As shown in Fig. 3, the PY motif of p53BP-2 by itself could interact with the YAP WW1 domain in both ways, whereas a mutant of the YAP WW1 domain, YAFE-LH10 (L190W and H192G), did not interact with the PY5 construct (data not shown) (24). As positive control, we repeated and confirmed the previous results reported by Field and co-workers (17) using p53 fused to the GAL4 DNA-binding domain (pVA3) and pGAD-Cterm, which corresponds to the carboxyl-

**Fig. 2.** The YAP WW1 domain precipitates four major proteins. Shown is a far Western blot of GST pull-down experiments with chicken brain extracts incubated with GST alone, GST-YAPWW1, or GST-YAPWW1-P202A. Upper panel, precipitated proteins were run on SDS-polyacrylamide gel (10.5%), electrotransferred to nitrocellulose membrane, and then probed with 32P-radiolabeled YAP WW1 domain. Lower panel, GST calibration is shown. Western immunoblotting was carried out with anti-GST antibody. Molecular mass markers are on the right side.

**Fig. 3.** The YAP WW1 domain interacts with p53BP-2 in a yeast two-hybrid assay. The PY5 motif was sufficient to interact with YAP WW1 domain. Yeast cotransfected with the YAP WW1 domain and the p53BP-2 SH3 domain increases the precipitation of YAP. A, GST, GST-WBP-1, GST-PY5, and GST-Cterm were incubated with chicken brain lysates. Upper panel, after running on SDS-polyacrylamide gel (10.5%) and electrotransfer to nitrocellulose, the blot was probed with anti-YAP antibody. Lower panel, GST calibration is shown. The same blot were stripped and reprobed with anti-GST antibody. B, the same experiment as in A was performed, except that different GST constructs were used for the pull-down experiment: GST-PY5, GST-Cterm, and GST-CtermpSH3.
terminal part of p53BP-2 from the PPXY motif to the end of the protein (amino acids 729–1005) (data not shown).

The PY Motif and SH3 Domain of p53BP-2 Are Required for Efficient YAP Precipitation—To map the region of p53BP-2 that interacts with YAP, two different GST fusion proteins of p53BP-2 (GST-PY5 and GST-Cterm) were expressed. As depicted in Fig. 4A, GST-PY5 and GST-Cterm precipitated the two YAP isoforms (doublet) from chicken brain extracts, whereas the PY motif of WBP-1 or GST alone could not. The same result was obtained with human cellular extracts. Therefore, the PY motif by itself is also sufficient to precipitate YAP. It is interesting to note that Cterm has a higher affinity for YAP than for PY5 alone, suggesting that another region in GST-Cterm can interact with YAP to stabilize this complex.

Since YAP interacts with the SH3 domain of c-Yes, we hypothesized that the SH3 domain of p53BP-2 might also interact with YAP. To address this, the SH3 domain was rendered inactive by deleting the last two β strands of the p53BP-2 SH3 domain from the GST-Cterm construct (GST-CtermΔSH3), and

![image]

**FIG. 5.** The p53BP-2 SH3 domain interacts directly with YAP through the VPMRLR peptide. A, 15-mer peptides representing full-length human YAP protein were synthesized on a SPOT membrane. Each spot overlaps the next one by 10 amino acids. The spot sequence is shown in the right panel. The first and last spots are in boldface. Numbers above the sequence indicate the beginning of the corresponding peptide. The membrane was probed with GST-Cterm radiolabeled with 32P in the left panel. B, shown are controls: SH3 domain-binding motifs. The SPOT membrane was probed with radiolabeled GST-Cterm. Sequences of the spots are shown on the right. I, III, and V correspond to wild-type sequences, and II, IV, and VI are mutant sequences (mutations are in boldface). Underlined prolines show the positions of the PPXY motifs. C, shown are the results from progressive alanine fill-up and alanine scan. The SPOT membrane was hybridized with GST-Cterm.

![image]

**FIG. 6.** Trp976 within the p53BP-2 SH3 domain is responsible for binding to non-consensus SH3 domain-binding motifs. Four SPOT membranes hybridized with four different probes: GST-Cterm (Cterm), GST-Cterm W976R (WR), GST-Cterm L990Y (LY), and GST-Cterm W976R-L990Y (WR-LY). The sequences of the three spots are shown. Underlined amino acids indicate the mutations within the SB2 motif.

