Merlin, a Tumor Suppressor, Interacts with Transactivation-responsive RNA-binding Protein and Inhibits Its Oncogenic Activity*

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The neurofibromatosis type 2 gene-encoded protein, merlin, is related to the ERM (ezrin, radixin, and moesin) family of membrane-cytoskeleton-associated proteins. Recent studies suggest that the loss of neurofibromatosis type 2 function contributes to tumor development and metastasis. Although the cellular functions of merlin as a tumor suppressor are relatively well characterized, the cellular mechanism whereby merlin controls cell proliferation from membrane locations is still poorly understood. During our efforts to find potential merlin modulators through protein-protein interactions, we identified transactivation-responsive RNA-binding protein (TRBP) as a merlin-binding protein in a yeast two-hybrid screen. The interaction between TRBP and merlin was confirmed by glutathione S-transferase pull-down assays, co-immunoprecipitation, and co-localization experiments. The carboxyl-terminal regions of each protein were responsible for their interaction. Cells overexpressing TRBP showed enhanced cell growth in cell proliferation assays and also exhibited transformed phenotypes, such as anchorage-independent cell growth and tumor development in mouse xenografts. Merlin efficiently inhibited these oncogenic activities of TRBP in our experiments. These results provide the first clue to the functional interaction between TRBP and merlin and suggest a novel mechanism for the tumor suppressor function of merlin both in vitro and in vivo.

Neurofibromatosis type 2 (NF2)1 is a predominantly inherited disorder that leads to the bilateral occurrence of vestibular schwannomas and other brain tumors, especially meningiomas and schwannomas of other cranial nerves and spinal nerve roots (1, 2). The NF2 gene was isolated by positional cloning and encodes merlin, which was named for its striking structural similarity with the ERM family proteins (moesin, ezrin, and radixin) linking the actin cytoskeleton to various membrane-associated proteins (3–6). Merlin also localizes at the membrane-cytoskeleton interface, which is unusual for tumor suppressors.

Merlin interacts with a number of protein partners and inhibits many growth signal pathways when overexpressed (5, 6). Studies using NF2 mutant mice demonstrated that the NF2 tumor suppressor regulates the proliferation of many cell types and that NF2 loss plays a wide role in both tumor development and metastasis (7–10). Recently, Lallemand et al. (11) showed that NF2 deficiency leads to defective cadherin-mediated cell-cell adhesion, abolishing the contact-dependent inhibition of cell growth. Their results indicated that merlin localizes at the adherens junctions and physically interacts with their components in confluent cells, suggesting that merlin normally controls adherence junctions assembly and contact-dependent growth inhibition directly from the sites of cell-cell contact.

For the better understanding of merlin function, merlin-associated proteins were screened using a yeast two-hybrid interaction trap strategy from a human brain cDNA library. We identified a human transactivation-responsive RNA-binding protein (TRBP) as a novel protein interacting with merlin via its carboxyl-terminal domain.

TRBP is encoded by the tarbp2 gene localized at human chromosome 13 and mouse chromosome 15 (12, 13). The two isoforms of TRBP, TRBP1 (original TRBP) and TRBP2, are expressed by alternative transcriptional initiation, resulting in the TRBP2 protein that is longer than TRBP1 by 21 amino acids at its amino terminus (14–16). TRBP was originally cloned as an HIV-1 transactivation-response RNA-binding protein and belongs to the family of double-stranded RNA (dsRNA)-binding proteins with two clearly defined dsRNA-binding domains (dsRBDS) and a carboxyl-terminal basic region (17–21). TRBP dsRBD2 binds transactivation-response with higher affinity than dsRBD1, because of the presence of a KR helix motif (16, 17).

