The ErbB-4 receptor protein-tyrosine kinase is proteolytically processed by membrane proteases in response to the ligand or 12-O-tetradecanoylphorbol-13-acetate stimulation resulting in the cytoplasmic fragment translocating to the cell nucleus. The WW domain-containing co-transcriptional activator Yes-associated protein (YAP) associates physically with the full-length Erb-B4 receptor and functionally with the Erb-B4 cytoplasmic fragment in the nucleus. The YAP-ErbB4 complex is mediated by the first WW domain of YAP and the most carboxy-terminal PPXY motif of Erb-B4. In human tissues, we documented the expression of YAP1 with a single WW domain and YAP2 with two WW domains. It is known that the COOH-terminal fragment of ErbB4 does not have transcriptional activity by itself; however, we show here that in the presence of YAP its transcriptional activity is revealed. There is a difference in the extent of transactivation activity among YAP isoforms: YAP2 is the stronger activator compared with YAP1. This transactivation is abolished by mutations that abrogate the YAP-ErbB4 complex formation. The unphosphorylatable mutation that increases the nuclear localization of YAP increases transcription activity. The COOH-terminal fragment of ErbB4 and full-length YAP2 overexpressed in cells partially co-localize to the nucleus and of the COOH-terminal fragment of ErbB4 that translocates to the nucleus to regulate transcription.

Cells are continuously exposed to diverse stimuli ranging from soluble paracrine and endocrine factors to signaling molecules on neighboring cells. These extracellular signals are transduced to cell nuclei to achieve an appropriate developmental or proliferative response. Receptor protein-tyrosine kinases play pivotal roles in this process. Upon binding of their cognate ligands, the intrinsic protein-tyrosine kinase activity of the receptor is significantly elevated and initiates a network of signaling pathways including the well characterized Ras/mitogen-activated protein kinase and the signal transducers and activators of transcription pathways (1–3).

Whereas many cell surface receptors transmit signals to the nucleus through complex protein cascades, several examples of membrane receptors translocating itself to the nucleus have been described (4, 5). In the case of ErbB-1, the epidermal growth factor receptor, addition of its cognate ligand causes translocation of the ligand-receptor complex to the nucleus and the complex binds to the cyclin D1 promoter activating its transcription (6). ErbB-3 (7) and fibroblast growth factor receptor 1 (8, 9) have been also reported to be present in nucleus.

Recently, it has been suggested that ErbB-4, the newest member of the epidermal growth factor protein-tyrosine kinase receptor family, activates gene expression in a more direct manner (10, 11). The binding of its ligand, heregulin, or activation of protein kinase C by 12-O-tetradecanoylphorbol-13-acetate provokes an ectodomain cleavage by a metalloprotease tumor necrosis factor-α-converting enzyme, followed by a subsequent cleavage by γ-secretase that release the ErbB-4 intracellular domain fragment from the membrane. The processing by γ-secretase facilitates the translocation of the COOH-terminal fragment of ErbB-4 (CTF) to the nucleus where it may affect the transcription of target genes. Similar mechanisms have been described for the proteolytic processing of the Notch receptor and the Alzheimer’s amyloid precursor protein (APP) (12–14). This process is called regulated intramembrane proteolysis and it represents a relatively new paradigm of signal transduction (15, 16). In Notch-1 signaling, the ligand binding causes intra-membrane cleavage of Notch-1 and generation of a transcriptionally active fragment. The Notch signaling pathway plays an important role in the cell fate specification process in multicellular organisms. The intracellular fragment of Notch is translocated to nucleus and binds directly to downstream transcription factors of the C promoter binding factor/ suppressor of hairless/Lag-1 family, to control transcriptional repression and activation of Notch target genes (12). In the case of APP, its intracellular fragment is produced by γ-secretase and translocates to the nucleus to form a multimeric complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60. This multicomponent complex is able to activate transcription via Gal4 or LexA reporters (14). In that experimental system, a robust transcriptional activation was observed only when the COOH-terminal fragment of APP was co-expressed with Fe65; the COOH-terminal fragment alone was not active. Fe65 is a typical adaptor protein composed of a WW domain and two PTB domains. The PTB1 domain binds to the histone acetyltransferase Tip60 and the PTB2 domain interacts with the cytoplasmic tail of APP (14, 17).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) A4316529.

