CALEB Binds via Its Acidic Stretch to the Fibrinogen-like Domain of Tenascin-C or Tenascin-R and Its Expression Is Dynamically Regulated after Optic Nerve Lesion*

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Recently, we described a novel chick neural transmembrane glycoprotein, which interacts with the extracellular matrix proteins tenascin-C and tenascin-R. This protein, termed CALEB, contains an epidermal growth factor-like domain and appears to be a novel member of the epidermal growth factor family of growth and differentiation factors. Here we analyze the interaction between CALEB and tenascin-C as well as tenascin-R in more detail, and we demonstrate that the central acidic peptide segment of CALEB is necessary to mediate this binding. The fibrinogen-like globe within tenascin-C or -R enables both proteins to bind to CALEB. We show that two isoforms of CALEB in chick and rodents exist that differed in their cytoplasmic segments. To begin to understand the *in vivo* function of CALEB and since *in vitro* antibody perturbation experiments indicated that CALEB might be important for neurite formation, we analyzed the expression pattern of the rat homolog of CALEB during development of retinal ganglion cells, after optic nerve lesion and during graft-assisted retinal ganglion cell axon regeneration by *in situ* hybridization. These investigations demonstrate that CALEB mRNA is dynamically regulated after optic nerve lesion and that this mRNA is expressed in most developing and in one-third of the few regenerating (GAP-43 expressing) retinal ganglion cells.

A complex network of molecular interactions regulates the differentiation of the nervous system. These interactions occur between neural cells as well as between neural cells and their extracellular surroundings, the extracellular matrix (ECM)[1] which is composed of a complex agglomerate of glycoproteins and proteoglycans with diverse functions (1). For example, some of these glycoproteins can support or inhibit axonal growth (2, 3), and others may modulate the function of proteins and peptides that are synthesized and released by neurons and glial cells (4). One such example of the latter is the binding of acetylcholine receptor-inducing activity (ARIA, a specific isoform of neuregulin-1) to the glycosaminoglycan portion of proteoglycans due to charged interactions with its amino-terminal portion (5). The EGF-like domain of ARIA that mediates its biological activity can be released by proteolysis from the transmembrane precursor and might become tethered to the ECM of developing synapses (6). Due to regulated proteolysis, the active EGF-like domain of ARIA might become accessible to the ErbB receptor tyrosine kinases and induce the synthesis of acetylcholine receptor subunits at the neuromuscular junction (7–10).

In a screen to isolate novel molecules presumed to be involved in nervous system differentiation, we identified the transmembrane protein CALEB (chicken acidic leucine-rich EGF-like domain containing brain protein) (11). We described three different forms of CALEB of molecular masses of 200, 140, and 80 kDa, all of which contain an EGF-like domain, which is most similar to the EGF-like domains of the members of the EGF family of transmembrane growth and differentiation factors. In addition to the EGF-like domain, a very acidic peptide segment is present in the extracellular part of all the three CALEB components. The 140- and 200-kDa components of CALEB additionally comprise an amino acid sequence enriched in leucines and prolines and potential attachment sites for chondroitin sulfate chains. CALEB is expressed in synapse and axon-rich areas in the developing nervous system, and *in vitro* antibody perturbation experiments revealed a participation of CALEB in neurite formation in a permissive growth environment. CALEB is able to interact both with tenascin-C (TN-C) and tenascin-R (TN-R), members of the tenascin family of ECM proteins. These are large glycoproteins composed of a cysteine-rich region at the amino terminus, multiple EGF-like repeats of the tenascin subtype, several fibronectin type III

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[1] The abbreviations used are: ECM, extracellular matrix; ARIA, acetylcholine receptor-inducing activity; CALEB, chicken acidic leucine-rich EGF-like domain containing brain protein; dac, days after crush; E, embryonic day; EGF, epidermal growth factor; FN, fibronectin; NGC, neuroglycan C; ONL, optic nerve lesion; P, postnatal day; RGC, retinal ganglion cell; RPTP, receptor protein-tyrosine phosphatase; TN-C, tenascin-C, TN-R, tenascin-R; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis; DIG, digoxigenin; mAb, monoclonal antibody; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

(FNIII)-like repeats, and a single carboxyl-terminal globe similar to the globular parts of the γ and β chains of fibrinogen (12–15). TN-C and TN-R associate to hexamers and trimers, respectively, mediated by heptad repeats within the amino-terminal cysteine-rich segment. For TN-C, several variants have been reported that are generated by alternative splicing of the FNIII-like repeats whereas TN-R contains two sites of alternative splicing. Multiple ligands have been described for TN-C and TN-R including the cell surface proteins F11, axonin-1, neurofascin, RPTPβ/ζ, the β_2 subunit of voltage-gated sodium channels, and different types of integrins (16–25). Furthermore, both TN-C and TN-R can bind to other ECM glycoproteins and proteoglycans such as neurocan, phosphacan (a specific isoform of RPTPβ/ζ), versican, brevican, fibronectin, as well as to heparin (26–28). In addition, the alternative spliced segment TnfnA–D is able to interact with annexin II (29). Many functional features have been ascribed to TN-C and TN-R in the nervous system. For example, TN-C and TN-R contain both adhesive and counteradhesive sites for cell attachment. TN-C is able to stimulate neurite outgrowth (30), and TN-R has been shown to enhance neurite growth mediated by other substrates and to modulate the cellular receptor usage that is responsible for mediating the outgrowth effect (31, 32).

