The EMBO Journal Vol.18 No.9 pp.2551–2562, 1999

A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator

Ryohei Yagi, Lin-Feng Chen, Katsuya Shigesada[1], Yota Murakami and Yoshiaki Ito[2]

Department of Viral Oncology and [1]Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan

[2]Corresponding author
e-mail: yito@virus.kyoto-u.ac.jp

A protein module called the WW domain recognizes and binds to a short oligopeptide called the PY motif, PPxY, to mediate protein–protein interactions. The PY motif is present in the transcription activation domains of a wide range of transcription factors including c-Jun, AP-2, NF-E2, C/EBPα and PEBP2/CBF, suggesting that it plays an important role in transcriptional activation. We show here that mutation of the PY motif in the subregion of the activation domain of the DNA-binding subunit of PEBP2, PEBP2α, abolishes its transactivation function. Using yeast two-hybrid screening, we demonstrate that Yes-associated protein (YAP) binds to the PY motif of PEBP2α through its WW domain. The C-terminal region of YAP fused to the DNA-binding domain of GAL4 showed transactivation as strong as that of GAL4–VP16. Exogenously expressed YAP conferred transcription-stimulating activity on the PY motif fused to the GAL4 DNA-binding domain as well as to native PEBP2α. The osteocalcin promoter was stimulated by exogenous PEBP2αA and a dominant negative form of YAP strongly inhibited this activity, suggesting YAP involvement in this promoter activity *in vivo*. These results indicate that the PY motif is a novel transcription activation domain that functions by recruiting YAP as a strong transcription activator to target genes.

Keywords: co-activator/PEBP2/PY motif/WW domain/Yes-associated protein

Introduction

Transcription factors are generally composed of two modules: the DNA-binding domain and the transcription activation domain. How transcription activation domains transmit their effects to target genes is currently a subject of intensive study. Several types of activation domains with characteristic amino acid compositions are known. These include the acidic domain rich in acidic amino acids, the proline-rich domain and the glutamine-rich domain, although there are many other domains which lack these characteristic amino acid compositions. It has been shown that certain activation domains interact directly with components of the basal transcription machinery including TFIIB, TATA-box binding proteins (TBP) and TBP-associated factors (TAFs) (Stringer *et al.*, 1990; Lin *et al.*, 1991; Tjian and Maniatis, 1994). In these cases, transcription factors bound to DNA are thought to function by recruiting the basal transcriptional machinery to the promoter for gene activation (Ptashne and Gann, 1997).

Other activation domains interact with the basal transcription machinery through indirect interactions mediated by 'co-activators'. For example, the acidic activation domain of yeast transcription factor GCN4 interacts with a co-activator complex containing ADA2, ADA3 and GCN5, which in turn makes contact with the basal transcription machinery (Barlev *et al.*, 1995). In mammalian cells, a large number of DNA-binding transcription factors use the co-activator p300/CBP (Janknecht and Hunter, 1996), which has a domain highly similar in sequence to a part of yeast ADA2, and associate with the factor p/CAF which shows significant homology to yeast GCN5 (Yang *et al.*, 1996). In addition to the recruitment of the basal transcription machinery to the promoter, recent studies indicate that co-activators including GCN5, p/CAF and p300/CBP possess histone acetyl transferase activity (Bannister and Kouzarides, 1996; Brownell *et al.*, 1996; Ogryzko *et al.*, 1996; Yang *et al.*, 1996). Core histone acetylation destabilizes the nucleosome so that the basal transcription machinery can gain access to promoters more effectively to facilitate transcription (Wade and Wollfe, 1997). These co-activator complexes can interact with more than one transcription factor at the same time, and, thereby, are thought to integrate the activities of distinct activation domains to increase the recruitment of basal transcription machinery to promoters.

Specific protein–protein interactions are important for a multitude of cellular processes and are mediated by functionally and structurally distinct domains, such as SH3, SH2, PTB, PH, PDZ and WW domains (Musacchio *et al.*, 1992; Marengere and Pawson, 1994; Bork and Margolis, 1995; Ferguson *et al.*, 1995; Songyang *et al.*, 1997). Among these protein modules, the WW domain (also called the WWP domain or Rsp5 domain) is a globular domain consisting of ~40 amino acids, of which two, tryptophan and an invariant proline, are highly conserved (Andre and Springael, 1994; Bork and Sudol, 1994; Hofmann and Bucher, 1995). This domain is present in numerous and unrelated proteins such as Yes-associated protein (YAP), Nedd4, Rsp5, Pub1, dystrophin, FE65, Pin1 and formin-binding proteins (FBPs) (Rotin, 1998). Like the SH3 domain, the WW domain can recognize certain proline-rich motifs represented by the sequence PPxY (Chen and Sudol, 1995; Linn *et al.*, 1997) or PPLP (Bedford *et al.*, 1997; Ermekova *et al.*, 1997) which are distinguishable from the ligand sequences of SH3 domains. The PPxY sequence, known as the PY motif, was originally identified in WBP (WW domain-binding protein) 1 and 2 (Chen and Sudol, 1995), and was shown to be recognized

by a subclass of WW domains present in YAP and Nedd4 *in vitro* (Chen and Sudol, 1995; Staub and Rotin, 1996; Staub *et al.*, 1996). Sometimes, the PY motif is conserved in several members of the same family including three ENaC subunits (epithelial sodium channel protein α, β and γ), retroviral Gag proteins (those of RSV, HTLV1 and AEV), interleukin receptors (IL-2R, IL-6R and IL-7R) and several Ser/Thr kinases (MAPKAP2 and CamKI) (Schild *et al.*, 1996; Rotin, 1998). Although this observation suggests the potential importance of the PY motif, its biological significance remains poorly understood.

It is interesting to note that the PY motif is found in the transcription activation domains of many transcription factors, including c-Jun (Baichwal and Tjian, 1990), AP-2 (Williams and Tjian, 1991), C/EBPα (Nerlov and Ziff, 1994), NF-E2 (Mosser *et al.*, 1998), KROX-20 (Vesque and Charnay, 1992), KROX-24 (Gashler *et al.*, 1993) and MEF2B (Molkentin *et al.*, 1996). This observation suggests that the PY motif plays a role in mediating transcription stimulation by interacting with WW domain-containing proteins. However, the actual target proteins of the PY motif which confer transcription stimulation activity have not yet been identified.

A transcription factor, polyomavirus enhancer binding protein 2 (PEBP2) (also called core binding factor, CBF) is a member of the Runt domain transcription factor family. Members of this family are composed of α and β subunits (Speck and Stacy, 1995; Ito, 1997). The α subunit is the mammalian homolog of *Drosophila* runt and contains an evolutionarily conserved region, the Runt domain, which is required for DNA binding and heterodimerization with the β subunit (Meyers *et al.*, 1993; Ogawa *et al.*, 1993b; Kagoshima *et al.*, 1996). The α subunit also contains the transactivation domain (Bae *et al.*, 1994; Kanno *et al.*, 1998). The β subunit does not bind to DNA but it enhances the affinity of the α subunit for DNA (Ogawa *et al.*, 1993a; Kagoshima *et al.*, 1996). In mammalian cells, the α subunit is encoded by three independent genes, *PEBP2αA/CBFA1/AML3*, *PEBP2αB/CBFA2/AML1* and *PEBP2αC/CBFA3/AML2* (Ito and Bae, 1997). *PEBP2αA* is essential for osteogenesis, and heterozygous mutations in this gene cause the human bone disease, cleidocranial dysplasia (Ducy *et al.*, 1997; Komori *et al.*, 1997; Mundlos *et al.*, 1997; Otto *et al.*, 1997; Zhang *et al.*, 1997a). *PEBP2αB/AML1* plays pivotal roles in hematopoieses and is the most frequent target of chromosome translocations in human leukemias (Ito, 1996; Okuda *et al.*, 1996; Wang *et al.*, 1996). We recently identified activation and inhibitory domains (AD and ID, respectively) in the C-terminal region of PEBP2αB1 (to be referred hereafter as αB1 for simplicity) (Kanno *et al.*, 1998). ID, which is contained within a 40 amino acid region and located next to the AD, keeps the full transactivation potential of the full-length protein below its optimal level probably through intramolecular masking of AD. AD is contained within an 80 amino acid region and does not have any of the amino acid clusters that characterize transactivation domains. We noted the presence of a sequence that completely matches the consensus sequence for the PY motif in AD. Moreover, the 10 amino acid sequence containing the PY motif, HTYLPPPYPG, is perfectly conserved among PEBP2α family members so far identified from *Xenopus* to human. These observations suggest the PY motif is potentially important for the stimulation of transcription by PEBP2.

In this study, we show that the PY motif of PEBP2α interacts with YAP and that YAP confers transcription stimulation activity on the PY motif fused to the GAL4 DNA-binding domain. These studies indicate that the PY motif and its binding partner, YAP, function together as a trans-activating motif and as a co-activator, respectively, in the regulation of transcription.

Results

The PY motif in the transcription factor PEBP2α acts as an activation motif

We previously mapped a transcription AD of αB1 to the region between amino acids 291 and 371. AD can be subdivided into two elements, TE1 and TE2, which function differently depending on the cell type (Kanno *et al.*, 1998). A 10 amino acid sequence (HTYLPPPYPG) within the TE2 subregion of AD [AD(TE2)] contains a candidate PY motif which is well conserved in PEBP2αA1 and PEBP2αC (to be referred to hereafter as αA1 and αC, respectively) as well as in chicken and *Xenopus* homologs of αB1. As will be shown below, the PPPY sequence within this conserved region is a genuine PY motif and interacts specifically with WW domains. We investigated the role of the PY motif in the stimulation of transcription using the 33 amino acid region containing the PY motif from αB1 fused to the minimum GAL4 DNA-binding domain (referred to as G-PY33). A previous study indicated that alanine substitution of the first, second or fourth amino acids of the PY motif abolished binding to the WW domain *in vitro*, whereas alanine substitution at the third position reduced but did not abolish the activity (Chen and Sudol, 1995). Thus, we introduced the same alanine substitutions (P1A, P2A, P3A and Y4A in Figure 1A) into the PY motif of G-PY33 to disrupt a possible interaction with a WW domain protein(s). In MC3T3-E1, NIH 3T3 and P19 cells, G-PY33 activated transcription from the tk promoter through the GAL4 binding site significantly. Furthermore, each of the P1A, P2A and Y4A mutations abolished this activity almost completely, while the P3A mutation was less effective (Figure 1B). Similar results were obtained in ROS17/2.8 cells (data not shown). These results agree well with the characteristics of the interaction between the PY motif and the WW domain, indicating that the PY motif is mainly responsible for AD(TE2) stimulatory activity in these cells. The results also indicated that a WW domain-containing protein probably interacts with the motif to stimulate transcription.