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Isoforms of the ErbB-4 receptor generated by alternative splicing have been described (Ref. 3 and Fig. 1a) including the JM-a isoform that is sensitive to the cleavage, whereas the JM-b isoform is insensitive to the cleavage because of the sequence difference in the juxtamembrane region (18). The other isoform contains a sequence change in the phosphatidylinositol 3-kinase (PI3K) binding region within the cytoplasmic domain (19, 20). This site is deleted in an isoform designated CYT-2 and the other isoform that contains PI3K site is alignments as CYT-1 form. The ErbB4-CTF that was shown to translocate to the nucleus contains a protein-tyrosine kinase domain, autophosphorylation sites, and the PDZ domain recognition site (21, 22). Within the ErbB4 sequence we identified several proline-rich motifs that represent potential binding sites for WW domains.

The WW domain is a protein-protein interaction module composed of 35–40 amino acids (23, 24). The domain binds ligands containing proline-rich sequences (25). The largest class of WW domains binds ligands containing PPXY motif. The three PPXY sequences in the COOH-terminal region of ErbB-4 completely match the consensus motif that is recognized by the Class I WW domains.

Here we report that the WW domain-containing proteins, Yes-associated protein (YAP) and its isoforms associate with the cytoplasmic region of ErbB-4 and transactivate the COOH-terminal fragment of ErbB-4-dependent transcription in the Gal4 system. YAP has been characterized as a co-transactivator for several transcription factors including the Runx family proteins (26), the TEAD/TEF family of transcription factors (27), and p73 (28, 29). Most recently, it has been reported that the localization of YAP in the nucleus is regulated by Akt kinase and subsequent binding to 14-3-3 protein (29, 30). However, none of the upstream or membrane signals that communicate with YAP have been previously described. This is the first report showing the stimulation of transcription by ErbB-4 in complex with YAP.

**MATERIALS AND METHODS**

**Plasmids**—The BomiIII-EcoRI fragments that include coding region of human YAP1 and YAP2 were excised from pcDNA3-YAP and cDNA clone HYAP5 (31), respectively, and ligated into pcDNA4HIS-MAX (Invitrogen).

The ErbB4-XhoI fragment was excised from the pcDNA4HIS-MAX and the fragments were ligated into the p2xFLAG-CMV2a vector to prepare expression vectors, p2xFLAG-YAP1 and p2xFLAG-YAP2. The expression construct encoding full-length ErbB-4 with COOH-terminal fragment of ErbB-4-dependent transcription in the Gal4 system. YAP has been characterized as a co-transactivator for several transcription factors including the Runx family proteins (26), the TEAD/TEF family of transcription factors (27), and p73 (28, 29). Most recently, it has been reported that the localization of YAP in the nucleus is regulated by Akt kinase and subsequent binding to 14-3-3 protein (29, 30). However, none of the upstream or membrane signals that communicate with YAP have been previously described. This is the first report showing the stimulation of transcription by ErbB-4 in complex with YAP.

**Cell Culture and Antibodies**—Human embryonic kidney 293T cells, COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. The polyclonal antibody that recognizes the COOH-terminal region of ErbB-4 (C18) was purchased from Santa Cruz. The M2-antibody and the HA antibody were purchased from Sigma and Roche Diagnostics, respectively.

**Immunoprecipitation and Immunoblotting**—For analysis of the interaction between YAP and ErbB-4, 293T cells transfected with p2xFLAG-YAP1 or YAP2 and pcDNA3.1-ErbB-4 or pEF6-3xErbB-4 using FuGENE 6 (Roche) were lysed with RIPA buffer (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 300 mM NaCl, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) 36–48 h after transfection and immunoprecipitated with anti-FLAG M2 affinity gel (Sigma). The immunoprecipitates were washed with the RIPA buffer and bound proteins were separated on a SDS-PAGE and immunoblotted by HA antibody.