![image]

**TABLE 1**

Amino acid comparison of SH3 domains

| Domain   | Cterm | WR | LY | WR-LY |
|----------|-------|----|----|-------|
| PLCγ     |       |    |    |       |
| KIAA0771 |       |    |    |       |
| Other    |       |    |    |       |

Shown is a partial listing of SH3 domains. Arrows indicate the positions of p53BP-2 β strands. Conserved amino acids are shown in gray; similar amino acids are inverted shown in black. The human (h) KIAA0771 clone is closely related to murine (m) p53BP-2. Asterisks localize the two major differences between the p53BP-2 SH3 domain and other SH3 domains. PLCγ, phospholipase Cy.
the GST pull-down experiments were repeated. As shown in Fig. 4B, the p53BP-2 SH3 domain interacted, directly or indirectly, with YAP since the precipitation efficiency of the GST-Cterm construct was lower compared with that of the GST-Cterm construct.

The SH3 Domain of p53BP-2 Interacts with YAP through a Non-consensus Binding Motif—To determine whether the SH3 domain of p53BP-2 interacts with YAP directly, 15-mer peptides that cover the entire sequence of human YAP with 10 amino acids overlapping between two consecutive peptides were synthesized. The peptides were covalently attached to a cellulose membrane through their C termini as described in the SPOT technique (31). The membrane was then probed with the radiolabeled GST-Cterm fusion protein containing the SH3 domain of p53BP-2. As shown in Fig. 5A, the p53BP-2 SH3 domain bound to YAP through two peptides, spots 16 and 17, whereas the GST-Cterm construct did not interact with any spot, as expected (data not shown). Spots 16 and 17 shared the PQTVPMRLRK sequence, which did not contain the general consensus sequence PXXP DY for binding to SH3 domains. As controls, we also synthesized the p53-derived peptide CNSSCMGGMNRRPIL, which was previously shown to bind to the p53BP-2 SH3 domain (18), and two peptides (LASRPLPLLNASPG and VPLGRPEIPLRKSLP) that were identified previously in a phage display screen as strong binders to the Src and p53BP-2 SH3 domains, respectively (34). As negative controls, we generated mutant variants of the same peptides with a Pro-to-Ala point mutation to destroy the PXXP motif. In addition, a hot spot mutation (R248W) within the p53-derived peptide was generated. This mutation was shown to abrogate the p53-p53BP-2 complex (18). As depicted in Fig. 5B, the Src-binding motif did not interact with the p53BP-2 SH3 domain, whereas both the phase-selected binding peptide for p53BP-2 and the p53 peptide were positive in this binding assay. Interestingly, the affinity of the p53BP-2 SH3 domain for YAP was higher than that for p53 (A and B in Fig. 5 come from the same experiment and the same autoradiogram). As expected, the point mutation R248W in the p53 sequence abrogated this interaction; but surprisingly, the mutation P6A in the artificial motif did not, and only a slight decrease was observed. Taken together, these data suggest that the p53BP-2 SH3 domain interacts with a non-consensus binding motif in which basic amino acids seem to be important.

To map the minimal binding motif for the p53BP-2 SH3 domain, progressive alanine substitution and alanine scan analyses on the YAP sequence were performed using the SPOT peptide assay. As shown in Fig. 5C, the minimal length sequence required for the interaction between the SH3 domain of p53BP-2 and YAP was the VPMRLR peptide. We noticed that the longer sequence, PQTVPMRLRK, bound better. Interestingly, a single point mutation (R87A) abolished the interaction with the p53BP-2 SH3 domain, reminiscent of the p53 R248W mutant (Fig. 5B), suggesting again that the basic amino acid at this position could be critical for binding.