To test whether the PY motif alone is sufficient for activation, we fused the 12 amino acid peptide, HTYLPPPYPGSS, to the GAL4 DNA-binding domain (referred to as G-PY12) and examined the capacity of the construct to stimulate transcription. In P19 cells, G-PY12 stimulated transcription 1.5-fold compared with the GAL4 DNA-binding domain alone. Alanine substitution of the first proline of the PY motif (G-PY12 P1A) abolished the activity (Figure 1B). These results suggested that the PY motif by itself acts as a transcription activation element and that it is primarily responsible for AD(TE2) activity in these cells.

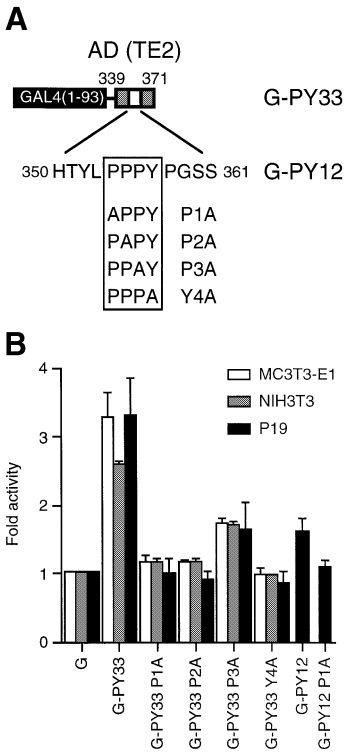

Fig. 1. The PY motif of PEBP2α proteins determines their AD(TE2) activity. (**A**) Schematic illustration of the minimum GAL4 DNA-binding domain, GAL4(1–93), fused to the PY motif-containing regions of αB1 from amino acid 339 to 371 (G-PY33). In G-PY12, 12 amino acids from amino acid 350 to 361 of αB1 were fused to the GAL4(1–93). The alanine substitutions introduced into the PY motif of each GAL4 fusion protein are indicated as P1A, P2A, P3A and Y4A. (**B**) PY motif activities in various cell lines. MC3T3-E1, NIH 3T3 and P19 cells were transfected with 0.2 μg of tk-GALpx3-LUC, 1 ng of pRL-EF, 0.2 μg of the pG-based plasmids expressing the indicated GAL4 fusion proteins and 0.6 μg of pEF-BOS as carrier DNA. The luciferase activities relative to those obtained with GAL4(1–93) are shown.

Isolation of YAP as a PY motif-binding protein

A PY motif-binding protein was identified by yeast two-hybrid screening using the AD of αB1 as a bait. From ~10^8 clones screened, 14 positive clones were obtained. Six of them encoded the mouse homolog of YAP. Chicken YAP was originally isolated as a protein that binds to the SH3 domain of c-Yes (Sudol, 1994) and subsequently mouse and human homologs were identified (Sudol *et al.*, 1995). Chicken YAP and human YAP have only one WW domain, whereas mouse YAP (mYAP) has two copies (Figure 2A). In addition to the WW domain, all YAPs reported so far contain a proline-rich motif, which serves as the SH3-binding motif, a glutamine-rich region and an acidic region (Figure 2A).

If YAP is involved in transcriptional regulation, YAP must be expressed in cells in which the PY motif plays an active role in transcription. We performed RT–PCR analysis using mRNAs from P19 and NIH 3T3 cells and detected mYAP transcripts in P19 and NIH 3T3 cells (Figure 2B) as expected from the data shown in Figure 1B. On the other hand, we did not detect a mYAP transcript in L1210 in which PY motif-dependent activities were not detected (see Figure 5A and B).

YAP is localized both in the cytoplasm and the nucleus

YAP interacts with the SH3 domain of c-Yes tyrosine kinase, a protein that is found attached to the cytoplasmic face of the plasma membrane. YAP also binds to other SH3 domain-containing proteins including c-Src and Crk *in vitro* (Sudol, 1994). In addition, YAP has been implicated in the maturation of retrovirus particles by associating with GAG protein (Garnier *et al.*, 1996). Because of these properties, YAP is thought to be a cytoplasmic protein. If YAP is a transcriptional co-activator, however, YAP must become localized to the nucleus at some stage. As shown in Figure 2C, YAP was found in the nucleus as well as in the cytoplasm of NIH 3T3 cells.

The WW domain of YAP interacts with PEBP2α through the PY motif

To confirm that the YAP-WW domain interacts directly with the PY motif of PEBP2α, we performed GST pull-down assays *in vitro*. αB1 and its PY motif mutants were translated *in vitro* in the presence of [^{35}S]methionine and their interaction with GST–mYAP-WW domain-1 (GST–WW1) and GST–mYAP-WW domain-2 (GST–WW2) was examined. The results of the experiments are shown in Figure 3A and B, and the data are quantified in Figure 3C.

αB1 and its derivatives did not bind to GST alone (Figure 3A, lanes 16–20). αB1 bound to WW1 twice as efficiently as to WW2 (compare lane 6 with lane 11). However, αB1(P1A), αB1(P2A) and αB1(Y4A) bound neither to WW1 nor to WW2 efficiently (compare lanes 7, 8 and 10 with lane 6 and lanes 12, 13 and 15 with lane 11, respectively). αB1(P3A), on the other hand, bound to WW1 and WW2 nearly as efficiently as to αB1 (compare lane 9 with lane 6 and lane 14 with lane 11, respectively). These results suggest that both mYAP-WW domains interacted with αB1 through the PY motif-containing region, although WW1, which is conserved between human, mouse and chicken YAPs, has higher affinity for the YAP-PY motif than WW2.

We also tested whether αA1 and αC were able to bind to the WW1 and WW2 domains (Figure 3B). Both GST–WW1 and GST–WW2 efficiently pulled down αC and again WW1 bound more strongly than WW2 (compare lanes 8 and 12). In the case of αA1, the full-length protein interacted with WW1 and WW2 poorly (lanes 5 and 9). Unexpectedly, the C-terminally truncated form, αA1(424), interacted with WW1 and WW2 more efficiently than full-length αA1 (compare lanes 6 with 5 and 10 with 9 and Figure 3C). Further truncation to amino acid 388 which removes the PY motif (see Figure 6A) eliminated binding to both WW1 and WW2 (lanes 7 and 11). The results indicate that both WW domains of mYAP bind to all members of the PEBP2α family of proteins and that WW1 binds more efficiently than WW2. The difference in binding efficiency between the full-length and C-terminally truncated PEBP2α will be discussed later.

We fused the 70 amino acid region of αB1 containing AD with or without alanine substitution of the PY motif to the DNA-binding domain of LexA [LexADBD-αB1 (302–371)] and analyzed the interactions of the resulting constructs with YAP using the yeast two-hybrid assay (Figure 3D). A strong interaction between LexADBD-

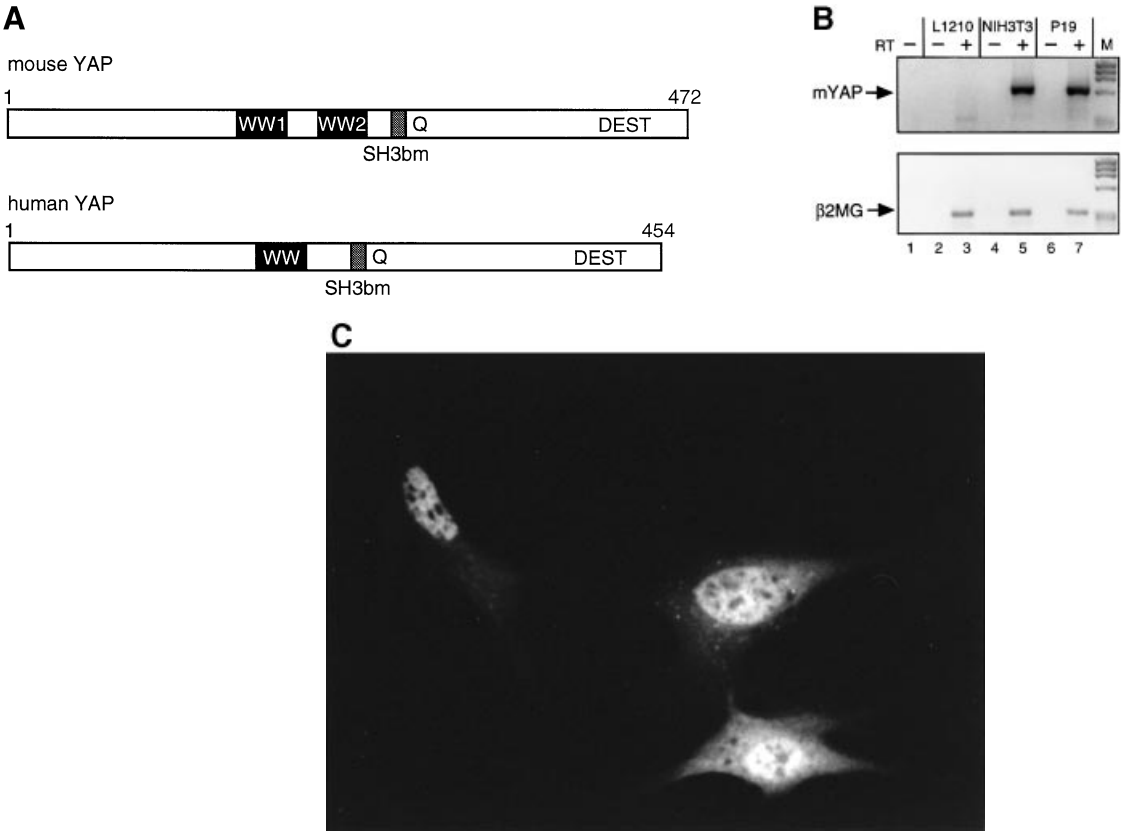

Fig. 2. Expression and subcellular localization of YAP. (**A**) Schematic illustrations of mouse and human YAP. WW, WW domain; SH3bm, SH3-binding motif; Q, glutamine-rich sequences; DEST, aspartic acid, glutamic acid, serine and threonine-rich region. (**B**) Expression of the mouse YAP transcripts in various cell lines. Poly(A)$^+$ RNA was isolated from L1210 cells (lanes 2 and 3), NIH 3T3 cells (lanes 4 and 5) and P19 cells (lanes 6 and 7). PCR products with mYAP primers (mYAP, upper panel) or mβ_2-microglobulin primers (β2MG, lower panel) as a control, using poly(A)$^+$ RNA (RT–; lanes 2, 4 and 6) or reverse-transcribed products (RT+; lanes 3, 5 and 7) as template were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide. The negative control without template is shown in lane 1. M, DNA size marker (ϕx174/*Hae*III). (**C**) Immunofluorescence staining of FLAG epitope-tagged YAP expressed in NIH 3T3 cells.