**Luciferase Assay**—Cells in 12-well dishes were transfected with the plasmids indicated in the figure legends using FuGENE 6 (Roche Diagnostics) harvested 36–48 h later. Firefly and Renilla luciferase activities were assayed with the dual luciferase assay system (Promega) and firefly luciferase activity normalized with respect to Renilla luciferase activity. All experiments were performed at least three times.

**Immunofluorescence Microscopy**—COS-7 cells on glass coverslips were transfected with p2xFLAG-YAP2 and/or pcDNA3-ErbB-4 (CTF-674–1292) using FuGENE 6 (Roche Diagnostics). After 36–48 h, cells were fixed with 4% formaldehyde and permeabilized in 0.25% Triton X-100 in phosphate-buffered saline, blocked in 10% goat serum. For the colocalization study of exogenously expressed YAP and the COOH-terminal fragment of ErbB-4, transfected cells were stained with FLAG M2 antibody (Sigma) and ErbB4-antibody (Santa Cruz) followed by incubation with rhodamine-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG. Images were acquired using Nikon microscope Eclipse TE2000-S equipped with a CCD camera.

**PCR**—Total RNA was isolated from tissues obtained from FVB/N mice (8 weeks old) using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (1 µg) was treated with DNase I (Amb grade, Invitrogen) to eliminate residual contamination of DNA. The DNase-treated RNA was subsequently transcribed to cDNA with Superscript II enzyme according to the manufacturer’s instructions (Strategene) using oligo-(dT)$_{12}$ primer. The cDNA was subjected to PCR analysis and primers were designed to be able to distinguish mouse YAP1 and YAP2 isoforms with primer pairs to sandwich both WW domains: 5’-cactgtagtacctgacg-3’ (nucleotides 654–674 of mouse YAP2) and 5’-cactgtagtacctgacg-3’ (nucleotides 1271–1251 of mouse YAP2). Glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified as a positive control with primers: 5’-caccacgcatcatc-3’ and 5’-tccacacgctgtgctg-3’. The PCR products were separated on a 2% agarose gel.
RESULTS

Human YAP1 and YAP2—It has been reported that human YAP contains a single WW domain and mouse YAP contains two WW domains. We found a splicing variant that encodes human YAP with two WW domains. YAP with a single WW domain, known previously as human YAP (31), is designated here as YAP1 and the new YAP with two WW domains is designated as YAP2. The difference in the nucleotide sequence between YAP1 and YAP2 is an insertion of the additional WW domain encoding region, its flanking sequence, and the deletion of the four amino acid sequence “QVRP” in YAP2 (Fig. 1b), suggesting that YAP2 is a splicing variant. The second WW domain of human YAP2 differs from the second WW domain of mouse YAP by one amino acid in the aromatic triplet located in the second β-strand of the domain. The amino acid sequence of human YAP2 shares 90.2% sequence homology with the mouse YAP, indicating that YAP2 is most likely the human homologue of mouse YAP.

YAP1 and YAP2 Associate with ErbB-4 through the WW Domain—To assess interaction between human YAPs and ErbB-4, co-immunoprecipitation experiments were performed. Both the HA-tagged CYT-2 isoform of the full-length ErbB-4 and FLAG-tagged YAP1 or YAP2 were transiently expressed in 293T cells, and the cell lysate was immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitant was probed with HA antibody to detect the coprecipitation of ErbB-4. In the presence of YAP1 or YAP2, ErbB-4 was pulled-down (Fig. 2b, lanes 2 and 5) indicating that YAP1 or YAP2 associates with ErbB-4 in cells. YAP2 seemed consistently more efficient in co-precipitation of ErbB-4 than YAP1 (Fig. 2a, lane 2 and 5), suggesting that YAP2 associates with ErbB-4 stronger than YAP1 does. Next, to determine whether this association is mediated by the WW domain of YAP1, a WW domain mutant