A Point Mutation (W976R) in the p53BP-2 SH3 Domain Switches the Binding Specificity to the PXXP Motif—By simple comparison of all SH3 domain sequences available in the database, two amino acid positions were identified that might explain why the SH3 domain of p53BP-2 does not require the PXXP motif to bind to YAP, but instead selects the VPMRLR core as its cognate ligand. First, the SH3 domain of p53BP-2 is unique in having three tryptophans in a row located...
in the βC strand (see Table I for a partial listing). Second, the SH3 domain of p53BP-2 does not have the consensus tyrosine (or aromatic amino acid) within the βE strand. Interestingly, the latter difference is also observed in the sequence of the SH3 domain of Eps8, which interacts with a unique binding motif, the PXXDY peptide, and represents the third group of specificity for SH3 domains (13). To address the potential role of these two singularities in the binding specificity of the SH3 domain of p53BP-2, point mutations in the βC and βE strands were introduced. Toward this end, we mutated Trp 976 to Arg and Leu990 to Tyr separately and together within the GST-C-term construct (containing the p53BP-2 SH3 domain). Trp976 was replaced by Arg due to the fact that charged amino acids are very often present at this position in other SH3 domains (Table I). The binding properties of these mutants were assayed by the SPOT technique. As depicted in Fig. 6, the GST-C-term W976R mutant, as well as the double mutant GST-C-term W976R/L990Y, interacted weakly with SH3 domain-binding peptide-2 of YAP (referred to as SB2; NVPQTVPMRLRKLP). In contrast, they bound strongly to a SB2 peptide in which a PXXP motif had been artificially introduced (NVPQTVPMRLRKLP). The L990Y mutant behaved similarly to the wild type, which bound SB2 as well as the SB2 mutant engineered for the presence of the PXXP motif. The mutation R87A in the SB2 motif abolished the interaction with the three SH3 domain mutants as well as with wild-type SH3. Therefore, constructs possessing the W976R mutation belong to Group II of SH3 domains (PXXP-XXP). Neither the wild type nor these three mutants interacted with the PXXP DY (Eps8 binder) or RXLPXXP (Src binder) motif (data not shown). In summary, these data suggest that a new binding specificity for SH3 domains, as represented by the SH3 domain of p53BP-2, could be determined by the third consecutive Trp within the βC strand of the domain. We suggest that the SH3 domain of p53BP-2 may represent a new group of specificity for SH3 domains (Group IV).

Mapping of the Minimal Sequence of p53BP-2 Required for Binding to YAP—To better understand the interaction between the YAP WW1 domain and the p53BP-2 PY motif, the PY5 core and its flanking sequences were further characterized. Using the SPOT technique, we showed that the nanopeptide
The interaction between YAP and DB-Cterm. The same ratio of HYAP6 (0.25 μg) to DB-Cterm (0.5 μg) was cotransfected with the pMIK-Neo vector alone (1 μg; Vector) or with different pMIK-Neo-Yes constructs. B, Yes Y535F does not reduce YAP transcriptional activity. DB-HYAP6 (0.25 μg) was cotransfected with pMIK-Neo (1 μg; Vector) or pMIK-Neo-Yes-Y535F.

Y$_{PP}P_{PP}P_{PP}$Y$_9$ represents the optimal sequence to interact with the WW1 domain of YAP (Fig. 7, A and B). To analyze the importance of each amino acid in the sequence, we performed an alanine scan using the SPOT peptide binding assay. We pointed to four essential amino acids, YPPY$_2$Y$_3$P$_4$P$_5$P$_6$Y$_7$P$_8$Y$_9$, that are covalently attached to a cellulose proline-rich insert in the GST-PY5 construct. The scan was repeated by performing site-directed mutagenesis on the construct DB-HYAP6 (DB-HYAP6(Y535F)). Since the GST-PY5 mutant proteins, when blotted on membranes and probed with radiolabeled YAP WW1 domain, displayed the same consensus sequence as that shown in the SPOT binding assay (data not shown), we think that the discrepancy observed between SPOT and pull-down experiments may reflect the differences in structural constraints of peptide ligands. For the SPOT technique, binding occurs on the membrane, whereas for the pull-down experiments, binding occurs in solution. The pull-down experiments seem to be closer to the physiological conditions. We conclude that the YPPPY$_2$Y$_3$Y$_4$Y$_5$Y$_6$Y$_7$Y$_8$Y$_9$ motif is the minimal sequence required for the YAP WW1 domain/p53BP-2 interaction in solution.

Fig. 9. Effect of an activated form of c-Yes on YAP/p53BP-2 interaction. CAT/ELISA was carried out on HEK 293 cells using the same conditions as described for Fig. 8. A, Yes Y535F disrupts the interaction between YAP and DB-Cterm. The same ratio of HYAP6 (0.25 μg) to DB-Cterm (0.5 μg) was cotransfected with the pMIK-Neo vector alone (1 μg; Vector) or with different pMIK-Neo-Yes constructs. B, Yes Y535F does not reduce YAP transcriptional activity. DB-HYAP6 (0.25 μg) was cotransfected with pMIK-Neo (1 μg; Vector) or pMIK-Neo-Yes-Y535F.