Our previous investigations demonstrated that CALEB can interact with TN-C and TN-R, but it has not been defined which regions of CALEB or the tenascins are required (11). Here we report that the acidic peptide segment of CALEB is necessary for binding to TN-C and TN-R. Furthermore, we show that the fibrinogen-like module of TN-C and TN-R is responsible for the interaction with CALEB. We have investigated whether other isoforms of CALEB are generated in the chick, and we compared these sequences with mammalian homologs. In all species examined, two isoforms of CALEB exist, which are generated by alternative pre-mRNA splicing. These splicing events lead to two different cytoplasmic tails of CALEB. Since our previous *in vitro* investigations suggested a participation of CALEB in neurite formation, we examined the expression pattern of the rat homolog of CALEB during development, after optic nerve lesion, and during graft-assisted axon regeneration in the retina of adult rats.

MATERIALS AND METHODS

Constructs and cDNA—CALEB constructs C1, C2, and C3 were generated using PCR and the CALEB cDNA (11). For construct C1, C2, and C3, the upstream primers 5′-cacactagtcgagatcatcgacgttgactactac-3′, 5′-cacactagtggccgatttctaccccaccacctcc-3′, and 5′-cacactagtgccggagaacagcagtgagtgccg-3′ were used, respectively. The downstream primer in all cases was 5′-cacctcgaggtcggtgacgatggcctcgcagcg-3′. PCR products were introduced into vector pDELF-1 (33) using *Spe*I and *Xho*I sites. mCALEB/NGC constructs mC2 and mC3 were generated using mCALEB/NGC cDNA and PCR. For construct mC2 and mC3 the upstream primers were 5′-cacactagtgtctgatttctaccccaccacatcc-3′and 5′-cacactagttctcctgccctctcaaaagcctgg-3′, respectively. The downstream primer was 5′-cacctcgaggtccgtgatgatggactcacagcg-3′ in both cases. PCR products were subcloned into vector pDELF-1 using *Spe*I- and *Xho*I-cloning sites.

The putative mouse homolog of CALEB was obtained by screening a cDNA library of P20 mouse brain (Stratagene, Heidelberg, Germany) using a probe composed of the sequence that encodes the acidic peptide segment, the EGF-like domain, and the transmembrane region of CALEB. Different independent cDNA clones were obtained, which cover most of the coding sequence but lacked the 5′-end. 5′-RACE technology using the "5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0" (Life Technologies, Inc.) with the following modification was employed to establish the 5′-end. 2 μg of poly(A)$^+$ RNA for cDNA synthesis was used instead of total RNA, and the "gene-specific primer" was 5′-cacactagtcactgcagccgatgcctctag-3′. PCR products of four independent reactions were cloned and sequenced. The presumed rat homolog of CALEB was amplified by RT-PCR from poly(A)$^+$ RNA prepared from rat brain using the upstream primers 5′-cacgaattctgggcagctctcccggggtcaccag-3′ and 5′-cacgaattcaagccgcgcgcaccggcaacagc-3′ and the downstream primer 5′-cacgaattctgcccgagaggagacaacagtccttc-3′, all of which were derived from the corresponding mouse sequence. Several independent amplification products were cloned and sequenced. A cDNA clone encoding CALEBb, the isoform not described so far, was obtained by screening an E16 chick brain library (34) using a probe, which is composed of the sequence encoding the EGF-like domain, the transmembrane region, and the cytoplasmic domain of CALEB.

Proteins and Antibodies—TN-C and TN-R were isolated from urea extracts of adult chicken brains by immunoaffinity chromatography as described using mAb M1 and mAb 23-13, respectively (35, 36). SDS-PAGE was performed with 7% acrylamide under reducing conditions followed by Coomassie Blue staining (37). Western blots of CALEB components were analyzed using mAb 4/1 to CALEB. Recombinant chick TN-C variants (TN-C/190, FF$^-$, EFb$^-$, and EFn$^-$) were cloned as detailed elsewhere and expressed in stably transfected HT 1080 cells (American Type Culture Collection, Manassas, VA) (28, 30). The encoded recombinant proteins were purified from the conditioned medium of the HT 1080 cells. The chick TN-R construct EFn$^-$ (TN-R) corresponding to EFn$^-$ (TN-C) was cloned into the vector pFLAG-CMV-1 (Sigma) and expressed in COS7 cells (American Type Culture Collection). For this, the cysteine-rich region of TN-R together with a His tag was amplified using PCR with the primers 5′-gcgaattcacatcaccatcaccatcacgaggggggcctggccaactgc-3′ and 5′-gggagatctacggcacgactccaggct-3′ and cloned into pFLAG-CMV-1 using *Eco*RI and *Bgl*II. In the next step the fibrinogen-like domain of TN-R was amplified by PCR using the primers 5′-gggagatctggtggccgtgtctttt-3′ and 5′-cgcggatcctcagagctgtaggaccg-3′ and introduced into this modified vector using *Bgl*II and *Bam*HI. Recombinant protein was purified from conditioned medium using nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany). The purity of proteins was analyzed by SDS-PAGE followed by silver staining.

Purified TN-C and TN-R as well as the different recombinant proteins were coupled to red fluorescent microspheres of 0.5 μm diameter according to the manufacturer's protocol (Bioclean: Duke Scientific Corp., Palo Alto, CA). Proteins to be coupled were used in a concentration range of 20–50 μg/ml. The coupling reactions were performed in phosphate-buffered saline for 2 h at 24 °C or overnight at 4 °C. Residual binding sites were blocked with bovine serum albumin.