FIG. 2. YAP1 and YAP2 associate with ErbB-4 through the WW domain. a, the WW domain mutants (1st WW* and 2nd WW*) and the unphosphorylatable (S127A) mutant used for the co-immunoprecipitation experiments are indicated. The original (wild type) sequences are indicated as WT. b and c, 293T cells were transfected with pcDNA3ErbB-4HA together with p2xFLAG-CMV2, p2xFLAG-YAP1, p2xFLAG-YAP2, p2xFLAG-YAP1WT, p2xFLAG-YAP1-S127A, p2xFLAG-YAP2-S127A, p2xFLAG-YAP2–1stWW*, or p2xFLAG-YAP2–2ndWW*. At 48 h post-transfection, whole cell lysates were prepared and subjected to anti-FLAG immunoprecipitation. Immune complexes were separated by SDS-PAGE and then subjected to anti-HA immunoblotting. Ten percent input of the lysates and the precipitated YAP are indicated.

ErbB-4 Requires YAP for Its Transcription Activity

FIG. 3. YAP1 and YAP2 associate with ErbB-4 through the WW domain and a PPXY motif present at the most COOH terminus. a, the PPXY motif mutants (PY1 and PY3) for ErbB-4 are indicated. The original (wild type) sequences are indicated as Org. b, 293T cells were transfected with pEF6-A2EyrbB-4 (WT), pEF6-A2EyrbB-4 (PY1), or pEF6-A2EyrbB-4 (PY3) together with pFLAG-CMV2, pFLAG-YAP1, and pFLAG-YAP2. Co-immunoprecipitation analysis was carried out as described in the legend to Fig. 2.
Erβ-B-4 Requires YAP for Its Transcription Activity

FIG. 4. The CTF requires YAP for stimulation of transcription via the Gal4 transactivation system. a, COS-7 cells were transfected with pCΔN3HA-ErbB4 CTF (right panel, residue 676–1292) and stained with anti-ErbB-4 polyclonal antibody (Santa Cruz, C18) followed by staining with fluorescein isothiocyanate-conjugated anti-rabbit IgG. The transfected cells were observed under a fluorescence microscope. Non-transfected cells were used as a negative control (left panel). b, the Gal4 DNA binding domain (Gal4BD, residue 1–142) was fused to the entire ErbB-4 cytoplasmic fragment (CTF, residues 676–1292) and ErbB-4 cytoplasmic fragment with a deletion of the kinase domain (CTFΔK, residues 988–1292). Each of these fusion constructs was co-transfected into COS-7 cells with a reporter plasmid encoding firefly luciferase controlled by a Gal4-responsive promoter in the presence of p2xFLAG-CMV2 (control), p2xFLAG-YAP1, or p2xFLAG-YAP2. A reporter plasmid encoding Renilla luciferase was also co-transfected to normalize transcription efficiencies. The amount of DNA is adjusted to 600 ng for all the transfection.

was created in which the second conserved tryptophan and the conserved proline were each substituted to alanine (Fig. 2b). Based on our previous studies (32, 33) and on the understanding of the WW domain molecular structure (34) such mutants should render the domain inactive in terms of ligand binding. The WW domain mutation in YAP1 completely abolished the binding to ErbB-4 (Fig. 2b, lane 4). Interestingly, the analogous mutation of the second WW domain in YAP2 did not have any effect on binding to ErbB-4 (Fig. 2c, lane 3). These results indicate that the first WW domain of YAPs is primarily responsible for the interaction with ErbB-4 but not the second WW domain in YAP2.

A Mutation of the 14-3-3-binding/Akt Phosphorylation Site in YAP Affects the Binding to ErbB-4—It has been reported that the serine 127 residue in YAP is phosphorylated by a protein kinase, Akt, and it is recognized by the 14-3-3 protein (29). The binding by 14-3-3 causes translocation of YAP from the cytoplasm to the nucleus. To determine the importance of this site for the binding to ErbB-4, the serine residue at position 127 was substituted to alanine (Fig. 2a) and binding was examined by co-precipitation experiments. The mutation of serine residues at position 127 in both YAP1 and YAP2 attenuated the binding to ErbB-4 (Fig. 2b, lanes 3 and 6).