αB1(302–371) and GAL4-AD-YAP was observed, whereas P1A, P2A and Y4A mutants barely bound to GAL4-AD-YAP. The P3A mutant, however, showed decreased but significant binding activity to GAL4-AD-YAP. These results are consistent with the results shown above, suggesting that YAP interacts with αB1 through the PY motif *in vitro* and *in vivo*.

YAP contains a strong, intrinsic transcription activation domain

To test whether YAP itself contains the transcription activation domain, we fused YAP to the minimum GAL4 DNA-binding domain, GAL4(1–93), and examined the effect of the fusion on GAL4 binding site-containing reporter activity. The GAL4–YAP fusion protein strongly stimulated GAL4 binding site-dependent transcription in L1210 cells at a level similar to that attained by GAL4–VP16 (Figure 4A). Similar results were obtained in P19 and NIH 3T3 cells (data not shown). These results indicated that YAP contains strong, intrinsic transactivation activity which activates transcription when it is artificially tethered to a promoter in mammalian cells. We also detected strong activation by the LexA–YAP fusion protein in yeast cells (data not shown).

We mapped the activation domain of YAP using the deletion derivatives of mYAP shown in Figure 4B. Each deletion mutant was fused to GAL4(1–93) and tested for its transcription stimulation activity in COS7 cells. The C-terminal deletion up to amino acid 301 abolished the stimulatory activity and further deletion had no further effect (lanes 3 and 4). Consistent with this observation, the C-terminal regions, amino acids 156–472 and 276–472, by themselves showed strong stimulatory activity (lanes 5 and 6). Deletion of the SH3-binding motif did not affect the activity (lane 2). The fragments containing one or two WW domains did not stimulate transcription (lanes 7 and 8). These results established that the C-terminal region between amino acids 276 and 472, which is rich in serine, threonine and acidic amino acids, is responsible for the strong transcription stimulation activity of mYAP and that this intrinsic transcription activation domain is distinct from the WW domains. Unlike the transcriptional co-activator, p300, YAP did not show any histone acetyltransferase activity (Figure 4C).

YAP stimulates transcription by binding to the PY motif

We next tested the possibility that YAP stimulates transcription by binding to the PY motif using exogenously expressed YAP and G-PY33 together with the GAL4 site-containing reporter. The activity was assayed in L1210 cells in which the YAP transcript was not detected. In this cell line, G-PY33 and the GAL4 DNA-binding domain showed essentially the same activity (Figure 5A).

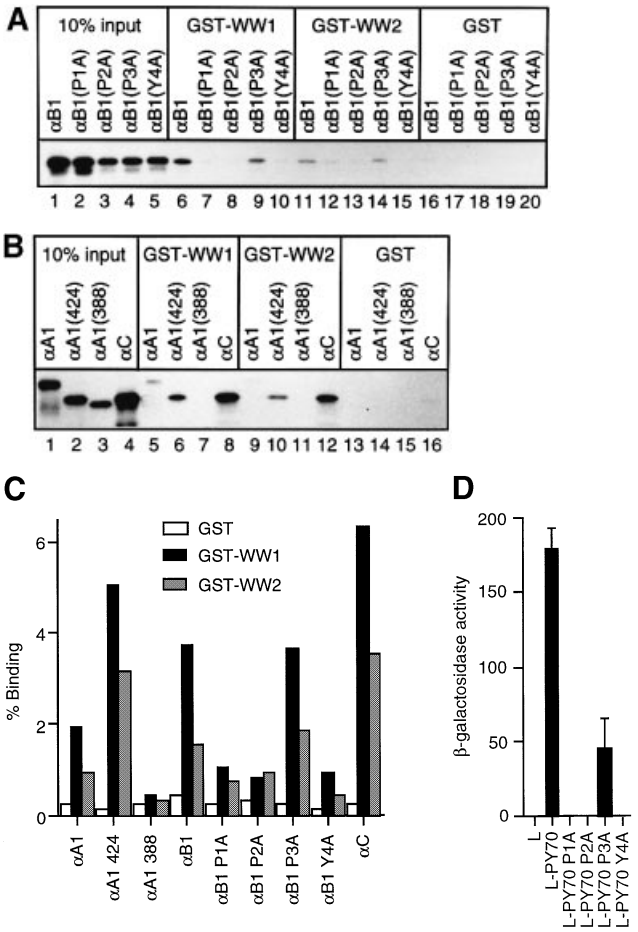

Fig. 3. YAP interacts with the PY motif of PEBP2α. (A), (B) and (C): GST pull-down assays. αB1 and its PY motif mutants (**A**), αC, αA1 and its derivatives (**B**) were translated *in vitro* and pulled down with GST, GST–mYAP–WW-domain-1 (GST–WW1) or GST–mYAP–WW-domain-2 (GST–WW2) bound beads. The proteins bound to the beads and 10% of the *in vitro* translated proteins used in the binding assay (10% input), were separated by 10% SDS–PAGE. (**C**) The band intensities in (A) and (B) were quantified and the relative binding efficiency (band intensity of the bound protein against that of the input protein) is indicated. (**D**) Yeast L40 strain was transfected with both pGAD-YAP and a pBTM116-based plasmid expressing the LexA DNA-binding domain fused to αB1(302–371) or its PY motif mutant as indicated. The β-galactosidase activities (units) of three independent transformants were determined. L, LexA DNA-binding domain; PY70, αB1(302–371).

Co-expression of increasing amounts of the YAP-expressing plasmid clearly increased the transactivation activity of G-PY33 by up to 5-fold in a dose-dependent manner (Figure 5A). Furthermore, mutants of G-PY33 harboring P1A, P2A or Y4A mutations no longer responded to YAP for transcription activation, while the P3A mutant was slightly stimulatory (Figure 5B). This transcription stimulatory activity in the presence of YAP correlated well with the binding activity of YAP to the PY motif described above, indicating that exogenously expressed YAP confers transcription stimulation activity by binding on the PY motif.

To test whether the PY motif alone is sufficient for YAP stimulation of transcription, we tested whether G-PY12 can be used by YAP as a docking site. G-PY12 activated transcription ~1.6-fold in the presence of YAP, while the GAL4 DNA-binding domain alone or the amino acid substitution mutant, P1A (G-PY12 P1A), did not (Figure 5B), indicating that YAP was able to bind to, and stimulate transcription through the PY motif on the 12 amino acid oligopeptide. G-PY33, having 21 amino acids more, responded to YAP better; presumably the longer peptide could form a more stable complex with YAP. The reason for stability could be either that the longer peptide assumes a more natural conformation to interact with YAP or that an additional protein is required for interaction with YAP.

Next, we tested whether YAP could co-activate transcription through the natural binding site present on the full-length PEBP2α protein. We obtained ambiguous results using several reporter systems in a number of differentiated cells, possibly reflecting the relative interplay between YAP and other factors involved in transcriptional regulation, as was observed in similar experiments performed for CBP/p300. For example, the activity of SV40 enhancer was not stimulated by exogenously expressed p300 unless the endogenous p300 was sequestered by adenovirus E1A (Eckner *et al.*, 1994). In P19 embryonal carcinoma cells, however, we were able to obtain clear-cut results. For this experiment, we employed a reporter containing three tandem copies of the TGFβ-responsive element from the Ig Cα promoter which contains the PEBP2 binding site (Lin and Stavnezer, 1992; Shi and Stavnezer, 1998). In this system, αA1, αB1 and αC mildly stimulated reporter activity (Figure 5C) and the activity was entirely dependent on the intact PEBP2 binding site (data not shown). Co-transfection of YAP further stimulated the activity in a dose-dependent manner in all cases, and especially in the case of αC (Figure 5C). A possible explanation for the higher activity of αC will be presented below. Stimulation of αB1 activity by YAP was virtually eliminated when the PY motif was mutated (Figure 5D). This was also shown to be the case for the mutated version of G-PY33 (Figure 5B).

The results presented above show that YAP stimulates transcription by binding to the PY motif of PEBP2αA1, αB1 and αC.

Endogenous YAP is partly responsible for the transactivation of the osteocalcin promoter by αA

To obtain evidence that endogenous YAP actually participates in transcription activation, the effect on G-PY33 activity of a C-terminally truncated YAP lacking the transcription activation domain was examined. As shown in Figure 6B, the C-terminally truncated YAP virtually abolished G-PY33 activity, suggesting that it inhibited endogenous activity by acting in a *trans*-dominant manner in NIH 3T3 cells.