p53BP-2 and YAP Interact in Vivo—Since p53BP-2 is a cytoplasmic protein (21) and YAP is present in both the cytoplasm and the nucleus (15, 16), these two proteins may interact in vivo. Recently, Ito and co-workers (16) have shown that YAP harbors a transcriptional activation domain within its carboxyl-terminal region. We decided to reproduce this result and consider a transcriptional assay as a biological “readout” for the YAP/p53BP-2 complex. The DNA-binding domain of GAL4 was fused to YAP (DB-HYAP6) and cotransfected with a reporter construct containing the CAT gene downstream of five GAL4-binding sites, p5G5CAT. As shown in Fig. 8A, DB-HYAP6 drove the CAT expression in a dose-dependent manner. This transcriptional activity was dependent on the activation domain of YAP since its deletion within the construct DB-HYAP6 (DB-HYAP6ΔAD) did not lead to CAT expression. As a control, the overexpression of HYAP6 did not activate CAT transcription.

In the same experimental system, we investigated whether p53BP-2 also possesses transcriptional activity. As shown in Fig. 8A, full-length p53BP-2 fused to the DNA-binding domain of GAL4 (DB-2BP-2) did not activate the transcription of CAT, suggesting that p53BP-2 does not possess transcriptional activity.

The cotransfection of DB-2BP-2 and HYAP6 led to CAT production (Fig. 8C), which was dose-dependent for YAP DNA (data not shown). This result suggests that these two proteins interact together in vivo. The reverse cotransfection, DB-HYAP6 with different amounts of p53BP-2 DNA, did not modify the amount of CAT produced by DB-HYAP6 alone (Fig. 8C).

Interestingly, the interaction of p53BP-2 and YAP in vivo was drastically increased when only the carboxyl-terminal part of p53BP-2 was used (DB-Cterm). This difference in activation of CAT by the C terminus of p53BP-2 versus full-length p53BP-2 proteins might be due to the instability of full-length p53BP-2, as previously documented (21). As expected, the strength of binding between YAP and p53BP-2 was dependent on the presence of the WW and SH3 domains (Fig. 8, D and E).

Overexpression of a Constitutively Active Form of Yes Modifies the Binding Affinity between YAP and p53BP-2—Since YAP was first described as a Yes-interacting protein, we investigated the effect of Yes overexpression on the interaction between YAP and p53BP-2. To address the issue concerning the involvement of the Yes kinase activity, a constitutively active form of c-Yes kinase, the mutant Y535F, was also used. The cotransfection of the DB-Cterm-HYAP6 complex with the Yes Y535F mutant led to a decrease in CAT production in a dose-dependent manner, whereas wild-type Yes, under the same conditions, did not change the amount of CAT (Fig. 9A). Since the Yes Y535F mutant did not change the transcriptional activity of DB-HYAP6 (Fig. 9B), we concluded that the kinase activity of Yes decreases the affinity between YAP and p53BP-2. The same results were obtained with a constitutively active Src mutant, Y527F (data not shown). Taken together,
these data suggest that at least two non-receptor tyrosine kinases, namely Yes and Src, can decrease, directly or indirectly, the interaction between YAP and p53BP-2.

The in Vitro Binding of YAP to p53BP-2 Is Negatively Regulated by Phosphorylation—Since non-receptor tyrosine kinases could directly modulate the binding affinity between YAP and p53BP-2, we investigated the tyrosine phosphorylation status of p53BP-2, knowing that YAP is not a phosphotyrosine, but a phosphoserine protein (29). As depicted in Fig. 10, p53BP-2 was highly phosphorylated on its tyrosine(s) when the active mutant of c-Yes, but not the wild type, was overexpressed. This observation suggests that non-receptor tyrosine kinases can phosphorylate p53BP-2 either directly or indirectly.

Since the PY5 motif of p53BP-2 possesses four phosphorylatable amino acids (YPYPPP), the consequences of their phosphorylation on binding affinity were investigated. We were also interested whether the MAPK cascade would be implicated in the consequences of the serine phosphorylation. Using the SPOT technique, each tyrosine or serine, alone or in combination, was replaced by its phosphorylated form, by an acidic amino acid to mimic the negative charge (Asp), or by a neutral amino acid (Asn). As shown in Fig. 11, substitution of Tyr9 by a similar peptide, YPYPPP, was unable to show any co-immunoprecipitation with this protein (17, 19–21). According to their data, one possible explanation would be the instability of p53BP-2 (21). In our CAT/ELISA, the difference in the CAT produced by DB-BP-2-HYAP6 or DB-Cterm-HYAP6 could also be explained by the full-length p53BP-2 instability (Fig. 8D). However, we cannot exclude other possibilities such as full-length p53BP-2 could exist in different states of conformations/complexations/phosphorylations during the cell cycle, leading to a narrow window where YAP and p53BP-2 could interact. A closed conformation of p53BP-2 involving its SH3 domain and a binding motif upstream of the PY motif would explain the difference observed in Fig. 8D between DB-BP-2 and DB-Cterm. This scenario is likely since a motif similar to the YAP SB2 peptide is present in the amino-terminal part of p53BP-2: VPLREK (amino acids 322–327). Such hypotheses need to be further investigated.