TRBP acts in synergy with functional Tat to stimulate the expression of the HIV-1 long terminal repeats in human and murine cells (14, 22). The murine homologue, Prbp, binds the 3′-untranslated region of Prm1 protamine RNA, represses its translation, and plays a physiological role in spermatogenesis (23, 24). TRBP also directly binds the double-stranded RNA-dependent protein kinase (PKR) that has anti-viral and anti-proliferative effects. TRBP inhibits the ability of PKR to phosphorylate eukaryotic translation initiation factor 2, leading to its inactivation (25, 26). In relation to the inhibition of PKR
activity, TRBP was recently demonstrated to play a growth-promoting role and has an oncogenic potential (26). Therefore, it is possible that the tumor suppressor Merlin interacts with oncogenic TRBP and inhibits its function. In this report, we present evidence for the direct interaction between Merlin and TRBP both in vitro and in vivo and show that Merlin reverses the growth-promoting and tumorigenic activities of TRBP.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The plasmids for wild type Merlin, its deletion mutants (M1–M4), and active Ras in the mammalian system were described in previous reports (27, 28). The yeast plasmids for the Merlin proteins were generated by PCR cloning in pGBT9, a GAL4 DNA-binding domain vector (Clontech, Palo Alto, CA) using a BamHI site. The full-length and derivatives of human TRBP were synthesized using PCR and inserted in the BamHI/XhoI sites of pACT2 yeast plasmids (Clontech) or in the cloning sites of pCDNA3.1-V5-His TOPO vector (Invitrogen) for mammalian expression. His and V5 epitopes were tagged at the carboxyl-terminus of TRBP proteins in the mammalian expression plasmids. The full-length TRBP was constructed in pGEX-KG (Amersham Biosciences) for GST fusion (pGST-TRBP) and in pEGFP-N1 (Clontech) for GFP fusion (pEGFP-TRBP), using the BamHI/XhoI sites in both cases. The RFP fusion of the full-length Merlin was generated by cloning RFP fragment from pDsRed-N1 (Clontech) into the BamHI site of pcDNA-NF2, a Merlin expression plasmid.

**Yeast Two-hybrid Screen**—The full-length human NF2 in pGBT9 (pGBT9-NF2) was used as bait in the yeast two-hybrid screens of a human brain cDNA library in pACT2 (Clontech). The bait and the library DNAs were co-transformed by the lithium acetate method as previously described (29). Twenty-five colonies of 2 × 106 transformants grew normally in the absence of histidine and showed detectable β-galactosidase activity within 3 h of reaction. The clones were selected to partial sequence determination.

**Northern Blotting**—Hybridizations were performed with the random-primed 32P-labeled probes, corresponding to the full-length human tarbp2 mRNA. The insoluble materials were removed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was then subjected to SDS-PAGE and Western blotting. The blots were blocked in phosphate-buffered saline containing 5% skim milk and 0.05% Tween 20 and incubated with the primary and horseradish peroxidase-conjugated secondary antibodies. Detection was performed according to the enhanced chemiluminescence protocol (Amersham Biosciences). For all of the Merlin detection procedures, a mixture of anti-merlin polyclonal antibodies sc331 and sc332 (Santa Cruz Biotechnology) was used as the primary antibody at 1:1,000 dilution.

**GST Pull-down Assay**—The GST-TRBP was expressed in Escherichia coli and purified as previously described (30). For the pull-down assay, 2 μg of each GST fusion protein was immobilized on the glutathione-Sepharose 4B beads and incubated with the Merlin-transfected NIH3T3 cell lysates in RIPA-B buffer for 2 h at 4 °C. After extensive washing with PBS, the samples were analyzed by Western blotting using the anti-merlin antibody.

**Confocal Fluorescence Microscopy**—NIH3T3 cells were seeded in a 4-well chamber slide (2 × 105 cells/well) and transfected with pRFP-merlin and/or pEGFP-TRBP. Twenty-four hours later, the localization of the expressed proteins was determined by means of their green and red fluorosciences using a confocal laser-scanning microscope.