We then studied the role of YAP at a natural promoter using the minimal osteocalcin promoter whose activity is controlled by αA (Ducy *et al.*, 1997). Osteocalcin promoter activity was enhanced by exogenously expressed αA1 and even more efficiently by the C-terminally truncated form, αA1(424) carrying the PY motif, but not by the still shorter one, αA1(388) lacking the PY motif (Figure 6C, lanes 1, 4, 7 and 10). This activation correlated well with the binding activity of αA1 to the WW domain of YAP examined in Figure 3B. The activities of αA1 and αA1(424) progressively decreased as the amount of the dominant negative form increased (Figure 6, lanes 4–9),

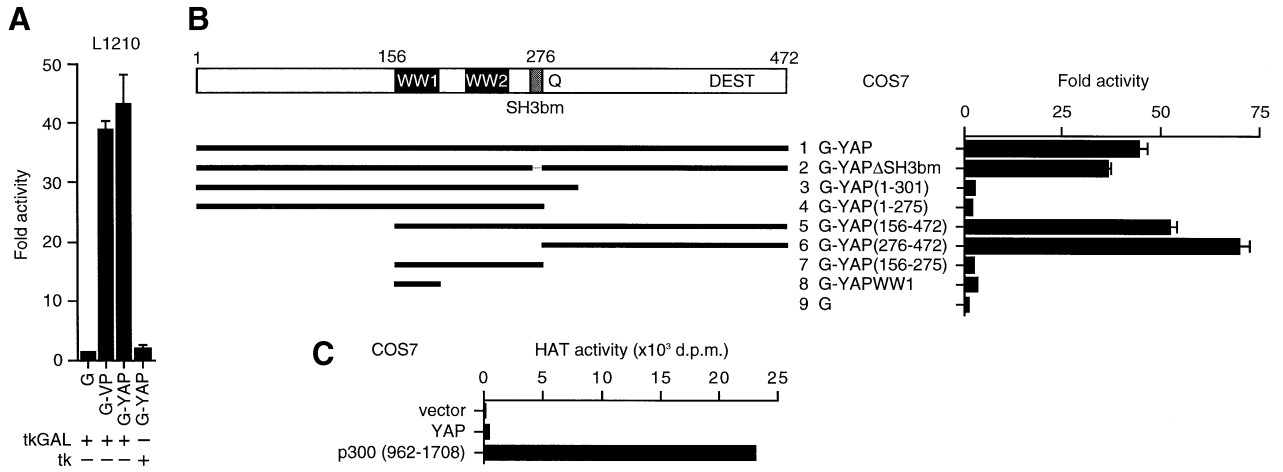

Fig. 4. YAP contains a strong, intrinsic transactivation domain. (**A**) L1210 cells were transfected with 2 μg of tk-GALpx3-LUC (tk-GAL) or tk-luc (tk), 2 ng of pRL-EF, 16 μg of pEF-BOS backbone vector and 2 μg of the plasmid expressing the indicated GAL4(1–93) fusion protein. The luciferase activities relative to those obtained with GAL4(1–93) are shown. (**B**) Schematic illustration of mouse YAP and its deletion constructs, and the transcriptional activity of each fragment fused to GAL4(1–93). COS7 cells were transfected with 0.2 μg of tk-GALpx3-LUC, 1 ng of pRL-EF, 0.6 μg of pEF-BOS backbone vector and 0.2 μg of the plasmid expressing the indicated GAL4(1–93)–YAP fusion protein. The luciferase activities relative to those of GAL4(1–93) are indicated. (**C**) COS7 cells were transfected the plasmids expressing the indicated Xpress-tagged protein and HAT activities were assayed as described in Materials and methods.

while the activity of αA1(388) and basal activity remained nearly the same (Figure 6, lanes 1–3 and 10–12). These results suggest that endogenous YAP or YAP-related protein stimulates the activity of the osteocalcin promoter.

Binding of YAP to PEBP2α is regulated by an auto-inhibitory domain, ID

We reported previously that the AD of αB1 is intramolecularly regulated by an adjacent auto-inhibitory region termed the inhibitory domain (ID) as illustrated in Figure 7A (Kanno *et al.*, 1998). This region is also well conserved in αA1. In the case of αC, only the N-terminal half of the region is highly conserved (Figure 7B). The results shown above in Figure 3B indicated that YAP binding was more efficient to the C-terminally truncated αA1(424) than to the full-length αA1. Therefore, we investigated whether the phenomenon observed in Figure 3B is related to the activity of the ID. As expected, stimulation of the reporter activity by YAP was strongly enhanced when the ID was removed from αB1 (Figure 7C). Again, this enhancement was entirely dependent on the intact PY motif (Figure 7D). Similarly, a deletion of the C-terminal region comprising the ID resulted in an increase in reporter activity in the presence of YAP (Figure 7E), indicating that αA1 also possesses an ID.

The results suggested that the ID of PEBP2 negatively regulates AD(TE2) activity by regulating the binding of YAP to the PY motif.

Discussion

Based on the following results, we conclude that the PY motif of PEBP2α represents a new transactivation domain which functions by recruiting the strong transactivator, YAP, to transcriptional promoters through a specific PY interaction element, the WW domain, present in the middle region of YAP. (i) Mutations in the PY motif of the TE2 subregion of the activation domain of PEBP2α abolished AD(TE2) activity. (ii) The WW domain of YAP interacted specifically with the PY motif of PEBP2α. (iii) YAP displayed strong transcription-stimulating activity and YAP conferred transcription activation through the PY motif. (iv) PY motif-dependent AD(TE2) activity could be observed only in cells where YAP was expressed and exogenous expression of YAP in cells lacking endogenous YAP expression stimulated PY motif-dependent AD(TE2) activity.

YAP is a co-activator of the PEBP2α family

Vertebrate PEBP2α contains a conserved PY motif within the TE2 subregion of its AD. YAP was able to bind to, and potentiate the transcription activity of all members of the mammalian PEBP2α family. We have provided evidence to show that the interaction of YAP with the PY motif of αA1 is largely responsible for the transcription stimulation of a natural αA1-dependent promoter identified in the osteocalcin gene, suggesting that αA1 uses YAP as a transcriptional co-activator during the process of osteogenesis. Indeed, we detected PY motif activity in bone-related cell lines MC3T3-E1 and ROS17/2.8. The major role of αB1 is carried out in hematopoietic cells. However, YAP appears not to be expressed in many hematopoietic cell lines. Furthermore, the PY motif of αB1 does not seem to be functional in several hematopoietic cell lines including L1210 (B cells), Jurkat (T cells) and U937 (monocyte) (data not shown). In addition, YAP transcripts were hard to detect in these cells. It will be necessary to determine whether YAP actually functions in hematopoietic cells, and if so, whether this expression correlates with a certain stage of differentiation. αB1 is also expressed in cells other than hematopoietic lineages. For example, both αB1 (Zhu *et al.*, 1994) and YAP (Sudol *et al.*, 1995) are known to be expressed in muscle cells. The Ig Cα promoter is stimulated specifically by αC to induce immunoglobulin class switching in B cells (Shi and Stavnezer, 1998), where YAP is not expressed. Given the fact that the PY motif is well conserved from *Xenopus* to human in all three PEBP2α subunits, αC is also likely to be a natural

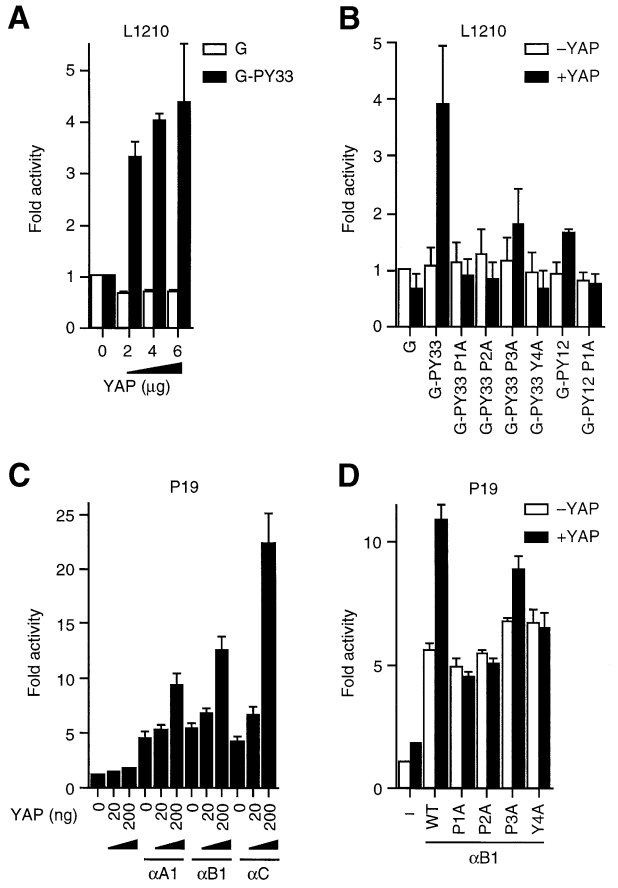

Fig. 5. Co-activation of transcription by PEBP2α and YAP. (**A**) L1210 cells were transfected with 2 μg of tk-GALpx3-LUC, 2 ng of pRL-EF and the indicated amounts of pEF-YAP together with 2 μg of the plasmid expressing GAL4(1–93) (G, open bars) or G-PY33 (filled bars). The luciferase activities relative to those obtained with GAL4 (1–93) are shown. (**B**) L1210 cells were transfected with 2 μg of tk-GALpx3-LUC, 2 ng of pRL-EF and 2 μg of the indicated pG-based plasmid coding for one of the GAL4(1–93)–PY motif fusion proteins and its derivative together with (filled bars) or without (open bars) 4 μg of pEF-YAP. The luciferase activities relative to those of GAL4(1–93) without YAP are indicated. (**C**) P19 cells were transfected with 0.2 μg of pFL56-3, 1 ng of pRL-EF, 0.2 μg of a pEF-BOS-based effector plasmid expressing full-length αA1, αB1 or αC, and the indicated amount of pcDNA-YAP. Luciferase activities relative to those obtained without effector plasmids are shown. (**D**) P19 cells were transfected with 0.2 μg of pFL56-3, 1 ng of pRL-EF, and 0.2 μg of the pEF-BOS-based effector plasmid expressing full-length αB1 or its PY motif mutant as indicated, together with (filled bars) or without (open bars) 0.2 μg of pcDNA-YAP. Luciferase activities relative to those obtained with no effector are shown.