Our data show that an efficient interaction between YAP and p53BP-2 requires both the YAP WW1 and p53BP-2 SH3 domains. By pull-down and SPOT experiments, we have found that the PPXY binding motif, previously established by us, is not sufficient for an efficient interaction with the YAP WW1 domain. In the p53BP-2 context, the YAP WW1 domain requires the PPXY peptide to interact with p53BP-2. Interestingly, a similar peptide, YLPPX, is present in polyomavirus enhancer binding protein-2 (PEBP-2a), a transcription factor that also binds to YAP (16). A BLAST search using the PPXY sequence showed that other proteins possess this very motif, such as the transcription factor Egr-1/KROX-24 (YPPPX), suggesting that these proteins could also interact with the YAP WW1 domain or related WW domains. This observation is in agreement with multiple protein bands observed in the pull-down experiments done with the YAP WW1 domain (Fig. 2). It is worthwhile to mention that p53BP-2 was also precipitated by GST-YAPWW1, but it was not one of the four major bands in Fig. 2 (data not shown). The fact that p53BP-2 did not appear as a major ligand in this assay supports our hypothesis that SH3 and WW domains are both required for a stable and strong interaction since only the WW1 domain of YAP (without the SB2 motif) was used in the pull-down experiments. The identification of these other partners would be helpful to fully understand the function of YAP.
Besides the WW domain/PY motif interaction, the p53BP-2 SH3 domain binds to YAP via the VPMRLR peptide, a non-consensus SH3 domain-binding motif (PXXP or PXXDY). The single substitution of the third tryptophan (Try976) by an arginine in the βC sheet prevents the mutant SH3 domain from interacting with a non-PXXP binding motif. This finding suggests that the presence of three tryptophans (or perhaps three aromatic amino acids) in a row within the βC sheet is responsible for the SH3 domain interaction with a non-PXXP binding motif. This result is not in contradiction to the RPXXPXXR consensus binding motif of the p53BP-2 SH3 domain previously obtained by phage display (34) because this consensus sequence was obtained from a biased library, in which the PXXP motif was fixed (XβPXXPβ). To confirm our data, it would be interesting to perform phage display with a non-biased library, on the W967R mutant as well as the wild type. It would also be very informative to extend the study to the few other SH3 domains possessing three aromatic residues in a row.

We have shown that the c-Yes kinase led to p53BP-2 tyrosine phosphorylation and to a diminution of the binding affinity between YAP and the carboxyl-terminal region of p53BP-2. From SPOT data, we found that tyrosine phosphorylation of the PY motif can either preserve (Tyr1 and Tyr4) or disrupt (Tyr9) binding to the YAP WW1 domain (Fig. 11), suggesting a sensitive regulation by tyrosine kinase(s). Considering that the MAPK cascade activated by non-receptor tyrosine kinases might also be involved, we showed that the serine phosphorylation within the PY motif (Ser11) decreases the interaction with the YAP WW1 domain. It would be interesting to identify which amino acid(s) is phosphorylated in vivo and to mutate it to study the effect of c-Yes overexpression on the YAP-p53BP-2 complex.

All evidence points to p53 as not being part of the YAP-p53BP-2 complex because of the following. (i) The p53BP-2 SH3 domain is required for its interaction with YAP and p53; and (ii) in a yeast three-hybrid system, we failed to demonstrate a tripartite complex: p53-Cterm-YAPWW1 (data not shown). Taken together, these data suggest that there would be a competition between YAP and p53 for binding to p53BP-2, as has been described with the other partners of p53BP-2 (17, 19–21).

The role of p53BP-2 is still confusing. Some authors propose that p53BP-2 is a pro-apoptotic protein (21), whereas others conclude that p53BP-2 overexpression does not lead to apoptosis, but impedes G2/M transition (20). This discrepancy could be due to the cell types used by the authors. Our data suggest a link between the activation of non-receptor tyrosine kinases and the release of p53BP-2 from its binding with YAP, allowing p53BP-2 to regulate p53 activity.