Wild type and NF2-deficient MEF cells in Lab-Tek II glass chamber slides (VWR International) were fixed with 2% formaldehyde in PBS for 15 min and permeabilized in ice-cold methanol. The chamber slides were incubated in PBS supplemented with 20 mM ammonium chloride and 10% fetal calf serum for blocking, and the slides were then incubated with rabbit polyclonal anti-TRBP antibody (diluted at 1:300 in PBS with 10% fetal calf serum) followed by highly cross-adsorbed goat anti-rabbit IgG antibody conjugated with Alexa Fluor 555 (Molecular Probes, Eugene, OR). The nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). The confocal laser scanning fluorescent microscopy and imaging were performed using a 545-nm HeNe laser for excitation and a 565–585-nm filter for emission.

**Cell Proliferation Assays**—The proliferation of stable NIH3T3 cells expressing pcDNA control, TRBP, NF2, or TRBP + NF2 was assessed by CellTiter 96 Aqueous kit according to the manufacturer’s instructions (Promega). Briefly, each stable cell line was seeded into the 96-well plates at 5 × 103 cells/well. Three hours after plating (when the cells were well attached), the combined MTS/PMS solution was added into each well, to control the initial amount of cells. After incubation for 30 min in the CO2 incubator, the absorbance was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader. Then cell
proliferation was chased at days 1, 2, 3, and 4 after seeding using the same procedure as that described above.

**Soft Agar Colony Formation Assay**—The NIH3T3 cells stably expressing the pcDNA control, active Ras, TRBP, NF2, or TRBP/H11001 NF2 (5 x 10^3 cells) were mixed with 1 ml of 0.35% molten agarose (Invitrogen) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Each mixture was plated on a 2-ml solidified layer of 0.7% agarose prepared in the same medium in a 6-well plate. The number and size of the resulting colonies were determined by microscopic visualization after 12–14 days.

**Tumorigenesis Assay in Nude Mice**—Five-week-old BALB/c-nu SLC mice were obtained from Jung-Ang Lab Animal, Inc. (Seoul, Korea). Three mice were used for each experimental group. Each mouse was inoculated subcutaneously with the same stable NIH3T3 cells (1 x 10^7 cells in 0.1 ml of PBS) as in the soft agar assay. Tumor growth was monitored twice a week and examined at 28 days after inoculation.

**RESULTS**

*Identification of TRBP as a Merlin-binding Protein*—To identify the proteins that associate with merlin, we performed a yeast two-hybrid screening with a human brain cDNA library and the full-length merlin as bait. Twenty-five clones were initially selected and subdivided into five different groups after DNA sequencing. One group of them contained parts of a cDNA encoding human TRBP. TRBP was initially identified as an HIV transactivation-response RNA-binding protein with two double-stranded RNA-binding domains, dsRBD1 (amino acids 31–79) and dsRBD2 (amino acids 160–222) (see Fig. 2B). It is currently implicated in counteracting PKR activity, which results in the proliferative and oncogenic effects in cells (26). Therefore, the direct interaction between merlin tumor suppressor and oncogenic TRBP might have biological significance in regulating cell proliferation.

The tissue distribution of TRBP expression was first determined using a human adult multiple tissue RNA blot (Clontech) and a full-length TRBP cDNA probe. The result showed that TRBP was present mainly as a single species of 1.5 kb in most of the tissues (Fig. 1). Its expression was high in the heart, skeletal muscle, kidney, liver, and placenta and was relatively low in the brain, colon, thymus, spleen, small intestine, and lungs. Two minor transcripts of 3.2 and 5.4 kb were also observed but were barely detected in most of the tissues, as

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**FIG. 2.** Schematic presentation of merlin and TRBP proteins. **A,** merlin contains three conserved protein-protein interaction domains: a FERM domain in its amino terminus, a coiled-coil region (CCR), and a carboxyl-terminal domain (C-terminal). The FERM domain of merlin contains three subdomains (labeled A, B, and C) and has a unique Blue Box (BB, residues 177–183). Deletion mutants of merlin are diagrammed with the amino acid positions used for deletion. For deletion notation, M1 lacks the FERM subdomain A; M2 lacks most of FERM subdomain B including BB; M3 lacks the coiled-coil region, the carboxyl-terminal domain and a small part of FERM subdomain C; and M4 lacks most of the FERM domain. **B,** TRBP contains three domains: two double-stranded RNA-binding domains (dsRBD1 and dsRBD2) and a carboxyl-terminal region. For deletion notation, TRBPΔdr1 lacks dsRBD1; TRBPΔdr1,2 lacks dsRBD1 and dsRBD2; and TRBPΔc lacks the carboxyl-terminal region. **TRBP partial** is a clone isolated from the yeast two-hybrid screening and contains most of the carboxyl-terminal region.

