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Association of ANA, a Member of the Antiproliferative Tob Family Proteins, with a Caf1 Component of the CCR4 Transcriptional Regulatory Complex

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A 35-kDa protein, ANA, belongs to an emerging family of antiproliferative proteins consisting of Tob, Tob2, ANA/BTG3, PC3B, PC3/TIS21/BTG2, and BTG1. All of these, except ANA and PC3B, have been shown to interact with the CCR4 transcription factor-associated protein Caf1. Here we show that ANA also associates with Caf1, ANA being the preferred partner of Caf1 among the Tob family proteins. Although ANA is likely to interact with Caf1 at its amino-terminal half, which is conserved among the family members, our data suggest that the carboxyl-terminal half of ANA plays a role in the interaction. Finally, *in situ* hybridization experiments revealed that expression of *Caf1* overlaps at least in part with that of *ANA*. Thus, ANA could function through its interaction with Caf1.

Key words: Tob family — Antiproliferative proteins — Caf1

The *Tob*, *Tob2*, *PC3B*, *PC3/TIS21/BTG2*, and *BTG1* genes, together with the *ANA* genes, form an antiproliferative gene family[1–8] termed the *Tob* family. *ANA* was identified as a new member of the Tob family by means of a polymerase chain reaction-mediated cloning procedure.[1] Exogenous expression of ANA suppresses growth of NIH3T3 cells by inhibiting cell cycle progression through the G1 phase,[1] as is the case with other Tob family members.[3, 4, 9] This suggests that *ANA* might be relevant to tumorigenesis. The *ANA* mRNA is expressed in a variety of adult tissues including testis, ovary, thymus, and liver. In the embryo, higher expression of *ANA* mRNA was detected in the neuroepithelial cells of the ventricular region of the neural tube,[1] suggesting that ANA plays a role in the regulation of growth and/or differentiation of the neuroepithelial cells. The expression of *PC3/TIS21/BTG2* is induced in a p53-dependent manner after DNA damage, and cells in which PC3/TIS21/BTG2 is ablated undergo apoptosis following DNA damage.[7] Therefore, PC3/TIS21/BTG2 is suggested to promote p53-induced cell cycle arrest. Although the underlying mechanisms of growth inhibition by the Tob family proteins remain to be fully established, recent studies strongly argue that Tob family proteins are involved in transcriptional control. For example, the homeodomain-containing transcription factor HoxB9 was identified as a possible functional partner of BTG1 and PC3/TIS21/BTG2.[10] It is also reported that overexpression of PC3/TIS21/BTG2 causes significant down-regulation of the cyclin D1 transcript by affecting cyclin D1 promoter activity.[9] Furthermore, Tob is associated with Smad proteins and inhibits BMP-induced, Smad-mediated transcription.[11] Finally, Tob, Tob2, BTG1, and PC3/TIS21/BTG2 are associated with Caf1 (CCR4-associated factor 1).[3, 12, 13] CCR4 (CCR: Carbon Catabolite Repression) is a component of a gigantic complex of transcription factors required for expression of number of genes including the gene coding for alcohol dehydrogenase II.[14] However, the specificity and mechanism of interaction between the Tob family proteins and Caf1 are unknown. As an initial step to address the issue, we examined the interaction of ANA with Caf1 and compared the degree of interaction between each Tob family protein and Caf1. Here, we report that ANA also associates with Caf1, ANA being the preferred partner of Caf1 among the Tob family proteins.

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). The cells were transfected with various combinations of plasmids. Twenty-four hours after transfection, cells were solubilized in a buffer containing 10 m*M* Tris-HCl (pH 7.8), 150 m*M* NaCl, 1% Nonidet P-40, 1% aprotinin, and 0.1% phenylmethylsulfonyl fluoride. Lysates were immunoprepicitated with the indicated immunoblotting antibody, and the proteins in the immunocomplex or the lysates were immunoblotted with the indicated immunoblotting antibodies as described.[1] Anti-FLAG M2 (Sigma Chemical Co., St. Louis, MO) and anti-Myc 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ANA[1] antibodies were used in the immunoprecipitation/immunoblotting experiments.