For affinity chromatography using recombinant proteins TNC/EFb$^-$ and TNC/EFn$^-$ immobilized to CNBr-activated Sepharose, embryonic day 20 (E20) chick brains were homogenized in homogenization buffer (250 mM sucrose in 20 mM Hepes, pH 7.2, with protease inhibitor mixture). The 10,000 rpm pellet was solubilized in homogenization buffer containing 2% Chaps. After centrifugation (100,000 $\times$ *g*), half the volume of the supernatant was passed over the TNC/EFn$^-$ and the TNC/EFb$^-$ columns, respectively. Both columns were washed with homogenization buffer containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.5% Chaps. Columns were eluted with the same buffer without cations but with 10 mM EDTA.

Transfection of COS7 Cells and Microsphere Binding Assay—COS7 cells were transiently transfected using the DEAE-dextran method as described previously (16, 38). At day 1 after transfection, the cells were trypsinized, washed, and replated on collagen-coated 8-well chamber slides (Nalge Nunc International Corp., Naperville, IL). At day 2 after transfection, the supernatant was removed and replaced by 200 μl of Dulbecco's modified Eagle's medium with 10% fetal calf serum. 1 μl of fluorescent microspheres was added per well. After a 2-h incubation at 37 °C, cells were washed, fixed, and stained for CALEB construct expression by indirect immunofluorescence using rabbit anti-human IgG, Fcγ fragment (Dianova, Hamburg, Germany). Binding reactions were analyzed with a confocal microscope (MRC1000, Bio-Rad or Zeiss LSM, Oberkochen, Germany).

Surgery and in Situ Hybridizations—Surgery and preparation of retinal explants were performed exactly as described (39). To generate a cRNA probe, a 200-base pair fragment of mCALEB was amplified by PCR using the mCALEB cDNA with the primers 5′-cacgaattcttggcaaagaagacagtgagcatg-3′ and 5′-cacggatccccagctactgccacaacggc-3′. The generation of cRNA and the procedure of the *in situ* hybridization were carried out as detailed (39). For double *in situ* hybridizations, the hybridization solution contained both a DIG- and a fluorescein-labeled riboprobe (150 ng/ml each). The DIG-labeled probe is detected first using anti-DIG-alkaline phosphatase reaction developed with BM purple. After the washing steps, the alkaline phosphatase is inactivated by heat (80 °C for 20 min). The fluorescein-labeled riboprobe was then detected by incubation with an alkaline phosphatase-conjugated antibody to fluorescein followed by a color reaction with Fast Red (Roche Molecular Biochemicals).

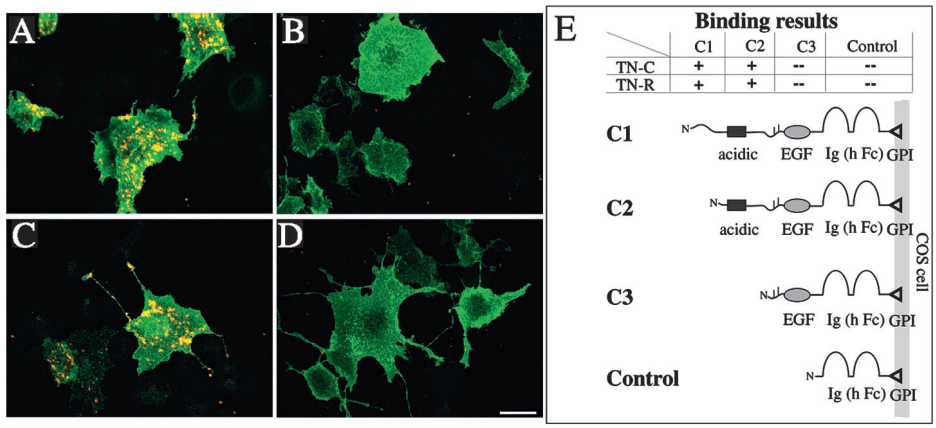


FIG. 1. **Binding of TN-C to COS7 cells expressing different CALEB constructs.** The CALEB mutant polypeptides shown in *E* were generated using the pDELF-1 vector that drives cell surface expression of these proteins in COS7 cells by the SV40 promoter and the signal peptide of the neural cell recognition molecule F11. The CALEB constructs were fused to the CH2 and CH3 domains of human IgG1 followed by the glycosylphosphatidylinositol (*GPI*) anchor attachment signal of F11. Red fluorescent microspheres loaded with TN-C were tested for binding to CALEB fusion proteins expressed on COS7 cells. Transfected cells were detected by their green fluorescence after labeling with an antibody that recognizes the human IgG-Fc portion of the fusion proteins followed by a fluorescein isothiocyanate-coupled secondary antibody. Red fluorescent microspheres loaded with TN-C bind to COS7 cells that express CALEB construct C1 (*A*) or C2 (*C*), both of which contain the acidic peptide segment as well as the EGF-like domain of CALEB. These microspheres do not bind to cells transfected with the control plasmid lacking any CALEB encoding sequences (*B*). *D* shows that TN-C-loaded microspheres are not able to interact with COS7 cells expressing CALEB construct C3, which contains the EGF-like domain of CALEB but lacks the acidic peptide segment. *E* summarizes the binding results and shows the different constructs in a schematic manner. TN-R reveals the same binding pattern as TN-C (Fig. 2*F* and data not shown). *Bar*, 50 μm.