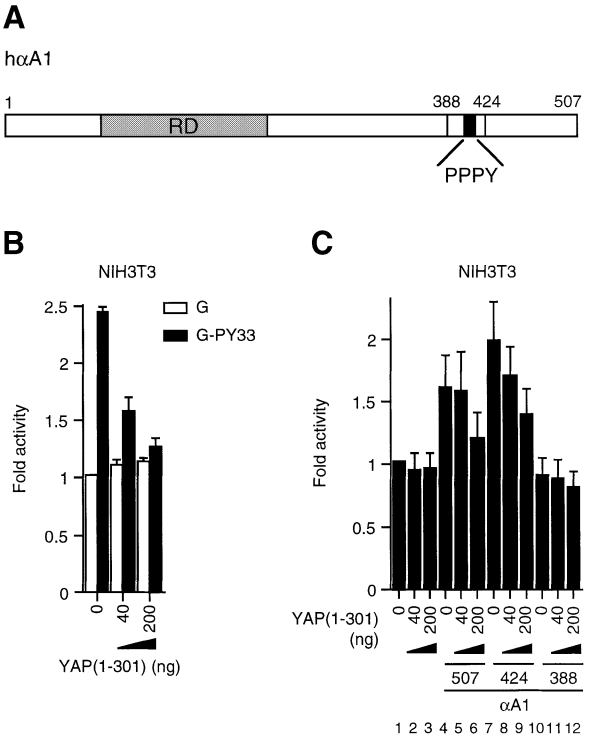

Fig. 6. Effects of a dominant negative form of YAP on endogenous PEBP2 activity. (**A**) Schematic illustration of human PEBP2αA1. RD, Runt domain. (**B**) NIH 3T3 cells were transfected with 0.2 μg of tk-GALpx3-LUC, 1 ng of pRL-EF, 0.2 μg of pG (open column) or pG-PY33 (filled bars), and the indicated amount of pcDNA -YAP (1–301). Luciferase activities relative to those obtained with GAL4 (1–93) are shown. (**C**) NIH 3T3 cells were transfected with 0.2 μg of p147-luc, 1 ng of pRL-EF, 0.1 μg of pEF-β2, 0.2 μg of the indicated pEF-BOS-based effector plasmid and the indicated amount of pcDNA-YAP(1–301). Luciferase activities relative to those obtained with no effector are shown.

partner of YAP for stimulation of certain promoters in certain cell types. Further studies are required to identify natural promoters which are stimulated by PEBP2α subunits in conjunction with YAP.

We showed that binding of YAP to the PY motif of αA1 and αB1 is inhibited by the ID, the adjacent autoinhibitory region in PEBP2α. We speculate that an external signal which activates PEBP2 activity would remove the ID and 'open' AD to allow the association of the potent transactivator, YAP, with the PY motif (Figure 8). In the case of other co-activators, this type of regulation of protein–protein association has been well documented. For example, CBP binds to the transcription factor CREB only when Ser133 in the activation domain is phosphorylated by cAMP-dependent kinase (Chrivia *et al.*, 1993). A co-activator of nuclear receptors, SRC-1/NCoA-1, binds to the C-terminal region of the receptors in a ligand-dependent manner (Onate *et al.*, 1998). It is noteworthy that αB1 is a substrate of MAP kinase and that phosphorylation by MAP kinase increases the transactivation activity of αB1 (Tanaka *et al.*, 1996). Thus, it seems possible that phosphorylation by MAP kinase is responsible for unmasking of the AD. From this point of view, it is intriguing to note that αC lacks the target sites of MAP kinase phosphorylation which are well conserved between αA1 and αB1 (Bae *et al.*, 1995). Interestingly, the amino acid sequences of ID are well conserved between αA1 and αB1, whereas the equivalent region of αC is less well conserved, suggesting that the ID of αC may not be as efficient as those of αA1 and αB1. Consistent with this notion, YAP potentiates the transcription activity of αC more efficiently than any other member of the PEBP2α family (Figure 6C).

Recently, a general transcriptional co-activator, p300, was shown to interact with AML1/αB1 between the Runt domain and the AD (Kitabayashi *et al.*, 1998). Thus, the relationship between p300 and YAP in potentiating the transcription activity of PEBP2 must be examined.

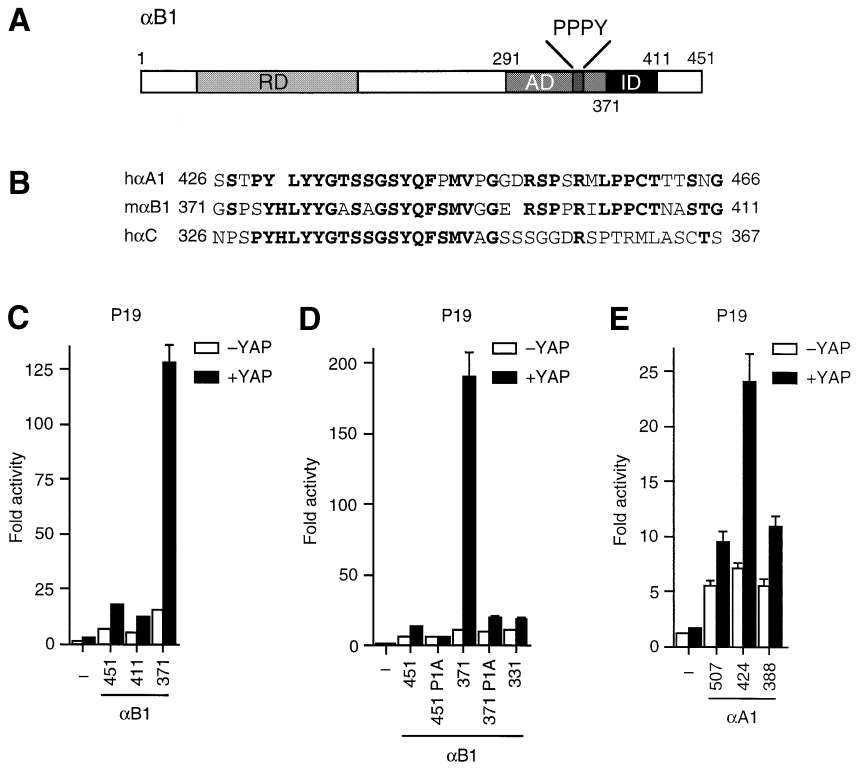

Fig. 7. Negative regulation of the interaction between PEBP2α and YAP by the ID. (**A**) Schematic illustration of mouse αB1. RD, Runt domain; AD, activation domain; ID, inhibitory domain. (**B**) Alignment of the ID regions of mammalian PEBP2α subunits. Bold type letters indicate identical amino acids in two α subunits. hαA1, human αA1; mαB1, mouse αB1; hαC, human αC. (**C**, **D** and **E**) P19 cells were transfected with 0.2 μg of pFL56-3, 1 ng of pRL-EF and 0.2 μg of the indicated pEF-BOS-based effector plasmid, together with (filled bars) or without (open bars) 0.2 μg of pcDNA-YAP. Luciferase activities relative to those obtained with no effector are shown.

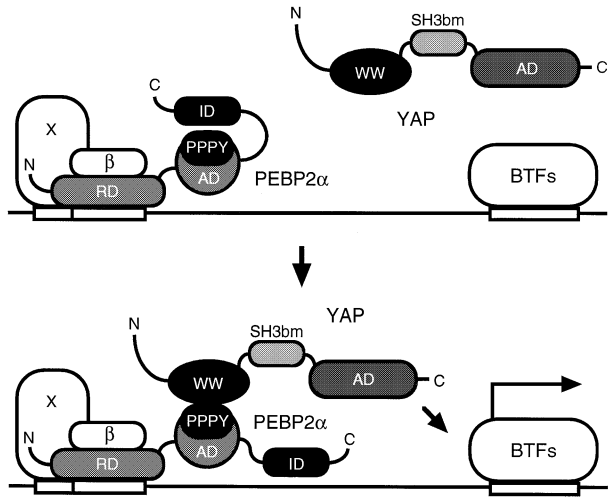

Fig. 8. A diagrammatic representation of the interaction between PEBP2α and YAP. For details, see the text. WW, WW domain; SH3bm, SH3-binding motif; N, amino-terminal; C, carboxy-terminal; RD, Runt domain; X, putative factor X which cooperatively acts with PEBP2; PY, PY motif; DBD, DNA-binding domain; BTFs, basal transcription factors.

YAP is a transcriptional co-activator reminiscent of VP16

CBP associates directly with transcription factors CREB (Chrivia *et al.*, 1993), c-Jun (Arias *et al.*, 1994; Bannister *et al.*, 1995), c-Fos (Bannister and Kouzarides, 1995) and STAT1 (Horvai *et al.*, 1997) through distinct portions of its polypeptide chain. CBP also forms a complex with other co-activators such as SRC-1/NCoA-1, p/CIP and p/CAF and associates with nuclear receptors through SRC-1/NcoA-1 (Torchia *et al.*, 1998). In contrast, interaction between the WW domain and the PY motif is highly specific, indicating that YAP is a more specialized co-activator of a subset of transcription factors. In this respect, YAP is reminiscent of herpesvirus VP16, which contains an intrinsic, strong activation domain and specifically binds to Oct1 to convert this relatively weak transactivator to a strong one (Kristie and Sharp, 1990; Stern and Herr, 1991). Since YAP contains a strong activation domain equivalent to that of VP16, binding of YAP would be expected to change weak activators to stronger activators. Indeed, the AD of PEBP2α has only weak transcription activation activity (Kanno *et al.*, 1998). The AD of VP16 is rich in acidic amino acids. The AD of YAP also includes a region rich in acidic amino acids. In addition, both ADs show strong activity in yeast cells. Interestingly, neither VP16 nor YAP show detectable HAT activity. These observations suggest that VP16 and YAP stimulate transcription by a similar mechanism. Since the AD of VP16 interacts directly with components of the basal transcription machinery including TFIIB to stimulate transcription (Lin *et al.*, 1991; Roberts *et al.*, 1993), it will be necessary to examine whether this is also the case for YAP.