Embryos were collected, fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose, embedded in OCT (Tissue-Tek, Miles Inc.) and stored at −80°C until analysis. Sec-

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tions (14 μm) were cut on a cryostat. Specimens were hybridized with riboprobes labeled with [α-^{35}S]UTP as described.[1)]

To investigate possible interaction between the ANA and Caf1 proteins, we performed coimmunoprecipitation experiments. The Flag-tagged Caf1 construct was transfected into COS7 cells together with an expression plasmid encoding the mouse ANA protein. The lysates of transfectants were subjected to anti-Flag immunoprecipitation followed by immunoblotting with anti-ANA antibodies. As shown in Fig. 1A, ANA was coimmunoprecipitated with Caf1. In the reciprocal coimmunoprecipitation experiments, the Flag-tagged full-length ANA or a Flag-tagged amino-terminal 116-amino-acid (Tob homology domain, THD) portion of ANA (ANA-N) construct was transfected into COS7 cells together with the Myc-tagged Caf1 expression vector. Fig. 1B showed that Myc-Caf1 was immunoprecipitated with Flag-tagged full-length ANA. Although Myc-Caf1 interacted with Flag-tagged ANA-N, the interaction was very weak as compared with that between Myc-Caf1 and Flag-tagged full-length ANA. Thus, it is likely that the carboxyl-terminal half of ANA strengthens the association of ANA with Caf1.

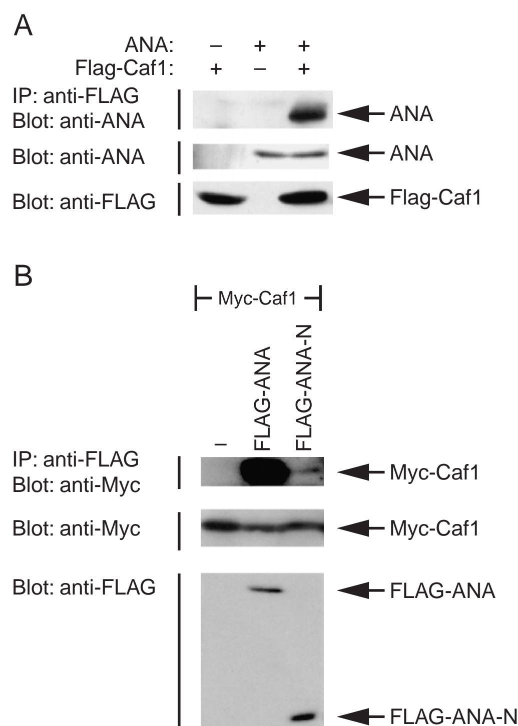

Fig. 1. Association of ANA with Caf1. The interactions of ANA with Flag-Caf1 (A) or those of Myc-Caf1 with Flag-ANA or Flag-ANA-N (B) in COS-7 cells were examined by immunoprecipitation (IP) followed by immunoblotting (Blot). The top panel shows the interaction, and the lower two panels show the expression of each indicated protein.

Next, we examined the specificity of the interaction between the Tob family proteins and Caf1. As we showed previously,[3)] Tob and Tob2 interacted with Caf1 (Fig. 2). Although other groups showed that BTG1 and BTG2 interacted with Caf1,[12, 13)] we did not detect the interaction between BTG1/2 and Caf1 in our coimmunoprecipitation experiments. Rouault *et al.*[12)] showed that BTG1/2 interacted with Caf1 in a yeast two-hybrid system, mammalian two-hybrid system, and *in vitro*. Bogdan *et al.*[13)] showed the association of BTG1 with Caf1 in a yeast two-hybrid system, and *in vitro*, as well as in coimmunoprecipitation experiments using lysates of rat aortic smooth-muscle cells (RSMCs). It is possible that some proteins expressed in RSMCs help Caf1 associate stably with BTG1 and that the proteins are missing in COS7 cells. It is also possible that interaction of THDs of the Tob family proteins with Caf1 is weak and strong interaction of the family proteins with Caf1 requires their carboxy-terminal proximal sequence. This is consistent with our present observation that interaction between THD of ANA and Caf1 is weaker than that between full-length ANA and Caf1. Thus, the mode of Caf1 interaction with ANA may be different from that with BTG1 and BTG2. The coimmunoprecipitation experiments shown in Fig. 2 also suggested that the association of Caf1 with ANA was stronger than that with Tob or Tob2. There may be a sequence located downstream of ANA THD that is responsible for the strong interaction between ANA and Caf1.