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| Merlin and TRBP interaction in yeast two-hybrid assay |
|------------------------------------------------------|
| **TRBP partial** | **Wild type TRBP** |
| **Merlin wild type** | **LacZ** | **His**, **Ade** | **LacZ** | **His**, **Ade** |
| M1 | ++ | + | + | + |
| M2 | + | + | + | + |
| M3 | + | + | + | + |
| M4 | ++ | + | + | + |

*Originally screened clone from human brain cDNA library.*
TRBP and Merlin Interact with Each Other via Their Carboxyl-terminal Regions in Yeast Two-hybrid Assays—The TRBP clone isolated from the screening lacked both of the RNA-binding domains and contained only the carboxyl-terminal region (cf. TRBP-partial in Fig. 2B). The partial and full-length TRBP obtained by PCR cloning were tested in the yeast system for the interaction with the full-length and deletion mutants of merlin (Fig. 2A). As summarized in Table I, both the full-length and partial TRBP specifically associated with the full-length merlin in the LacZ assay and yeast growth test. They were associated with the M1, M2, and M4 deletion mutants of merlin but not with the M3 mutant, which does not contain the carboxyl-terminal region. These results suggest that the carboxyl-terminal regions of each protein are responsible for the interaction of merlin and TRBP in the yeast system.

In Vitro Interaction between TRBP and Merlin in the GST Pull-down Assay—To further confirm the direct interaction of TRBP and merlin in vitro, the GST pull-down assays were performed with the GST fusion of full-length TRBP. The lysates of NIH3T3 cells expressing full-length merlin or its deletion mutants were incubated with the resin-bound GST-TRBP, and the pulled down samples were detected using anti-merlin antibodies (Fig. 3). Consistent with the yeast-two hybrid results (Table I), GST-TRBP, but not GST alone, selectively pulled down full-length merlin (Fig. 3A). Only the M1, M2, and M4 merlin deletions containing the carboxyl-terminal region of merlin were selectively pulled down by GST-TRBP, but the M3 merlin mutant was not (Fig. 3B). The amount of the various merlin proteins in the input sample was also checked by Western blotting. Various forms of the M2 deletion mutant of merlin were observed in the input and GST pull-down samples. The M2 deletion mutant of merlin seems to have some sites susceptible to cleavage. Nonetheless, all of them bound TRBP in the GST pull-down assay.

Notably, merlin was present mainly in a phosphorylated form in the input cell lysates. However, a significant amount of the hypophosphorylated merlin was detected in the GST-TRBP-pulled down assays, and the quantity of hypophosphorylated merlin was slightly greater than that of the phosphorylated merlin. This result suggests that TRBP might interact preferentially with the hypophosphorylated form of merlin in vitro.