YAPs Associate with ErbB-4 through the PPXY Motif Located in the Cytoplasmic Region—To make sure that YAPs bind to PPXY motifs in ErbB4, the tyrosine residues in the PPXY motifs were substituted to alanines. There are two PPXY motifs in the cytoplasmic region of ErbB-4 at positions 1031–1040 (NIPPPΨYTSR) and 1280–1288 (LPFPPYRHK). Two mutants were created, the first PPXY mutant was designated as PY1 and the second mutant was designated as PY3 (Fig. 3a). The co-immunoprecipitation experiments showed that the substitution of tyrosine residue in the most COOH-terminal PPXY region (PY3) completely abolished the binding to both YAP isoforms (Fig. 3b, lanes 6 and 9). On the contrary, the binding to YAPs was not affected by the mutation of the first PPXY sequence (Fig. 3b, lanes 5 and 8). All the above co-precipitation experiments indicated that the association between both YAPs and ErbB-4 is through the WW domain (the first WW domain in YAP2) in YAP and the most COOH-terminal PPXY sequence in ErbB-4. These data are in full agreement with the results of the optimal binding of PPXY-containing 10-mer peptides selected for YAP1 WW domain from phage display and SPOT membrane peptide repertoires (35).

The CTF Requires YAP for Stimulation of Transcription via the Gal4 Transactivation System—To make sure that the CTF is localized in the nucleus, the CTF was expressed in COS-7 cells and stained with ErbB-4 polyclonal antibody. Overexpression of the CTF showed prominent nuclear localization and weak cytosolic/membrane localization in COS-7 cells (Fig. 4a, right panel). The localization is distinct compared with the control, although this antibody showed low background of nuclear staining in non-transfected cells (Fig. 4a, left panel). This data confirms a previous result from the Carpenter laboratory obtained with the CTF-GFP fusion and cell fractionation study (10).

It was also shown that the CTF with the deleted kinase domain has an ability to transactivate transcription in the Gal4 system in contrast to the intact CTF. Here we have used the same Gal4 transactivation system (10), however, in the presence of YAP1 or YAP2. The Gal4 DNA binding domain (1–47) fused with the entire COOH-terminal fragment or with the deletion of the kinase domain of ErbB-4 was coexpressed with the Gal4-luciferase reporter in COS-7 cells and the luciferase activity was measured. As shown previously, only the COOH-terminal fragment with the kinase domain deletion could stimulate transcription 3–5-fold over the Gal4 control but the intact CTF fragment or the vector control were negative in the assay (Fig. 4, lanes 1–3). As we expected, coexpression of YAP1 or YAP2 significantly transactivated both the intact and kinase-deleted versions of CTF-mediated transcription: YAP1-CTF, 3-fold, and YAP1-CTF δ kinase: 17-fold; YAP2-CTF, 10-fold, and YAP2-CTF δ kinase, 78-fold over the Gal4 control. These results indicate that both YAPs co-activate transcription and that YAP2 is a stronger co-activator of transcription than YAP1.

The Association of YAP with the ErbB-4 Cytoplasmic Fragment Is Essential for Transactivation—In the subsequent experiments, we mainly used the intact version of CTF in the Gal4 assay, because the kinase-deleted fragment has not been detected under physiological conditions. To confirm that the transactivation by YAP is because of the direct association with the CTF-ErbB-4 fragment, we used YAP2 with a mutation in the WW domain that does not associate with ErbB-4 in the Gal4 assay. Although YAP2 with the mutation in the second WW domain activated less than the wild type (WT) YAP2, YAP2 with the mutation in the first WW domain did not activate transcription at all (Fig. 5a). Furthermore, mutation of the
PPXY sequence (PY3) of the CTF that is essential for binding to YAP also abolished the stimulation of transcription either on the entire COOH-terminal fragment or the kinase domain CTF mutant. As expected the PY1 mutant did not have any effect in the assay (Fig. 5, b and c). These data indicate that the transactivation is caused by the YAP CT complex mediated by the WW domain of YAP and the most COOH-terminal PPXY sequence of the ErbB-4 CTF.