To compare mRNA expression of *Caf1* with that of Tob family genes, we carried out *in situ* hybridization experiments using histological sections of the whole embryo at

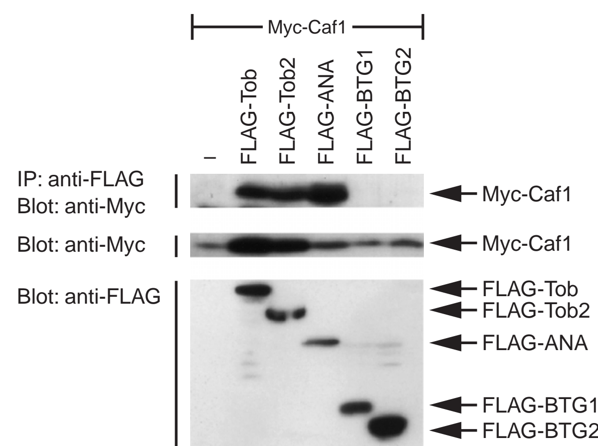

Fig. 2. Association of Tob family proteins with Caf1. The interactions of Flag-tagged Tob family proteins with Myc-Caf1 in COS-7 cells were examined by immunoprecipitation (IP) followed by immunoblotting (Blot). The top panel shows the interaction, and the lower two panels show the expression of each indicated protein.