TRBP Associates with Merlin in Vivo—The physical interaction between merlin and the full-length or deletion mutants of TRBP (TRBPΔdr1 and TRBPΔdr1,2; Fig. 2B) was examined in vivo by co-immunoprecipitation in NIH3T3 cells (Fig. 4). The V5 epitope-tagged TRBP or its deletion mutants were expressed together with merlin in NIH3T3 cells, and the cell lysates were subjected to immunoprecipitation with the anti-V5 antibody. Subsequent Western blotting with anti-merlin antibody revealed that the level of the hypophosphorylated and phosphorylated forms of merlin interacted with all of the TRBP proteins in vivo (Fig. 4A, middle panel). Again, the level of the hypophosphorylated merlin was quite low in the input lysates (Fig. 4A, top panel) and in the immunoprecipitated samples (Fig. 4B, bottom panel). Consistent with the result obtained from the yeast two-hybrid assay, merlin was co-immunoprecipitated with both of the TRBP deletion mutants containing the carboxyl-terminal region of TRBP (Fig. 4A, middle panel). Only a small amount of merlin was detected with TRBPΔdr1,2, probably because the TRBPΔdr1,2 protein level was relatively low in the immunoprecipitated sample (Fig. 4A, bottom panel) and in the input lysate (data not shown). It is likely that the TRBPΔdr1,2-V5 protein is relatively unstable compared with the other forms of TRBP. Reciprocally, TRBP was co-immunoprecipitated with merlin from the NIH3T3 cell lysates by the anti-merlin antibody (Fig. 4B). However, the TRBP mutant without the carboxyl-terminal region (TRBPΔc) was not co-immunoprecipitated with merlin (Fig. 4B, lane 5). Taken together, these results suggest that TRBP and merlin interact in vivo via the carboxyl-terminal regions of each protein and that both of the dsRBDs of TRBP are dispensable for the interaction with merlin.

The interaction between endogenous TRBP and merlin was investigated by immunoprecipitation in SK-N-Be (2), a human neuroblastoma cell line, because we used human TRBP sequence. As shown in Fig. 4C, TRBP and merlin were co-immunoprecipitated each other in SK cell lysates, whereas the pre-immune serum showed no detectable immunoprecipitates. This
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The localization of TRBP was further investigated in NF2-wild or -deficient MEF cell lines (MEF wt and MEF −/−). As shown in Fig. 5F, endogenous TRBP was detected mainly in the nucleus and partially in the perinuclear region and cytoplasm in the NF2-deficient MEF cells. Interestingly in wild type MEF cells with a physiological level of merlin, TRBP was also detected in the cellular membrane region as well as in the nucleus and perinuclear regions (Fig. 5G). As Lallemand et al. (11) reported, we also detected endogenous merlin in the membrane region of wild type MEF cells (Fig. 5H). This result suggests at least that merlin is necessary for TRBP to localize at the cellular membrane in MEF cells.

TRBP-induced Cell Growth Is Inhibited by Merlin—Previously, it was shown that NIH3T3 cells overexpressing TRBP exhibited transformed phenotypes, such as the loss of contact inhibition, colony formation in soft agar, and tumor formation in animals (26). To investigate the biological function of the interaction between TRBP and merlin, the effect of merlin on TRBP-induced cell proliferation was investigated. Stable NIH3T3 cell lines expressing TRBP, merlin, or both were constructed, and the growth rate of several clones for each cell line was examined by MTS/PMS assay at 1 day after plating. Stable cell lines containing pcDNA3.1 plasmid (pcDNA) were used as a control. As shown in Fig. 6A, all of the TRBP-expressing stable clones (TRBP) showed significantly higher growth rates than the control clones (pcDNA). In contrast, the stable cell clones expressing both merlin and TRBP (TRBP+NF2) showed less growth enhancement than the TRBP-expressing stable clones. The inhibition of TRBP-induced cell growth by merlin was further confirmed by determining the growth curve of each cell clone over time. As shown in Fig. 6B, the TRBP stable cells grew more quickly than the control cells (pcDNA), whereas the NF2 stable cells grew more slowly than the control. Two stable cell lines co-expressing TRBP and NF2 (TRBP+NF2) showed an intermediate growth rate, similar to that of the pcDNA control cells. The TRBP stable cells reached

the wild type and deletion mutants of TRBP (bottom panel). The merlin overexpression in the cell lysates was confirmed by direct immunoblotting with merlin antibody (top panel). The closed and open arrows refer to two different forms of the merlin protein. B, cell lysates were immunoprecipitated (IP) with anti-V5 antibody and immunoblotted (IB) with anti-merlin antibody (middle panel). The same membrane was immunoblotted with anti-merlin antibody (bottom panel) to check the immunoprecipitated merlin. The TRBP and TRBPΔC overexpression in the input cell lysates was confirmed by direct immunoblotting with anti-V5 antibody (top panel). C, the SK cell lysates were immunoprecipitated (IP) separately with rabbit preimmune serum and anti-merlin and anti-TRBP antibodies. The immunoprecipitated proteins were detected by immunoblotting with anti-merlin (upper panel) and anti-TRBP antibodies (lower panel).
a maximum cell density on day 3 after plating, and the cell number decreased on day 4.