It has been reported that YAP is mostly localized in the cytoplasm and its nuclear localization is inhibited by the binding of 14-3-3 upon Akt phosphorylation (29). It has also been known that the mutation of serine 127 to alanine increases transactivation activity of YAP because of a greater nuclear localization of YAP and therefore decided to assess the non-phosphorylatable mutant (YAP-S127A) in the same Gal4 assay. Although, in the immunoprecipitation experiments using the full-length ErbB-4, the mutation attenuated the binding of YAP, this mutation caused a transactivation increase about 1.9-fold compared with the WT (Fig. 5a) in agreement with the previously reported data in the slightly different "read-out," the p73 mediated transcription assay (29). The attenuation of YAP association described here may be because of the change in localization of YAP to the nucleus and its inaccessibility to the membrane localized full-length receptor.

Expression of YAP Transcripts in Several Tissues—Because the human YAP1 and YAP2 isoforms were described here for the first time, and the differences in their transcriptional activity was documented, we decided to investigate the expression pattern of each YAP transcript in various tissues. Because of the difficulties in obtaining normal human tissues, freshly dissected mouse tissues were used instead. Accordingly, we designed the mouse RT-PCR primers so that mouse YAP1 and YAP2 transcripts could be unequivocally amplified and distinguished. The sequence of the mouse YAP2 homologue and the topology of human YAP1 and YAP2 sequences were considered in the RT-PCR strategy (Fig. 7a). As we expected, doublet
bands derived from two isoforms were observed, but the expected size of 500 bp for the mouse YAP1 homologue transcript was not detected (Fig. 7b). Instead, the slower migrating band with 700 bp was amplified in most mouse tissues together with the 600-bp band expected to be the mouse YAP2 homologue transcript. We confirmed the PCR product by direct sequence analysis and identified the 600-bp band as previously known YAP (mouse YAP2 homologue). The 700-bp band turned out to represent a new isoform with an insertion of 48 nucleotides; we designated this isoform as YAP2L. This sequence has been already submitted to the NCBI data base with the accession number BC038235. The 700-bp bands were observed under a fluorescent microscope using the rhodamine-fluorescin filter for the double staining to get the co-localization images.

**Discussion**

We report that the transcriptional co-activator YAP associates with the cytoplasmic region of ErbB-4 and co-activates transcription of the CTF, the naturally occurring proteolytic fragment of the carboxyl end of ErbB4. We also showed the physical interaction between YAP and the full-length ErbB-4 in the co-immunoprecipitation experiments and functional interaction between YAP and the CTF of ErbB-4 in the nucleus using the Gal4 reporter system. These results indicate that YAP may associate with the ErbB-4 receptor at the membrane and also with the CTF in the nucleus. Furthermore, based on the presented data we suggest that the CTF of ErbB4 produced by y-secretase cleavage, translocates together with YAP to the nucleus upon ligand or TPA stimulation. YAP may act not only as a transcriptional co-activator for the CTF, but also may act as a carrier protein for the translocation of the CTF from the membrane to the nucleus. The following aspects of the work deserve further comments: (i) the localization of YAP and the networking with other signaling pathways; (ii) the role of various isoforms of YAP and isoforms of ErbB4 in signaling; and (iii) the emerging models of YAP-dependent transcriptional activation of the CTF-ErbB4 and its nuclear function.