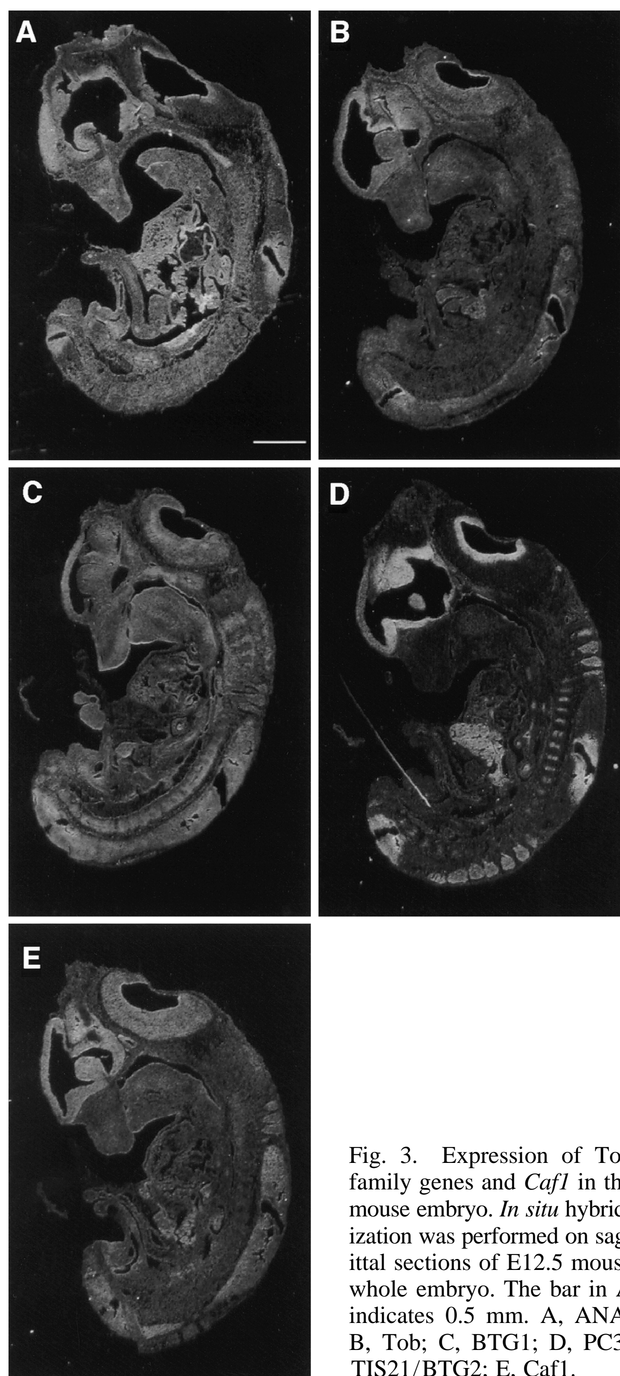

Fig. 3. Expression of Tob family genes and *Caf1* in the mouse embryo. *In situ* hybridization was performed on sagittal sections of E12.5 mouse whole embryo. The bar in A indicates 0.5 mm. A, ANA; B, Tob; C, BTG1; D, PC3/TIS21/BTG2; E, Caf1.

12.5 days postcoitum (E12.5). As we showed previously,[1] *ANA* mRNA is present in several tissues of the E12.5 embryo, being prominent throughout the entire ventricular zone, where neuroepithelial cells proliferate and commitment to a specific neural phenotype proceeds (Fig. 3A). The *Caf1* mRNA was also expressed in the ventricular zone (Fig. 3E). In addition to the expression in the ventricular zone, *Caf1* was expressed in the intermediate and marginal zones, where neuroepithelial cells that are no longer participating in DNA synthesis migrate and differentiate into neurons or glial cells. *BTG1*, *Tob*, and *Tob2* were expressed in the ventricular, intermediate, and marginal zones, like *Caf1* (Fig. 3, B and C; data not shown for Tob2). As previously reported,[15, 16] high expression of *PC3/TIS21/BTG2* mRNA was detected in the ventricular zone, as in the case of *ANA* (Fig. 3, A and D). In the mouse ventricular zone, expression of *PC3/TIS21/BTG2* (1) starts at the onset of neurogenesis, (2) is confined to a subpopulation of neuroepithelial cells that increases concomitantly with the progression of neurogenesis, and (3) is not detected in newborn neurons.[16] Expression of the *PC3/TIS21/BTG2* mRNA in the neuroepithelial cells occurs transiently in the G1 phase.[16] Therefore, like PC3/TIS21/BTG2, other Tob family members may regulate G1 arrest of neuroepithelial cells through their association with Caf1. Furthermore, expression of *Caf1* in the intermediate and marginal zones suggests that Caf1 functions in both neuroepithelial cells and differentiated neurons or glial cells. The pattern of expression of each Tob family mRNA overlaps with, but is distinct from that of *Caf1* mRNA, suggesting that Tob family proteins function with or without Caf1, depending on the cell types. Furthermore, Caf1 may be involved in tumor development because it is localized on chromosome 8p21.3–p22 that is often deleted in human tumors.[17] Therefore, the ANA interaction with Caf1 may play a role in the development of human tumors, although further studies in this direction are needed. Because accumulating evidence shows that Tob family proteins interact with transcription factors such as Caf1, Hoxb9 and, Smads,[3, 10–13] ANA may function as a regulatory component of transcription machineries.

There are reports showing that Caf1 is a component of a 1.9 mDa transcription machinery called CCR4-NOT complex,[18] and that Caf1 affects the initiation of transcription of several genes.[19, 20] It is likely that each Tob family protein is complexed with CCR4-NOT complex. Nonetheless, data are accumulating to show that Tob family proteins interact with other transcription factors, such as Smads,[11] and Hoxb9.[10] The biological significance of the interaction between Tob family proteins and these transcription machineries remains to be addressed. It should be also noted that Caf1 is reported to regulate mRNA function by participating in mRNA deadenylation.[21] This suggests possible involvement of Tob family proteins in regulation of mRNA turnover through the interaction with Caf1 as well as poly A tails of mRNA. Interestingly, our unpublished data show that Tob interacts with a poly A binding protein, PABP. Thus, it is likely that Tob family proteins and Caf1 function in both the cytoplasm and nucleus. Future studies on Caf1 and Tob family proteins may

uncover some link between transcription and translation machineries. Because ANA association with Caf1 is relatively strong as compared with that of the other family members, characterization of ANA function in transcriptional and translational controls is of importance.

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