RESULTS

The Acidic Peptide Segment of CALEB Is Necessary to Mediate the Binding to TN-C or TN-R—In a screening procedure applied to characterize novel binding proteins of known axon-associated glycoproteins, we identified CALEB due to its interaction with the ECM glycoproteins TN-C and TN-R (11). Further independent assays confirmed the binding between CALEB and TN-C or TN-R. To map regions of CALEB that might be responsible for these interactions, we used different deletion constructs of the extracellular portion of CALEB cloned into the pDELF-1 vector (Fig. 1). This vector is a derivative of plasmid pSG5, which contains the SV40 early promoter/origin of replication and carries the signal peptide sequence of the neural cell adhesion molecule F11, a multiple cloning site, and the CH2 and CH3 domains of human IgG1 followed by the glycosylphosphatidylinositol anchor attachment signal of F11 (33). The signal peptide of F11 results in a strong cell surface expression of CALEB domains, and the constant domains of IgG1 allowed us to compare the expression efficiency of the different CALEB constructs in COS7 cells by indirect immunofluorescence. Construct C1 contains amino acid residues 286 (EIIDV) to 476 (AIVTD) of CALEB (11) and therefore composes the acidic peptide segment and the EGF-like domain. In addition, it contains at its amino terminus a potential tyrosine sulfation motif, which might be important for binding TN-C or TN-R. Construct C2 lacks this potential tyrosine sulfation motif, starts with amino acid 338 of CALEB (ADFYP), and ends at position 476 as construct C1. Construct C3 is composed of the amino acid sequence starting from residue 411 (PENSS) and ending with residue 476 of CALEB. This construct encodes the EGF-like domain but not the acidic peptide segment of CALEB. These vectors were transfected into COS7 cells which, after 2 days of cultivation, were incubated with red fluorescent microspheres coated with purified TN-C or TN-R, respectively. Transfected COS7 cells were identified using an antibody to the Fcγ portion of human IgG1, followed by a secondary antibody, which contains a green fluorochrome. Red/yellow fluorescence indicates binding of the microspheres to the transfected cells. As shown in Fig. 1, microspheres coated with TN-C bind to COS7 cells transfected with CALEB construct C1 (Fig. 1*A*), or with construct C2 (Fig. 1*C*), but do not bind to COS7 cells transfected with construct C3 (Fig. 1*D*). No binding was observed to untransfected COS7 cells or to COS7 cells transfected with the pDELF plasmid on which the IgG1 domains without CALEB sequences are expressed (Fig. 1*B*). These observations further demonstrate the specificity of the assay. The same results were obtained when using microspheres coated with TN-R instead of TN-C (Fig. 2*F* and data not shown). These results demonstrate that the acidic peptide segment of CALEB is required for binding between CALEB and the tenascins, whereas the EGF-like domain of CALEB alone or the potential tyrosine sulfation motif is not important for this interaction.

The Fibrinogen-like Module of TN-C and TN-R Mediates the Interaction with CALEB—TN-C and TN-R are large ECM glycoproteins composed of a cysteine-rich region at the amino terminus followed by multiple EGF-like and FNIII-like domains. A fibrinogen-like module is located at the carboxyl terminus (12–15). To identify domains of TN-C and TN-R responsible for binding to CALEB, we expressed CALEB construct C1 (Fig. 1*E*), which does bind purified TN-C and TN-R, on COS7 cells. We then coupled proteins derived from different TN-C constructs (Fig. 2*E*) onto red fluorescent microspheres, and we incubated the transfected COS7 cells with these microspheres. Microspheres coated with recombinant TN-C/190, the smallest occurring splice variant of TN-C in chick lacking the FNIII-like domains A–D, are able to bind to COS7 cells that had been transfected with CALEB construct C1 (Fig. 2*A*). Microspheres coated with protein FF$^-$ (TN-C), which lacks both the FNIII-like domains as well as the fibrinogen-like module of TN-C, do not bind to COS7 cells transfected with CALEB construct C1 (Fig. 2*B*). The same result was observed for microspheres coated with EFb$^-$ (TN-C), which composes the cysteine-rich region and the FNIII-like domains 1–5 and 6–8 of TN-C but lacks the fibrinogen-like module (Fig. 2*C*). In contrast, recombinant protein derived from a construct that encodes only the cysteine-rich region and the fibrinogen-like globe of TN-C (EFn$^-$ (TN-C); Fig. 2*E*) is able to mediate the binding to transfected COS7 cells, when coupled to red fluorescent microspheres (Fig. 2*D*). On the basis of these findings using TN-C mutant polypeptides, we generated a similar deletion mutant of TN-R containing the cysteine-rich segment and the fibrino-