Merlin Inhibits TRBP-mediated Anchorage-independent Colony Formation of NIH3T3 Cells—A notable feature of TRBP is its ability to confer anchorage-independent growth ability on NIH3T3 cell lines, which can induce tumors in nude mice (26). Therefore, we wondered whether the concurrent expression of merlin would alter these oncogenic activities of TRBP. To investigate this, we tested the abilities of the TRBP- and/or merlin-expressing NIH3T3 cell lines described above to form colonies in the soft agar assay. A stable NIH3T3 cell line expressing oncogenic Ras was used as a control. As shown in Fig. 7, the TRBP stable cells showed significant colony forming ability in soft agar, in terms of the number and size of the colonies, although they were less active than the Ras stable cells. The colony forming ability of the TRBP+NF2 stable cells was reduced almost to zero compared with that of the TRBP stable cells. It is noteworthy that, whereas only about 40% of the TRBP stable cell colonies were <100 μm in diameter, all of the colonies of the TRBP+NF2 stable cells had this small size (Fig. 7A). Therefore, it can be concluded that the concurrent expression of merlin attenuates both the average number and size of the NIH3T3 cell colonies in soft agar formed by TRBP overexpression.

Merlin Inhibits TRBP-induced Tumorigenesis in Nude Mouse Model—To reproduce in vivo effect of merlin on TRBP observed in the soft agar assay, each of the stable cell lines used above was tested for its tumorigenic ability in nude mice. The stable cell lines were injected into the nude mice, and the tumor formation abilities were examined after 4 weeks. As seen in Fig. 8, the Ras or TRBP stable cells led to the formation of rapidly growing tumors in all three mice, whereas none of the three mice injected with the NF2 stable cells showed tumor formation. Only one of the three mice injected with the TRBP+NF2 stable cells developed a tumor-like protrusion. However, it was slightly smaller in size than the tumors developed by TRBP or Ras and showed signs of inflammation. Taken together with the results obtained from the cultured cells (Figs. 6 and 7), these findings confirmed that TRBP induces unregulated cell growth when overexpressed and indicate that merlin plays a regulatory role in the oncogenic activity of TRBP.

DISCUSSION

Previously, we reported that merlin inhibited the Ras signaling pathway strongly related to cell transformation and tumor development (27, 34). Moreover, we recently observed that merlin increased p53 activity by inducing MDM2 degradation.

Fig. 5. Subcellular localizations of merlin and TRBP. NIH3T3 cells were transfected with the TRBP-GFP and/or merlin-RFP fusion constructs (A–E). The cells were then observed for GFP (green) and RFP (red) fluorences using a confocal fluorescence microscope. The merged image of TRBP-GFP and merlin-RFP in the co-transfected cells was shown. The wild type and NF2-deficient mouse embryonic fibroblasts (MEF wt and MEF −/−) were immunostained with anti-TRBP antibody and counterstained for nucleus with 4',6-diamidino-2-phenylindole (F and G). The subcellular localization of merlin was investigated in wild type MEF cells (H). The results are representative images of three independent experiments. The expression of TRBP and merlin in MEF cell lines was checked by immunoblotting (I).
in NIH3T3 cells (35). Considering all of these results and related papers, it would appear that merlin has multiple activities for tumor suppression, one of which might be the suppression of TRBP function reported herein.

In this study, we showed that TRBP-mediated anchorage-independent cell growth and tumorigenesis in the nude mouse model were suppressed by merlin (Figs. 7 and 8). It was also demonstrated that the cell proliferation (Fig. 6) and focus formation (data not shown) induced by TRBP was inhibited by the co-expression of merlin in NIH3T3 cells. These findings suggest that merlin plays a role in the regulation of the oncogenic activity of TRBP.