The PY motif as a transcription activation domain

In this study, we showed that the PY motif functions as a transcription activation domain. As described in the Introduction, the PY motif is present in the ADs of many

transcription factors besides those of the PEBP2α family. Mosser *et al.* (1998) have recently shown that a mutation in one of the two PY motifs in the transcription activation domain of the p45 subunit of mouse NF-E2 reduces its transactivation activity. In addition, Gavva *et al.* (1997) have shown that the WW domains of YAP bind to the PY motif of NF-E2 *in vitro*. In AP2, the PY motif is present in the 60 amino acid proline-rich AD (Williams and Tjian, 1991). Furthermore, the PY motif is conserved in the AP2 family comprising AP2α, AP2β and AP2.2 in mouse, suggesting the importance of the motif for AP2 function. Interestingly, the expression of AP2.2 is induced during the neural differentiation of P19 cells which express YAP (Oulad-Abdelghani *et al.*, 1996).

Smad family proteins which are involved in the transcriptional regulation mediated by TGFβ/BMP signaling pathways also contain the conserved PY motif, although the motif is located outside of the previously determined transcription activation domain. Since the motif is well conserved in all members of the Smad family in mammalian cells with the exception of Smad4 (Rotin, 1998), it will be worth examining the possible involvement of YAP in Smad activities. We also noticed that the recently identified p73 (Kaghad *et al.*, 1997), p63 (Yang *et al.*, 1998) and p51B (Osada *et al.*, 1998) homologs of the tumor suppressor protein p53 contain a PY motif in their C-terminal regions which are not conserved in p53. Since these p53 homologs are thought to function as DNA-binding transcription factors, it will be interesting to examine whether these PY motifs function as transactivation domains. In any event, we propose that the PY motif functions as a transcription activation domain in a subset of transcription factors and utilizes YAP as a co-activator for the efficient stimulation of transcription.

Does YAP transmit a signal from Yes/Src/Crk to transcription factors?

YAP was originally isolated as a c-Yes-associated protein. *In vitro*, YAP was found to bind to the SH3 domain of c-Yes, c-Src and Crk, suggesting that YAP plays a role in a signaling pathway that transmits signals from proteins located in the cytoplasm to transcription factors in the nucleus (Sudol, 1994). Together with our results, we speculate that the behavior of YAP is reminiscent of STAT and Smad family proteins. STAT proteins are activated by tyrosine kinase associated with growth factor receptors and are translocated into the nucleus to form complexes with transcription factors (Darnell, 1997). Likewise, Smad proteins are activated by TGFβ/BMP/activin receptors having Ser/Thr kinase activity and are translocated into the nucleus where they participate in transcription stimulation (Massague, 1998). YAP may also be signal-regulated and transmit signals from Yes/Src/Crk to the nucleus to regulate the expression of specific target genes. This intriguing possibility will be a subject of future studies.

Since there are many WW domain-containing proteins with different functions in the cell (Rotin, 1998), it is possible that a WW domain-containing protein(s) other than YAP associate with the PY motif of transcription factors to perform functions distinct from those of YAP in certain situations. Indeed we isolated several WW domain-containing proteins besides YAP in our two-hybrid screening. We speculate that the PY motif–WW domain interaction constitutes a part of a complex network of multiple signal transduction pathways. Further efforts to identify the binding partners of the PY motif of various transcription factors should contribute to our understanding of the overall network of cellular signaling involved in transcriptional regulation.

Materials and methods

Plasmids

For the expression of minimum GAL4 DNA-binding domain [GAL4 (1–93)] fusion proteins in mammalian cells, the pG vector was constructed by deleting a fragment containing amino acids 94–147 of the GAL4 DNA-binding domain of pCMX-GAL4 (Willy *et al.*, 1995). Mouse YAP (mYAP) cDNA, the DNA fragment coding the AD of VP16 (amino acids 413–490) derived from pSGGAL4-VP16 (Fujii *et al.*, 1991), the PCR-amplified 33 amino acid PY motif-containing fragment of PEBP2αB1 (amino acids 339–371) and the synthetic oligonucleotides coding for the peptide sequences HTYLPPPYPGSS and HTYLAP-PYPGSS were inserted into pG in-frame with the coding sequence of the GAL4(1–93), resulting in pG-YAP, pG-VP16, pG-PY33, pG-PY12 and pG-PY12 P1A, respectively. The deletion mutants of pG-YAP were constructed by either exonuclease III/mung bean nuclease digestion or PCR amplification of the corresponding region. For expression of the LexA DNA-binding domain fusion protein in yeast cells, PCR products encompassing amino acids 292–371 and 302–371 of PEBP2αB1 were inserted into pBTM116 (Bartel *et al.*, 1993), resulting in pBTM-αB1(292–371) and pBTM-αB1(302–371), respectively. The alanine substitution mutations were introduced into the PY motif of pG-PY33 and pBTM-αB1(302–371) by PCR-based mutagenesis. mYAP cDNA was subcloned into pGAD424 (Clontech), resulting in pGAD-YAP which expresses the GAL4 activation domain fused to mYAP. pEF-αB1 series [αB1, αB1(1–411), αB1(1–371) and αB1(1–331)] (Kanno *et al.*, 1998), pEF-hαA1 series [hαA1, hαA1(1–424), hαA1(1–388)] (a gift from Y.W.Zhang), pEF-αC (Bae *et al.*, 1995) and pEF-β2 (Lu *et al.*, 1995) express PEBP2αB1, human PEBP2αA1, PEBP2αC and PEBP2β2, respectively, in mammalian cells. The PY motif mutants of pEF-αB1 and pEF-αB1(1–371) were constructed by replacing the PY motif-containing fragments with the appropriate fragments from pBTM-αB1(302–371) PY motif mutants. mYAP cDNA was subcloned into pEF-BOS lacking the SV40 origin (Mizushima and Nagata, 1990; Kanda *et al.*, 1994), resulting in pEF-YAP. To express FLAG-tagged YAP, a synthetic oligonucleotide encoding the FLAG epitope was fused to the N-terminus of YAP cDNA, and then inserted into pEF-BOS resulting in pEF-YAP-N-FLAG. pKS-αB1 (Ogawa *et al.*, 1993b), pKS-αC (Bae *et al.*, 1995) and the pKS-hαA1 series [hαA1, hαA1(1–424), hαA1 (1–388)] (a gift from Y.W.Zhang) were used for *in vitro* translation. A series of pSK(+)-αB1 PY motif mutants were made by subcloning the cDNAs from the pEF-αB1 PY motif mutants into pBluescript SK(+) (Stratagene). The pcDNA3.1/His (A, B and C) series (Invitrogen) were used for the expression of Xpress-tagged protein in mammalian cells. mYAP cDNA was subcloned into pcDNA3.1/HisC (Invitrogen), resulting in pcDNA-YAP. A fragment containing amino acids 1–301 of YAP derived from pG-YAP(1–301) was inserted into pcDNA3.1/HisC to make pcDNA-YAP(1–301). An *Eco*RI–*Aat*I fragment encoding p300(962–1708), which contains histone acetyl transferase activity (Ogryzko *et al.*, 1996), was cut out from pCMV-p300 (Eckner *et al.*, 1994) inserted into pcDNA3.1/HisB, resulting in pcDNA-p300(962–1708). pGEX-5X-2 (Pharmacia Biotech.), pGEX2TKmYAPWW1 and pGEX2TKmYAPWW2 (Gavva *et al.*, 1997) were used for bacterial expression of GST fusion proteins for *in vitro* GST pull-down assays. p147-luc (Ducy and Karsenty, 1995), ptk-GALpx3-LUC (Kanno *et al.*, 1998), ptk-luc (Zhang *et al.*, 1997b) and pFL56-3 (Lin and Stavnezer, 1992), which express Firefly luciferase, were used as reporter plasmids in the reporter assays. pRL-EF (Kim *et al.*, 1999), which expresses *Renilla* luciferase, was used as an internal control in the reporter assay.

Cell culture

P19 cells, a mouse embryonal carcinoma cell line, were cultured at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). NIH 3T3 mouse fibroblasts and COS7 cells were cultured at 37°C in DMEM supplemented with 10% FBS. MC3T3-E1 calvarial cells were cultured at 37°C in Minimum Essential Medium Alpha Medium supple-

mented with 10% FBS. L1210 mouse B-cell lines were maintained at 37°C in RPMI 1640 supplemented with 10% FBS.

cDNA library constructions

Poly(A)$^+$ RNA was isolated from P19 cells by using an RNA extraction kit (Pharmacia Biotech.) and an mRNA purification kit (Pharmacia Biotech.). Either oligo(dT) or random primed cDNAs were synthesized by the Gubler–Hoffmann method (Gubler and Hoffman, 1983) and were ligated with *Eco*RI–*Not*I adopters (Pharmacia Biotech.). The adopter-ligated cDNAs were inserted into pGAD424 at the *Eco*RI site, and then the products were transformed into *Escherichia coli* DH10B. The number of independent clones/insert size of oligo(dT) and random primed cDNA libraries were ~2×10^7 clones/0.5–7 kbp and 2×10^6 clones/0.3–5 kbp, respectively.

Yeast two-hybrid screen and assays

pBTM116-αB1(292–371) transformed into the L40 yeast strain (Bartel *et al.*, 1993) was used for the screening of the mixture of oligo(dT) and random primed mouse P19 cell cDNA libraries described above using Matchmarker™ Two-Hybrid System (Clontech) according to the manufacturer's instructions. From ~1×10^8 original transformants, 163 HIS$^+$/lacZ$^+$ clones were isolated. Library plasmids extracted from these positive clones were retransformed into the L40 strain along with the yeast expression plasmid for LexA-αB1(292–371) or LexA. Of these, 14 clones were HIS$^+$/lacZ$^+$ only when co-transfected with LexA-αB1(292–371) and were sequenced. The liquid β-galactosidase assay was performed using the Matchmarker™ Two-Hybrid System (Clontech) according to the manufacturer's instruction for quantitative analysis.