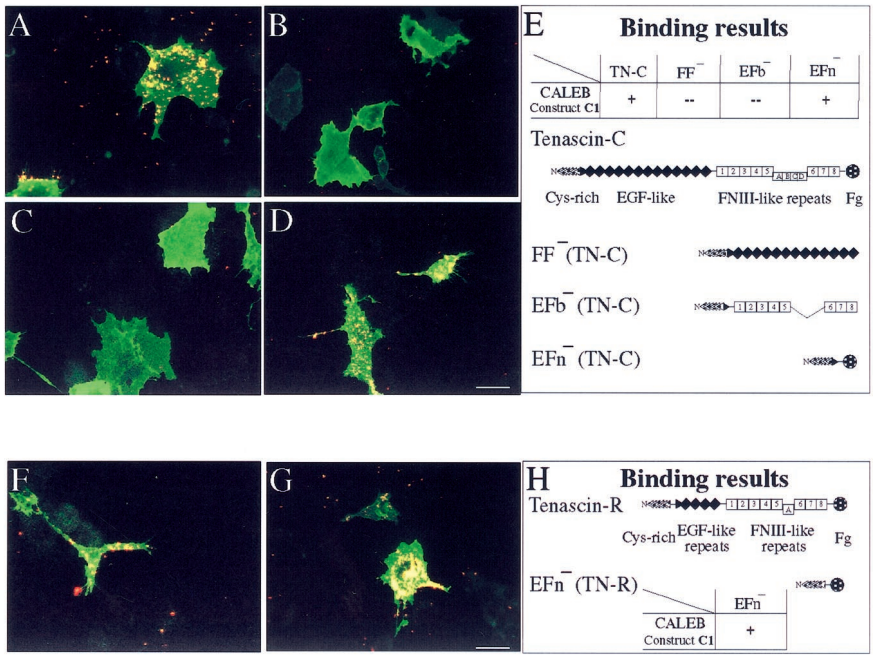


FIG. 2. **Binding of recombinant TN-C (*A–E*) or TN-R (*F–H*) mutant polypeptides to CALEB fusion protein expressed on COS7 cells.** In these experiments COS7 cells were transfected with CALEB construct C1 (see Fig. 1). The transfected cells were incubated with red fluorescent microspheres loaded with different recombinant proteins encoded by TN-C or TN-R constructs (see *E* and *H*). *A* reveals that recombinant TN-C (TN-C/190) binds to CALEB-transfected COS7 cells. Neither protein derived from construct FF⁻ of TN-C (*B*), which lacks the FNIII-like repeats as well as the fibrinogen-like module (*Fg*), nor from the construct EFb⁻ of TN-C (*C*), which does not contain both the EGF-like segment and the fibrinogen-like module, bind to CALEB-transfected cells. However, recombinant protein encoded by construct EFn⁻ of TN-C, which contains the cysteine-rich domain (Cys-rich) as well as the fibrinogen-like module, but not the EGF-like repeats or FNIII-like repeats, does bind to COS7 cells that were transfected with CALEB construct C1 (*D*). *F* and *G* show the binding of purified TN-R and the polypeptide derived from construct EFn⁻ of TN-R, respectively. Both interact with COS7 cells transfected with CALEB construct C1. The results are summarized in *E* and *H*. *Bars*, 50 μm.

gen-like module, and we coated microspheres with the recombinant protein (EFn⁻ (TN-R); Fig. 2*H*). These beads were found to bind to COS7 cells that were transfected with CALEB construct C1 (Fig. 2*G*). In summary, our investigations indicate that the fibrinogen-like module of either TN-C or TN-R mediates the interaction with the acidic peptide segment of CALEB.

CALEB Components Can Be Purified from Detergent Extract by Affinity Chromatography Using EFn⁻ (TN-C)—To confirm the importance of the fibrinogen-like globe in binding of TN-R or TN-C to CALEB, we prepared affinity columns with the recombinant polypeptides EFn⁻ or EFb⁻ of TN-C mentioned above. Equal amounts of a detergent extract from E20 chick brain as indicated by Coomassie Blue-stained gels in Fig. 3*A* were passed over both columns (load). After washing, the columns were eluted with 10 mM EDTA, and the resulting fractions (load (*L*), flow-through (*F*), wash (*W*), and eluate (*E*)) were separated on SDS-PAGE, blotted to nitrocellulose, and probed with mAb 4/1 to CALEB (Fig. 3*B*). Two immunopositive bands (*arrows*) characteristic for CALEB from embryonic brain could be detected in the load. The flow-through of column EFn⁻ (TN-C) contained a small amount of immunopositive CALEB bands, whereas most could be detected in the eluate. In contrast no CALEB components were observed in the eluate of the column EFb⁻ (TN-C) but were present in the flow-through. The EDTA elution step followed by 1 M NaCl did not result in elution of any additional CALEB components (data not shown). Taken together, these results demonstrate that the fibrinogen-like domain of TN-C or TN-R mediates the interaction to CALEB and support the binding studies using COS7 cells.

CALEB Is Generated in Two Isoforms and Is Related to Neuroglycan C in Mouse and Rat—Several members of the EGF family of differentiation factors such as the neuregulins are generated as multiple isoforms. We have therefore extensively screened for alternative forms of CALEB in cDNA libraries of embryonic chicken brain. By using a probe that encodes the EGF-like domain, the transmembrane region, and a part of the cytoplasmic domain of CALEB, we detected two isoforms of CALEB, designated as a and b forms, which differed in their cytoplasmic tails. The a form is identical to our original published sequence of CALEB (11), and the b form lacks the eight carboxyl-terminal residues (REAQHRAL) and instead contains an additional sequence of 50 amino acid residues (Fig. 4). As previously discussed by us the EGF-like domain, the trans-

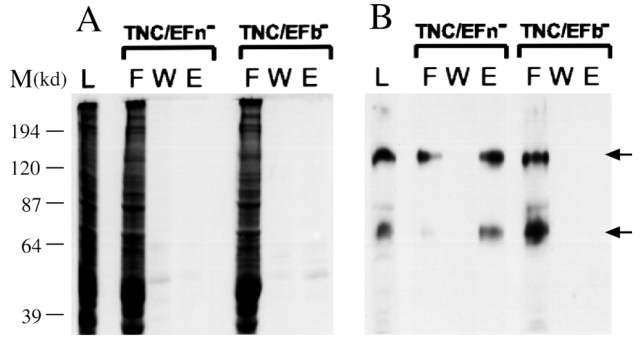


FIG. 3. **CALEB from brain detergent extract binds to immobilized recombinant protein encoded by construct EFn⁻ of TN-C.** A detergent extract of chicken brain (embryonic day 20) was passed over columns containing immobilized protein derived from either construct EFb⁻ of TN-C (TNC/EFb⁻) or EFn⁻ of TN-C (TNC/EFn⁻). After washing with homogenization buffer (see "Materials and Methods") containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.5% Chaps, the columns were eluted with 10 mM EDTA in homogenization buffer. *L* indicates the detergent extract that was applied to the column (load); *F* indicates the flow-through; *W* indicates the wash; and *E* indicates the eluate. Samples were resolved by 7% SDS-PAGE and stained with Coomassie Blue (*A*) or probed on Western blot with the mAb 4/1 specific for CALEB (*B*). Molecular mass markers (*M*) are indicated to the *left*. The two bands, which were recognized by the mAb 4/1, are marked by *arrows*.