It has been reported that YAP localizes to the apical membrane in epithelial cells associating with the EBP50 PDZ domain through its COOH-terminal PDZ domain binding sequence (37). It is therefore possible to assume that YAP and ErbB-4 colocalize at the plasma membrane in epithelial cells. The localization of YAP is regulated by the binding of 14-3-3 to the phosphorylation site and the phosphorylated YAP does not enter the nucleus (29, 30). It follows that co-transcriptional activity of the CTF/ErbB4 complex may be regulated by YAP phosphorylation and the ultimate level of YAP protein in the cell nucleus. Similar to the YAP-mediated transactivation of p73 and the YAP-related protein TAZ (29, 30), the co-transcriptional activity for the ErbB4-CTF is also increased by the mutation of the phosphorylation site of YAP (Fig. 5a). Through its ability to phosphorylate YAP, Akt may inhibit a number of transcription factors by blocking rate-limiting transcriptional co-activation. Neither the Carpenter laboratory (10) nor our laboratory were able to detect transcriptional activity of the ErbB4-CTF itself using the Gal4 system in COS-7 cells, in which the endogenous YAP is present. This may because of the absence of YAP in the nucleus caused by the relatively high activity of Akt in COS-7 cells (29); however, the exogenously expressed YAP strongly stimulated the transcription. Although the unphosphorylatable mutation (S127A) of the crucial Ser127 in YAP increases the localization of YAP in the nucleus, this mutation was not sufficient to allow the translocation of all available YAP to the nucleus (data not shown). Among possible mechanisms that regulate translocation of YAP to the nucleus by retaining the protein in the cytoplasm or at the plasma membrane, one may propose the anchoring role of PDZ domain-containing proteins such as EBP50 (37) and MUPP1 (27, 38). Further research should uncover the conditions and factors that stimulate the translocation of YAP to the nucleus.

In this study, we have used the alternatively spliced CYT-2 isoform of ErbB-4 that does not activate the PI3K pathway because of the lack of PI3K binding site (19). We have chosen this isoform in our experiments arguing that the COOH-terminal fragment produced from the CYT-1 isoform (with PI3K site) might not associate with YAP in the nucleus under physiological conditions. In cells expressing the CYT-1 isoform, upon the ligand stimulation in which the CTF should translocate to the nucleus, Akt is activated through the PDK-PI3K pathway (39, 40), but Akt is “silent” in the CYT-2 isoform-expressing cells because of the absence of the activation of the PI3K pathway.
We presume that after ligand stimulation, the cytoplasmic fragment of the CYT-2 isoform is cleaved from the membrane by γ-secretase and forms a complex with YAP in the nucleus. However, in the case of the CYT-1 isoform, the PI3K pathway-activated Akt would phosphorylate YAP and retain it in the cytoplasm for other functions. Alternatively, because of the PI3K binding site in CYT-1 and its flanking sequence contain the PPXY (PY2) sequence, this region might work as a binding site for YAP to inhibit the PI3K pathway so that YAP could escape from phosphorylation by Akt. These interesting but putative scenarios of signaling switches are being now investigated.

The expression of ErbB-4 is ubiquitous in several tissues. The CYT-1 isoform is predominant in heart, breast, and abdominal aorta, whereas the expression of the CYT-2 is abundant in neural tissues including cerebellum, cerebral cortex, spinal cord, and medulla oblongata (19). The expression of YAP is also ubiquitous and similar to the CYT-2 isoform, YAP2 isoform is predominantly expressed in neural tissues, suggesting a possible CYT-2-YAP2 complex functioning in neural tissues. Furthermore, the expression of YAP in heart could not be detected in the RT-PCR analysis and this correlates with the absence of expression in the CYT-2 but not the CYT-1 in the same assay (19). We also have found a new transcript of YAP2 with insertion of 16 amino acids. This transcript is widely expressed in various tissues but not detected in cerebellum. The inserted sequence of the 16-amino acid "QAIRNIPSTANAPKC" does not have any homology (or sequence similarity) to known motifs or domains. Interestingly, we could not identify the YAP1 homologue in mouse tissues using the RT-PCR approach. Furthermore, we could not find YAP1 homologue in the mouse and even in the human EST data base using human