RT–PCR

Poly(A)$^+$ RNA was isolated from batches of 10^7 cells by using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech.). mRNA was reverse-transcribed by Superscript II RNase H$^-$ reverse transcriptase (Gibco-BRL) and treated with RNase H. The PCR was carried out using the first strand cDNA or the poly(A)$^+$ RNA as template. PCR products obtained by using 1% of original amount of poly(A)$^+$ RNA isolated from 10^7 cells were separated by electrophoresis on 2% agarose gel, and stained with ethidium bromide. The primers used were as follows; mYAP sense primer, 5′-CCCTGATGATGTACCACTGCC-3′ [nucleotides (nt) 654–674 of mouse YAP]; mYAP antisense primer, 5′-CCACTGTTAAGAAAGGGATCGG-3′ (nt 1271–1251 of mouse YAP); mβ_2-microglobulin sense primer, 5′-GACCGGCTTGTATGCTATCC-3′ (nt 97–116 of mouse β_2-microglobulin); mβ_2-microglobulin antisense primer, 5′-TCTCGATCCCAGTAGACGGT-3′ (nt 405–386 of mouse β_2-microglobulin).

Indirect immunofluorescence staining

NIH 3T3 cells were transfected with FuGENE™6 (Boehringer Mannheim). The transfected cells were seeded onto chamber slides (Nalge Nunc, Naperville, IL). After 24 h incubation, the transfected cells were fixed, permeabilized and successively stained with anti-FLAG M2 antibody (Kodak) and FITC-conjugated goat anti-mouse antibody (Biosource).

In vitro protein–protein binding assays

[^{35}S]methionine-labeled proteins were synthesized *in vitro* by using a transcription–translation kit (Promega). GST fusion proteins were expressed in *E.coli* and immobilized on glutathione–Sepharose 4B beads (Pharmacia Biotech.). Then the beads were incubated for 2 h at 4°C with ^{35}S-labeled proteins in 500 μl of GPD buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40). Beads were washed four times with 500 μl of GPD buffer. Bound proteins were eluted by boiling in standard SDS gel loading buffer, separated by 10% SDS–PAGE and visualized by autoradiography. The band intensities were quantified by BAS2000 (Fuji).

Transient transfection and reporter gene assays

For reporter assays, the cells were transfected with the plasmids indicated in the figure legends. In Figures 5, 6 and 7, the total amount of transfected DNA was set to 20 μg (L1210 cells) or 1 μg (P19 and NIH 3T3 cells) by adding pEF-BOS (L1210 cells) or pcDNA3.1/HisA (P19 and NIH 3T3 cells). P19, NIH 3T3, COS7 and MC3T3-E1 cells in 6-well microplates were transfected by FuGENE™6 and the cells were harvested 36 h after transfection. L1210 cells (1×10^7 cells in 300 μl of serum-free RPMI 1640 medium in a 0.4 cm cuvette) were transfected by electroporation at a setting of 950 μF/250 V and at room temperature using Gene Pulser (Bio-Rad) and the transfected cells were harvested 24 h after transfection. Firefly and *Renilla* luciferase activities were assayed with the dual luciferase assay system from Promega with Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer's instruction. Firefly luciferase activity was normalized with respect to *Renilla* luciferase activity. All experiments in 'transfection and reporter gene assays' were performed at least three times.

IP HAT assay

The IP HAT assay was preformed by the essentially the same method as that described by Bannister and Kouzarides (1996) using anti-Xpress antibody (Invitrogen) and COS7 cells cultured in a 15 cm dish and transfected by FuGENE™6 with 2 μg of a pcDNA3.1/His-based plasmid.

Acknowledgements

We thank M.Sudol for pGEX2TKmYAPWW1 and pGEX2TKmY-APWW2; G.Karsenty for p147-luc; K.Umesono for pCMX-GAL4; and K.K.Yokoyama for pCMV-p300. This work was supported by a Grant-in-aid for Priority Areas in Cancer Research from the Ministry of Education and Science, Japan to Y.I. (No. 09253220).

References

Andre,B. and Springael,J.Y. (1994) WWP, a new amino acid motif present in single or multiple copies in various proteins including dystrophin and the SH3-binding Yes-associated protein YAP65. *Biochem. Biophys. Res. Commun.*, **205**, 1201–1205.

Arias,J., Alberts,A.S., Brindle,P., Claret,F.X., Smeal,T., Karin,M., Feramisco,J. and Montminy,M. (1994) Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature*, **370**, 226–229.

Bae,S.C. *et al*. (1994) PEBP2αB/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. *Mol. Cell. Biol.*, **14**, 3242–3252.

Bae,S.C., Takahashi,E., Zhang,Y.W., Ogawa,E., Shigesada,K., Namba,Y., Satake,M. and Ito,Y. (1995) Cloning, mapping and expression of PEBP2αC, a third gene encoding the mammalian Runt domain. *Gene*, **159**, 245–248.

Baichwal,V.R. and Tjian,R. (1990) Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain δ: differences between v- and c-Jun. *Cell*, **63**, 815–825.

Bannister,A.J. and Kouzarides,T. (1995) CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J.*, **14**, 4758–4762.

Bannister,A.J. and Kouzarides,T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature*, **384**, 641–643.

Bannister,A.J., Oehler,T., Wilhelm,D., Angel,P. and Kouzarides,T. (1995) Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation *in vivo* and CBP binding *in vitro*. *Oncogene*, **11**, 2509–2514.

Barlev,N.A., Candau,R., Wang,L., Darpino,P., Silverman,N. and Berger,S.L. (1995) Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein. *J. Biol. Chem.*, **270**, 19337–19344.

Bartel,P., Chien,C.-T., Sternglanz,R. and Fields,S. (1993) Using the two-hybrid system to detect protein–protein interactions. In Hartley,D.A. (ed.), *Cellular Interactions in Development: A Practical Approach*. Oxford University Press, Oxford, UK, pp. 153–179.

Bedford,M.T., Chan,D.C. and Leder,P. (1997) FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands. *EMBO J.*, **16**, 2376–2383.

Bork,P. and Sudol,M. (1994) The WW domain: a signalling site in dystrophin? *Trends Biochem. Sci.*, **19**, 531–533.

Bork,P. and Margolis,B. (1995) A phosphotyrosine interaction domain. *Cell*, **80**, 693–694.

Brownell,J.E., Zhou,J., Ranalli,T., Kobayashi,R., Edmondson,D.G., Roth,S.Y. and Allis,C.D. (1996) *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, **84**, 843–851.

Chen,H.I. and Sudol,M. (1995) The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc. Natl Acad. Sci. USA*, **92**, 7819–7823.

Chrivia,J.C., Kwok,R.P., Lamb,N., Hagiwara,M., Montminy,M.R. and Goodman,R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*, **365**, 855–859.

Darnell,J.E.,Jr (1997) STATs and gene regulation. *Science*, **277**, 1630–1635.

Ducy,P. and Karsenty,G. (1995) Two distinct osteoblast-specific *cis*-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.*, **15**, 1858–1869.

Ducy,P., Zhang,R., Geoffroy,V., Ridall,A.L. and Karsenty,G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*, **89**, 747–754.

Eckner,R., Ewen,M.E., Newsome,D., Gerdes,M., DeCaprio,J.A., Lawrence,J.B. and Livingston,D.M. (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.*, **8**, 869–884.

Ermekova,K.S., Zambrano,N., Linn,H., Minopoli,G., Gertler,F., Russo,T. and Sudol,M. (1997) The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of *Drosophila* enabled. *J. Biol. Chem.*, **272**, 32869–32877.

Ferguson,K.M., Lemmon,M.A., Sigler,P.B. and Schlessinger,J. (1995) Scratching the surface with the PH domain. *Nature Struct. Biol.*, **2**, 715–718.

Fujii,M., Tsuchiya,H. and Seiki,M. (1991) HTLV-1 Tax has distinct but overlapping domains for transcriptional activation and for enhancer specificity. *Oncogene*, **6**, 2349–2352.

Garnier,L., Wills,J.W., Verderame,M.F. and Sudol,M. (1996) WW domains and retroviral budding. *Nature*, **381**, 744–745.

Gashler,A.L., Swaminathan,S. and Sukhatme,V.P. (1993) A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. *Mol. Cell. Biol.*, **13**, 4556–4571.

Gavva,N.R., Gavva,R., Ermekova,K., Sudol,M. and Shen,C.-K.J. (1997) Interaction of WW domains with hematopoietic transcription factor p45/NF-E2 and RNA polymerase II. *J. Biol. Chem.*, **272**, 24105–24108.

Gubler,U. and Hoffman,B.J. (1983) A simple and very efficient method for generating cDNA libraries. *Gene*, **25**, 263–269.

Hofmann,K. and Bucher,P. (1995) The rsp5-domain is shared by proteins of diverse functions. *FEBS Lett.*, **358**, 153–157.

Horvai,A.E., Xu,L., Korzus,E., Brard,G., Kalafus,D., Mullen,T.-M., Rose,D.W., Rosenfeld,M.G. and Glass,C.K. (1997) Nuclear integration of JAK/STAT and Ras/AP-1 signaling by CBP and p300. *Proc. Natl Acad. Sci. USA*, **94**, 1074–1079.

Ito,Y. (1996) Structural alterations in the transcription factor PEBP2/CBF linked to four different types of leukemia. *J. Cancer Res. Clin. Oncol.*, **122**, 266–274.

Ito,Y. (1997) The runt protein and its companion PEBP2: a close link between this transcription factor and AML. *Leukemia*, **11**, 279–280.

Ito,Y. and Bae,S.-C. (1997) The Runt domain transcription factor, PEBP2/CBF, and its involvement in human leukemia. In Yaniv,M. and Ghsdael,J. (eds), *Oncogenes as Transcriptional Regulators*. Birkhauser Verlag, Basel, Switzerland, Vol. 2, pp. 107–132.

Janknecht,R. and Hunter,T. (1996) Transcription. A growing coactivator network. *Nature*, **383**, 22–23.