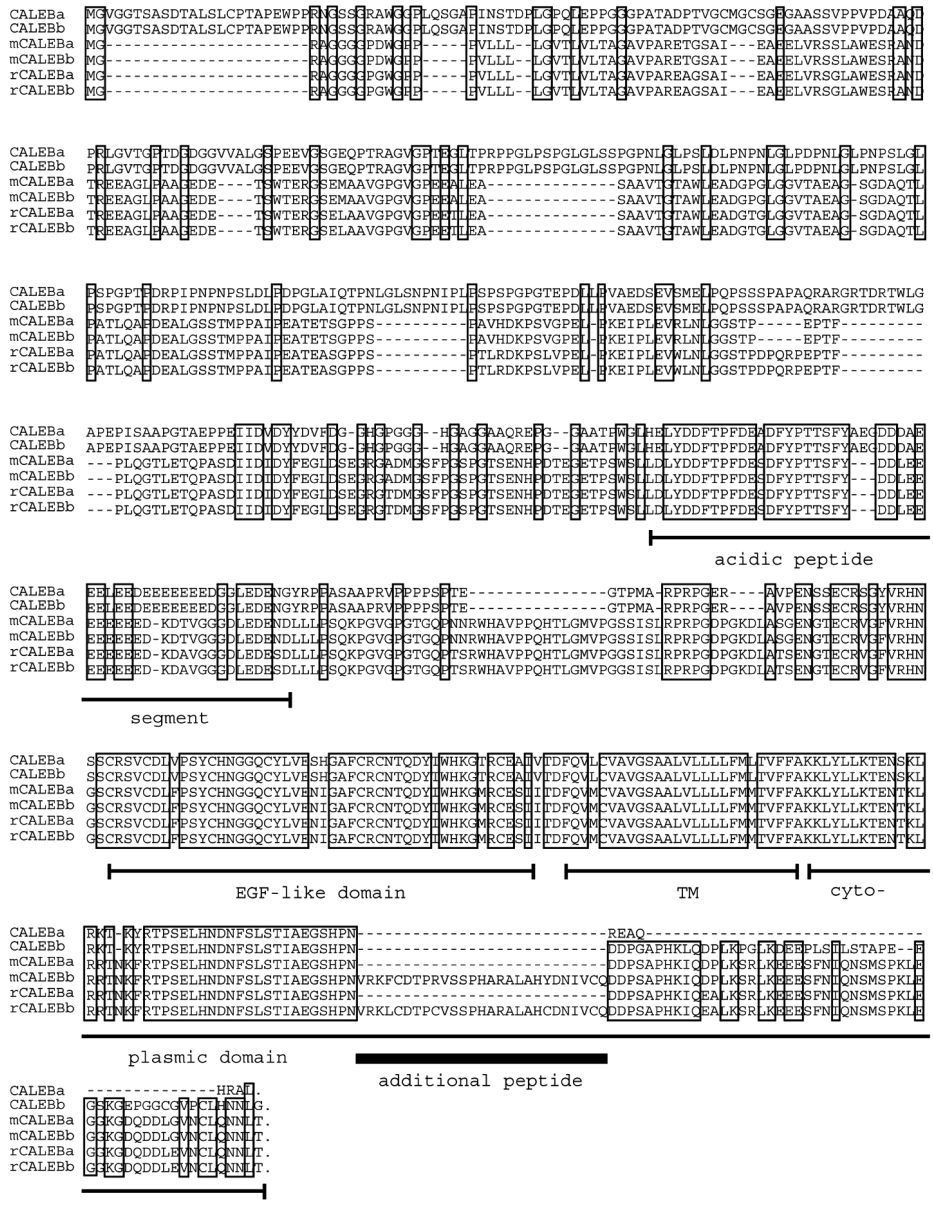


FIG. 4. **Alignment of CALEB isoforms and its putative species homologs in mouse (mCALEB/NGC) and rat (rCALEB/NGC).** A novel isoform of CALEB was identified in the developing chick nervous system that contains an additional segment of 50 amino acid residues at the carboxyl terminus (form b). The two forms of CALEB were aligned with the two forms identified in rodents. The putative mouse homolog of CALEB was cloned by screening a mouse cDNA library and by the RACE technique, whereas the putative rat homolog of CALEB was obtained by RT-PCR. The rodent forms were found to be identical to NGC. The b-form in mouse and rat contains an additional sequence of 27 amino acid residues (*additional peptide*) in the cytoplasmic domain. The splice site occurs at the same position in all three species. Identical amino acid residues are *boxed*. Sequences of the acidic peptide segment, the EGF-like domain, the transmembrane region (*TM*), and the cytoplasmic domain are *underlined*.

membrane segment, and most of the cytoplasmic stretch of the a form of CALEB are related to neuroglycan C (NGC), a chondroitin sulfate containing proteoglycan originally described with a cluster of basic amino acid residues in the rat (40), whereas the amino-terminal half and the carboxyl-terminal portion of rat NGC appeared completely unrelated to CALEB (11). This comparison suggested to us that a family composed of at least two members, CALEB and NGC, might exist in the developing nervous system. However, extensive screening in chick cDNA libraries as well as PCR studies and the identification of the b form which extends the relationship of CALEB to NGC did not result in further support of this interpretation. It remained conceivable, however, that the two species chick and rat diverged with respect to the number of CALEB-related genes or with respect to the amino- and carboxyl-terminal segments of CALEB.