Acknowledgments—We thank Sonny Cheang and Hillary Linn for preparation of the SPOT membranes, Sahng-June Kwak for the Yes and Yes Y535F constructs, and Marie Kosco-Vilbois and Kate Biblowitz for valuable comments on the manuscript.

REFERENCES

1. Bork, P., and Sudol, M. (1994) Trends Biochem. Sci. 19, 531–533
2. Chen, H. L., and Sudol, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7819–7823
3. Sudol, M. (1996) Prog. Biophys. Mol. Biol. 65, 113–132
4. Macias, M. J., Hyvonen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M., and Ushkaryov, H. (1996) Nature 382, 646–649
5. Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X., and Sudol, M. (1999) Biol. Chem. 380, 431–442
6. Kryukov, G. V., Zamboni, D., Minopoli, G., Gertler, F., Russo, T., and Sudol, M. (1997) J. Biol. Chem. 272, 32869–32877
7. Bedford, M. T., Chan, D. C., and Leder, P. (1997) EMBO J. 16, 2576–2583
8. Bedford, M. T., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M. B. (2000) J. Biol. Chem. 275, 10359–10369
9. Lu, J. P., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) Science 283, 1325–1328
10. Komuro, A., Sacki, M., and Kato, S. (1999) J. Biol. Chem. 274, 36513–36519
11. Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R., and Fields, S. (1994) EMBO J. 13, 6912–6923
12. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9166–9170
13. Mongiovì, A. M., Romano, P. R., Panni, S., Mendoza, M., Wong, W. T., Musacchio, A., Cesaroni, G., and Di Fiore, P. P. (1999) EMBO J. 18, 5300–5309
14. Sudol, M., Chen, H. I., Bougeret, C., Einbond, A., and Berk, P. (1995) FEBS Lett. 368, 67–71
15. Mohler, P. J., Kreda, S. M., Boucher, R. C., Sudol, M., Stutters, M. J., and Milgram, S. L. (1999) J. Cell Biol. 147, 879–890
16. Yagi, R., Chen, L. F., Shigesada, K., Murakami, Y., and Ito, Y. (1999) EMBO J. 18, 2551–2562
17. Ishiwaki, K., Bartel, P. L., Li, B., Marraccino, R., and Fields, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6098–6100
18. Gorina, S., and Pavletich, N. P. (1996) Science 274, 1001–1005
19. Helps, N. R., Barker, H. M., Elledge, S. J., and Cohen, P. T. (1995) FEBS Lett. 377, 295–300
20. Naumovski, L., and Cleary, M. L. (1996) Mol. Cell. Biol. 16, 3884–3892
21. Yang, P., Hori, M., Takahashi, N., Kawate, T., Hata, K., and Okamoto, T. (1999) Oncogene 18, 5177–5186
22. Nakagawa, H., Koyama, K., Murata, Y., Morito, M., Akiyama, T., and Yokota, J. (2000) Cancer Res. 60, 101–105
23. Chen, H. I., Einbond, A., Kwik, S. J., Linn, H., Koorpe, E., Peterson, S., Kelly, J. W., and Sudol, M. (1997) J. Biol. Chem. 272, 17070–17077
24. Espanel, X., and Sudol, M. (1999) J. Biol. Chem. 274, 17284–17289
25. Espanel, X., Le Cam, L., North, S., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 499–503
26. Herbomel, P., Bourachot, B., and Yaniv, M. (1998) Cell 93, 653–662
27. Sudol, M., and Hanafusa, H. (1986) Mol. Cell. Biol. 6, 2839–2846
28. Sudol, M. (1994) Oncogene 9, 2145–2152
29. Frank, R., and Doring, R. (1988) Tetrahedron 44, 6031–6040
30. Blankenmayer-Menge, B., Nimitz, B., and Frank, R. (1990) Tetrahedron 31, 1701–1704
31. Piruzzi, G., McConnell, S. J., Uveges, A. J., Carter, J. M., Sparks, A. B., Kay, B. K., and Fowlkes, D. M. (1997) J. Biol. Chem. 272, 14611–14616
32. Linn, H., Ermekev, K. S., Rentschler, S., Sparks, A. B., Kay, B. K., and Sudol, M. (1997) Biol. Chem. 378, 531–537
33. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quillam, L. A., and Kay, B. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1540–1544