Kaghad,M. *et al*. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*, **90**, 809–819.

Kagoshima,H., Akamatsu,Y., Ito,Y. and Shigesada,K. (1996) Functional dissection of the α and β subunits of transcription factor PEBP2 and the redox susceptibility of its DNA binding activity. *J. Biol. Chem.*, **271**, 33074–33082.

Kanda,T., Segawa,K., Ohuchi,N., Mori,S. and Ito,Y. (1994) Stimulation of polyomavirus DNA replication by wild-type p53 through the DNA-binding site. *Mol. Cell. Biol.*, **14**, 2651–2663.

Kanno,T., Kanno,Y., Chen,L.F., Ogawa,E., Kim,W.Y. and Ito,Y. (1998) Intrinsic transcriptional activation-inhibition domains of the polyomavirus enhancer binding protein 2/core binding factor α subunit revealed in the presence of the β subunit. *Mol. Cell. Biol.*, **18**, 2444–2454.

Kim,W.-Y., Sieweke,M., Ogawa,E., Wee,H.-J., Englmeier,U., Graf.T. and Ito,Y. (1999) Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains. *EMBO J.*, **18**, 1609–1620.

Kitabayashi,I., Yokoyama,A., Shimizu,K. and Ohki,M. (1998) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. *EMBO J.*, **17**, 2994–3004.

Komori,T. *et al*. (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, **89**, 755–764.

Kristie,T.M. and Sharp,P.A. (1990) Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV α-trans-activator protein. *Genes Dev.*, **4**, 2383–2396.

Lin,Y.C. and Stavnezer,J. (1992) Regulation of transcription of the germ-line Igα constant region gene by an ATF element and by novel transforming growth factor-β1-responsive elements. *J. Immunol.*, **149**, 2914–2925.

Lin,Y.S., Ha,I., Maldonado,E., Reinberg,D. and Green,M.R. (1991) Binding of general transcription factor TFIIB to an acidic activating region. *Nature*, **353**, 569–571.

Linn,H., Ermekova,K.S., Rentschler,S., Sparks,A.B., Kay,B.K. and Sudol,M. (1997) Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP. *Biol. Chem.*, **378**, 531–537.

Lu,J., Maruyama,M., Satake,M., Bae,S.-C., Ogawa,E., Kagoshima,H., Shigesada,K. and Ito,Y. (1995) Subcellular localization of the α and β subunits of the acute myeloid leukemia-linked transcription factor PEBP2/CBF. *Mol. Cell. Biol.*, **15**, 1651–1661.

Marengere,L.E. and Pawson,T. (1994) Structure and function of SH2 domains. *J. Cell Sci. Suppl.*, **18**, 97–104.

Massague,J. (1998) TGF-β signal transduction. *Annu. Rev. Biochem.*, **67**, 753–791.

Meyers,S., Downing,J.R. and Hiebert,S.W. (1993) Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein–protein interactions. *Mol. Cell. Biol.*, **13**, 6336–6345.

Mizushima,S. and Nagata,S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, **18**, 5322.

Molkentin,J.D., Firulli,A.B., Black,B.L., Martin,J.F., Hustad,C.M., Copeland,N., Jenkins,N., Lyons,G. and Olson,E.N. (1996) MEF2B is a potent transactivator expressed in early myogenic lineages. *Mol. Cell. Biol.*, **16**, 3814–3824.

Mosser,E.A., Kasanov,J.D., Forsberg,E.C., Kay,B.K., Ney,P.A. and Bresnick,E.H. (1998) Physical and functional interactions between the transactivation domain of the hematopoietic transcription factor NF-E2 and WW domains. *Biochemistry*, **37**, 13686–13695.

Mundlos,S. *et al*. (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*, **89**, 773–779.

Musacchio,A., Gibson,T., Lehto,V.P. and Saraste,M. (1992) SH3—an abundant protein domain in search of a function. *FEBS Lett.*, **307**, 55–61.

Nerlov,C. and Ziff,E.B. (1994) Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-α on the serum albumin promoter. *Genes Dev.*, **8**, 350–362.

Ogawa,E., Inuzuka,M., Maruyama,M., Satake,M., Naito-Fujimoto,M., Ito,Y. and Shigesada,K. (1993a) Molecular cloning and characterization of PEBP2β, the heterodimeric partner of a novel *Drosophila* runt-related DNA binding protein PEBP2α. *Virology*, **194**, 314–331.

Ogawa,E., Maruyama,M., Kagoshima,H., Inuzuka,M., Lu,J., Satake,M., Shigesada,K. and Ito,Y. (1993b) PEBP2/PEA2 represents a family of transcription factors homologous to the products of the *Drosophila* runt gene and the human AML1 gene. *Proc. Natl Acad. Sci. USA*, **90**, 6859–6863.

Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H. and Nakatani,Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87**, 953–959.

Okuda,T., van-Deursen,J., Hiebert,S.W., Grosveld,G. and Downing,J.R. (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, **84**, 321–330.

Onate,S.A., Boonyaratanakornkit,V., Spencer,T.E., Tsai,S.Y., Tsai,M.J., Edwards,D.P. and O'Malley,B.W. (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.*, **273**, 12101–12108.

Osada,M. *et al*. (1998) Cloning and functional analysis of human p51, which structually and functionally resembles p53. *Nature Med.*, **4**, 839–843.

Otto,F. *et al*. (1997) *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*, **89**, 765–771.

Oulad-Abdelghani,M., Bouillet,P., Chazaud,C., Dolle,P. and Chambon,P. (1996) AP-2.2: a novel AP-2-related transcription factor induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Exp. Cell Res.*, **225**, 338–347.

Ptashne,M. and Gann,A. (1997) Transcriptional activation by recruitment. *Nature*, **386**, 569–577.
Roberts,S.G.E., Ha,I., Maldonado,E., Reinberg,D. and Green,M. (1993) Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature*, **363**, 741–744.
Rotin,D. (1998) WW (WWP) domains: from structure to function. *Curr. Top. Microbiol. Immunol.*, **228**, 115–133.
Schild,L., Lu,Y., Gautschi,I., Schneeberger,E., Lifton,R.P. and Rossier,B.C. (1996) Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. *EMBO J.*, **15**, 2381–2387.
Shi,M.-J. and Stavnezer,J. (1998) CBFα3 (AML2) is induced by TGF-β1 to bind and activate the mouse germline Ig α promoter. *J. Immunol.*, **161**, 6751–6760.
Songyang,Z. *et al.* (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science*, **275**, 73–77.
Speck,N.A. and Stacy,T. (1995) A new transcription factor family associated with human leukemias. *Crit. Rev. Eukaryot. Gene Expr.*, **5**, 337–364.
Staub,O. and Rotin,D. (1996) WW domains. *Structure*, **4**, 495–499.
Staub,O., Dho,S., Henry,P.C., Correa,J., Ishikawa,T., McGlade,J. and Rotin,D. (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *EMBO J.*, **15**, 2371–2380.
Stern,S. and Herr,W. (1991) The herpes simplex virus trans-activator VP16 recognizes the Oct-1 homeo domain: evidence for a homeo domain recognition subdomain. *Genes Dev.*, **5**, 2555–2566.
Stringer,K.F., Ingles,C.J. and Greenblatt,J. (1990) Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature*, **345**, 783–786.
Sudol,M. (1994) Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene*, **9**, 2145–2152.
Sudol,M., Bork,P., Einbond,A., Kastury,K., Druck,T., Negrini,M., Huebner,K. and Lehman,D. (1995) Characterization of the mammalian YAP (Yes-associated protein) gene and its role in defining a novel protein module, the WW domain. *J. Biol. Chem.*, **270**, 14733–14741.
Tanaka,T. *et al*. (1996) The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability. *Mol. Cell. Biol.*, **16**, 3967–3979.
Tjian,R. and Maniatis,T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.
Torchia,J., Glass,C. and Rosenfeld,M.G. (1998) Co-activators and co-repressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.*, **10**, 373–383.
Vesque,C. and Charnay,P. (1992) Mapping functional regions of the segment-specific transcription factor Krox-20. *Nucleic Acids Res.*, **20**, 2485–2492.
Wade,P.A. and Wollfe,A.P. (1997) Histone acetyltransferases in control. *Curr. Biol.*, **7**, 82–84.
Wang,Q., Stacy,T., Binder,M., Marin-Padilla,M., Sharpe,A.H. and Speck,N.A. (1996) Disruption of the *cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl Acad. Sci. USA*, **93**, 3444–3449.
Williams,T. and Tjian,R. (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev.*, **5**, 670–682.
Willy,P.J., Umesono,K., Ong,E.S., Evans,R.M., Heyman,R.A. and Mangelsdorf,D.J. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.*, **9**, 1033–1045.
Yang,A., Kaghad,M., Wang,Y., Gillett,E., Fleming,M.D., Dotsch,V., Andrews,N.C., Caput,D. and McKeon,F. (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell*, **2**, 305–316.
Yang,X.J., Ogryzko,V.V., Nishikawa,J., Howard,B.H. and Nakatani,Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature*, **382**, 319–324.
Zhang,Y.W., Bae,S.-C., Takahashi,E. and Ito,Y. (1997a) The cDNA cloning of the transcripts of human PEBP2αA/CBFA1 mapped to 6p12.3-p12.1, the locus for cleidocranial dysplasia. *Oncogene*, **15**, 367–371.
Zhang,Y.W., Bae,S.C., Huang,G., Fu,Y.X., Lu,J., Ahn,M.Y., Kanno,Y., Kanno,T. and Ito,Y. (1997b) A novel transcript encoding an N-terminally truncated AML1/PEBP2αB protein interferes with transactivation and blocks granulocytic differentiation of 32Dcl3 myeloid cells. *Mol. Cell. Biol.*, **17**, 4133–4145.
Zhu,X., Yeadon,J.E. and Burden,S.J. (1994) AML1 is expressed in skeletal muscle and is regulated by innervation. *Mol. Cell. Biol.*, **14**, 8051–8057.

Received January 22, 1999; revised and accepted March 15, 1999