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**Fig. 7.** RT-PCR analysis of the distribution of YAP isoforms in mouse tissues. a, PCR primers were designed so that transcripts of YAP isoforms could be amplified. The forward primer (FW) encodes the flanking sequence of the first WW domain and the reverse primer (RV) encodes the region in the middle of the transcription activation site. The insertion of the 16-amino acid in YAP2L is indicated. b, total RNA was isolated and subjected to RT reaction with RT (+) or without RT (-) and to PCR using the primer set as indicated. As an internal standard, all the templates were also analyzed by PCR using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bottom panel). Mouse YAP RT-PCR product with the size of 600 bp was indicated as YAP2 and the 700-bp PCR product derived from the new transcript was indicated as YAP2L.

**Fig. 8.** The proposed models for YAP-CTF complex in the nucleus. In this model, CTF translocates together with YAP to the nucleus associating with some transcription factor on the specific promoter to perform gene regulation (left). Alternatively, the CTF may translocate to the nucleus where YAP already forms the complex with the transcription factors to affect the gene regulation (right).
YAP1 sequence, although many of YAP2 and YAP2L sequences are present in the EST data bases. Altogether the data showing that YAP1 is not detected in mouse tissues and the absence of YAP1 in the human and mouse EST data base suggest that YAP2 (or YAP2L) is the major isoform and YAP1 is a minor isoform expressed in human tissues. Alternatively, it is possible that YAP1 is only expressed under special conditions or during limited stages of development. In the kidney of an HIV-1 transgenic mouse that has severe renal failure (41), in addition to the transcript of YAP2 and YAP2 with the 16-amino acid insertion, we uncovered a different transcript in which the WW domain is deleted in half.2 This unusual isoform may act as a dominant negative form of YAP in renal disease. In summary, we have found three variants, most likely splicing variants, that encode YAP1, YAP2, and YAP2L 16-amino acid insertions in human and two variants YAP2 and YAP2L 16-amino acid insertion in mouse. We documented that YAP2 is a stronger transcriptional co-activator than YAP. The difference between YAP1 and YAP2 in the structure is the number of WW domains and the insertion of four amino acids in the transcription activation domain of YAP1. The presence of the additional WW domain might have a positive effect and the insertion might have an inhibitory effect on transcription. In fact, the mutation of the second WW domain slightly affected the transcription activity by an unknown mechanism. In this context the identification of the protein that associates with the second WW domain of YAP2 might be important. The precise signaling differences among YAP splicing variants remain unclear.

The YAP-dependent transcriptional activation of the COOH-terminal fragment of ErbB-4 described here invites modeling for new signaling route. Two simple models for the role of YAP-CTF complex in the nucleus are proposed. The CTF might translocate to the nucleus together with YAP and associate with several transcription factors on the promoter to activate transcription (Fig. 8, left). In this case, the CTF might work as a carrier of YAP for the specific transcription factor. This is quite similar to the COOH-terminal fragment of APP and a WW domain protein F6E5 complex described by Cao and Sudhof (14). The COOH-terminal fragment of APP produced by γ-secretase itself does not have any transcriptional activity and requires F6E5 as a co-activator. Furthermore, the transcription factor recruits the complex to the specific promoter has not been identified in APP-F6E5-Tip60 nor ErbB-4CTF-YAP so far. Identification of the transcription factors as binding partners for ErbB-4 or F6E5 in the nucleus is of importance.

Another possible scenario stipulates that the CTF alone might translocate to the nucleus where YAP already exists in complex with transcription factors, such as p73 (28, 29), Runx2 (26), or TEAD/TEF (27) (Fig. 8, right). In the case of p73 and Runx2, the complex could be of an inhibitory nature, because the CTF may compete with these transcription factors for binding to the WW domain of YAP. In contrast in the case of TEAD/TEF, the translocation of the CTF to the nucleus might result in a ternary protein complex composed of YAP-TEAD/TEF-CTF because the binding is not through the WW domain-

2 A. Komuro and M. Sudoh, unpublished data.