TRBP was initially known as a cellular protein that binds HIV-1 transactivation-response RNA and increases viral expression from the long terminal repeat in synergy with functional Tat (14, 22). TRBP is also known to play a role in the cellular down-regulation of the PKR that has anti-viral and anti-proliferative effects. TRBP directly binds PKR and inhibits its ability to phosphorylate eukaryotic translation initiation factor 2, leading to its inactivation (25, 26). In relation to the inhibition of PKR activity, TRBP was recently demonstrated to have a growth promoting role and oncogenic potential; cells that overexpress TRBP exhibit transformed phenotypes (26).

Therefore it is possible that the inhibition of TRBP function by merlin leads to PKR activation, which in turn inhibits cell proliferation and transformation. However, the exact role of each protein and the precise mechanism of their interaction in tumor development need further investigation.

The functional counteraction between TRBP and merlin appears to be mediated via direct interaction of these proteins. The physical interaction of merlin and TRBP was demonstrated in vitro, in vivo, and in the physiological condition (Figs. 3 and 4), and domain analysis identified the carboxyl-terminal regions of each protein as being responsible for their interaction (Table I and Figs. 3 and 4).

Interestingly in our study, TRBP interacted preferentially with an active form of the hypophosphorylated merlin, as compared with the phosphorylated form (Figs. 3 and 4). Both the endogenous and overexpressed merlins in cells seem to exist predominantly in the phosphorylated form, suggesting that the level of the hypophosphorylated merlin is tightly controlled. Previously, it was reported that the active hypophosphorylated merlin associates with β-catenin, a potential oncoprotein, and functions as a tumor and metastasis suppressor by controlling cadherin-mediated cell-cell contact (11). Merlin associates with β-catenin at sites of cell-cell contact and the loss of merlin at these locations may directly impair the formation of adherens junctions or the stability of their components, leading to the loss of contact-dependent inhibition of cell growth. In view of the results obtained so far, it is plausible to assume that active merlin also interacts with TRBP and inhibits its ability to control cell growth.

It is not yet clear what the regulation mechanism and downstream events of the interaction between TRBP and merlin are.
injected with the indicated stable cell lines. The only one nude mouse among the three test models. TRBP produce tumors of the indicated size are listed as the latency period. *, using the average values of width and length. The times required to TRBP was detected in the cellular membrane region only in the partly in the cell membrane (Fig. 5). In MEF cells, endogenous GFP was observed mainly in the cytoplasm and partly in the B mic membrane (Fig. 5)

FIG. 8. Merlin suppresses TRBP-induced tumorigenesis. The BALB/c-nu/nu mice were subcutaneously injected with the indicated stable cell lines (1 x 10^7). A, photographs of mice 4 weeks after being 107). B, photographs of mice 4 weeks after being

Notably in this study, Merlin seemed to have a role in the membrane subcellular localization of TRBP. Consistent with previous reports (32, 36, 37), this study showed that the overexpressed Merlin-RFP was observed primarily at the cytoplasmic membrane (Fig. 5B) and other previously reported sites, such as the membrane ruffling region and granules/vesicles in the perinuclear region (data not shown). Overexpressed TRBP-GFP was observed mainly in the cytoplasm and partly in the nucleus (Fig. 5A), as previously reported (22, 38). When expressed in the same cells, however, Merlin-RFP and TRBP-GFP were co-localized mainly in the perinuclear region and partly in the cell membrane (Fig. 5). In MEF cells, endogenous TRBP was detected in the cellular membrane region only in the wild type but not in the NF2-deficient MEF cells, which supports further the possible role of Merlin in the membrane localization of TRBP.

In conclusion, we demonstrated that TRBP, which was previously described as a double-stranded RNA-binding protein and was involved in tumorigenesis, also interacts specifically with the carboxyl terminus of Merlin via its carboxyl-terminal region. The consequences of their interaction include inhibitory effects on TRBP-mediated cell proliferation, anchorage-independent cell growth, oncogenic transformation, and tumor development in nude mice.

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