To distinguish between these possibilities and to study the function of CALEB-related proteins in the mouse and rat, we screened a cDNA library of P20 mouse brain with a probe that encompasses the acidic peptide segment, the EGF-like domain, and the transmembrane region of CALEB. In combination with the 5′-RACE technique we identified only two open reading frames of cDNA sequences, which encode proteins highly related to CALEB in the chick and identical to NGC in mouse ((41), GenBank™ accession number AF133700). The putative rat homolog of CALEB cloned by RT-PCR contains an acidic peptide segment and is identical to the corrected sequence of rat NGC ((42), GenBank™ accession number U33553). The CALEB isoforms are aligned with the different isoforms of the putative mouse and rat homologs of CALEB, which have been identified in Fig. 4. In all three species two isoforms of CALEB exist, which differ with respect to their cytoplasmic domains. When comparing these sequences it is obvious that a large part of CALEB has been highly conserved during evolution. In particular the acidic peptide segment, the EGF-like domain, the transmembrane region, and most of the cytoplasmic domain are highly related with regard to their amino acid sequences (Fig. 4). The mammalian proteins, however, contain an insert of 27 amino acid residues in their cytoplasmic tail that has not been detected in the chick. Curiously, the amino-terminal peptide segment is different between CALEB and its proposed species homologs in mouse and rat. The reason for this is not clear, but we have no indication that this difference could be due to an alternative pre-mRNA splicing event nor that two different but similar genes might be involved.

The Fibrinogen-like Module of TN-R, Which Mediates the

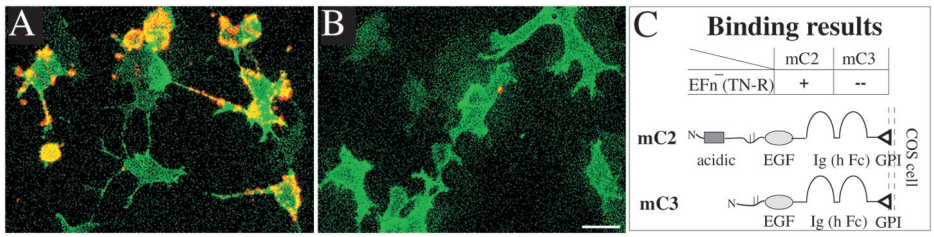


FIG. 5. **Binding of the fibrinogen-like module of TN-R to different mCALEB/NGC constructs.** COS7 cells were transfected with two different constructs (mC2 and mC3), derived from the cDNA sequence encoding the putative mouse homolog of CALEB. The construct mC2 resembles CALEB construct C2 (Fig. 1), whereas construct mC3 is similar to CALEB construct C3 (Fig. 1). The transfected COS7 cells were incubated with red fluorescent beads loaded with purified protein derived from construct EFn^{-}(TN-R) (Fig. 2). This polypeptide, which contains the cysteine-rich part and the fibrinogen-like domain of TN-R, binds to COS7 cells transfected with construct mC2 (*A*) but not with mC3 (*B*). *C* summarizes the binding results. *Bar*, 50 μm. *GPI,* glycosylphosphatidylinositol.

Interaction to CALEB, Also Binds to an mCALEB/NGC Fusion Protein Containing the Acidic Peptide Segment—Our studies indicate that the putative species homologs of CALEB in mouse (mCALEB/NGC) and rat (rCALEB/NGC) contain an acidic peptide segment close to the EGF-like domain as was established for CALEB. To analyze whether the acidic segment of mCALEB/NGC is also important in binding to TN-R, we transfected COS7 cells with mCALEB/NGC constructs that encode the EGF-like domain either joined to (Fig. 5*C*, *construct mC2*) or lacking (Fig. 5*C, construct mC3*) the acidic peptide stretch. As detailed above for CALEB, COS7 cells were then tested for their ability to bind microspheres coated with EFn^{-} (TN-R) (see Fig. 2*H*). Similar to the results obtained with CALEB constructs C2 and C3 (see Fig. 1), only COS7 cells that were transfected with construct mC2 bound microspheres (Fig. 5*A*). In contrast, microspheres coated with EFn^{-} (TN-R) do not bind to COS7 cells transfected with construct mC3 (Fig. 5*B*) thus indicating a similar mechanism of binding between TN-R and CALEB or its putative species homolog mCALEB/NGC. This result extends their structural similarity to a functional relationship.

rCALEB/NGC mRNA Is Present in Retinal Ganglion Cells (RGCs) of Embryonic, Postnatal, and Adult Rats—Our previous published *in vitro* studies indicated that CALEB might be important for neurite formation in a permissive growth environment. To extend these studies we made use of an *in vivo* model system that analyzes the importance of proteins for RGC axon growth and regeneration in rats (43, 44, 52). This includes analysis of mRNA expression during RGC development, after optic nerve lesion (ONL), and during RGC axon regeneration following sciatic nerve transplantation (45). Earlier results have shown that only some of the mRNAs and proteins involved in axon growth in the embryo are (re-)expressed during regeneration (*i.e.* L1, F11/F3, Gap-43), whereas other mRNAs and proteins are down-regulated directly after lesion (*i.e.* TAG-1; the netrin receptors DCC, UNC5H1, and UNC5H2 (39, 46–48)) and are not re-expressed in axon-regenerating RGCs. Against this background, we first determined the presence of rCALEB/NGC mRNA in embryonic, postnatal, and adult rats using *in situ* hybridization. Changes following axotomy or axotomy followed by a sciatic nerve graft (grafted rats) were evaluated subsequently.

In situ hybridization with an antisense cRNA probe of rCALEB/NGC resulted in staining of the RGC layer in E17 rat embryos, and of RGCs at P0 and P15 (Fig. 6, *A–C*) indicating that RGCs produce rCALEB/NGC mRNA during the time of RGC axon growth and target contact formation. Adult rats continued to express rCALEB/NGC mRNAs (Fig. 6*D*). The density of cells carrying the *in situ* hybridization signal was markedly reduced in the adult compared with retinae at P15.

RGCs Dynamically Regulate the Expression of rCALEB/NGC mRNA after Optic Nerve Lesion—To analyze whether rat RGCs regulate the synthesis of rCALEB/NGC mRNA in response to ONL, *in situ* hybridization experiments were performed using retinae between 2 and 28 days after crush. Labeled RGCs were counted in 10 1-mm^2 quadrants of 8 pie-shaped segments per retina (at least 4 retinae per mRNA) and averaged (Fig. 7*A*). In the unlesioned adult rat retina ~800 RGCs per mm^2, which is roughly 30–50% of all RGCs present (39, 43, 49–51), synthesize rCALEB/NGC mRNA. 5 days after crush (dac), when the number of RGCs begins to decline (to ~75% of those present normally) due to lesion-induced cell death, the number of rCALEB/NGC synthesizing RGCs decreases to ~120 cells per mm^2 which amounts to roughly 10% of cells present. In other words, RGCs down-regulate rCALEB/NGC mRNA. By 14–28 dac only ~10–15% of the RGCs survive (39, 43, 49–51), and counts of cells synthesizing rCALEB/NGC mRNA indicate that the mRNA is synthesized by the majority of the surviving cells (Fig. 7*A*).

To determine whether axon-regenerating RGCs express rCALEB/NGC mRNA, the retinae of grafted rats (28 days after surgery) were subjected to the *in situ* hybridization procedure. Counts of rCALEB/NGC mRNA producing RGCs in grafted rats were performed as before (in 10 1-mm^2 quadrants of 8 pie-shaped segments per retina in 3 retinae per mRNA; Fig. 7*B*). RGCs, able to regenerate an axon into the sciatic nerve graft, express GAP-43 mRNA (39). rCALEB/NGC mRNA synthesizing RGCs represent a fraction (approximately one-third) of RGCs, which produce GAP-43 mRNA (Fig. 7*B*). This is confirmed by double *in situ* hybridization with cRNA probes of rCALEB/NGC and GAP-43 (three retinae) showing the existence of RGCs that produce both rCALEB/NGC mRNA and GAP-43 mRNA (data not shown). This indicates that at 28 days after grafting, a subpopulation of RGCs with axons in the graft produce rCALEB/NGC mRNA thus implying either that axon regeneration occurs in the presence and absence of rCALEB/NGC or that rCALEB/NGC mRNA is transiently expressed by all axon-regenerating RGCs at earlier time points but is more rapidly down-regulated than GAP-43 mRNA.

DISCUSSION

To further our understanding of the molecular functions of CALEB, in this report we have investigated the interaction between CALEB and TN-C or -R, have characterized isoforms of CALEB in chick and rodents, and have studied its *in vivo* expression in an axonal regeneration model system. For binding analysis of CALEB, we have used deletion mutant polypeptides expressed on the surface of cells (CALEB) or generated by a eukaryotic expression system (TN-C or TN-R). As discussed elsewhere these methods have been proven to be reliable for analysis of the interactions of several multidomain transmembrane and extracellular matrix proteins (16, 18, 30–32) and thus appropriate for use in beginning to dissect the molecular function of CALEB. In the design of the deletion constructs, we

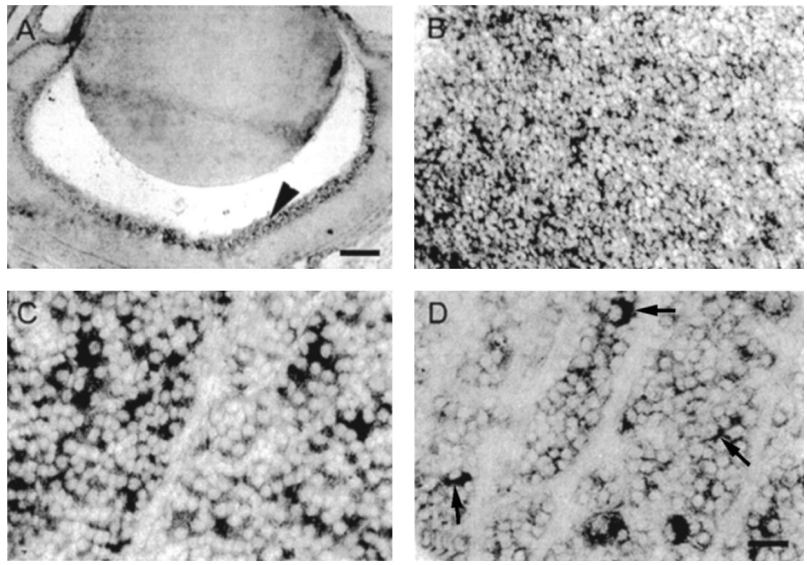
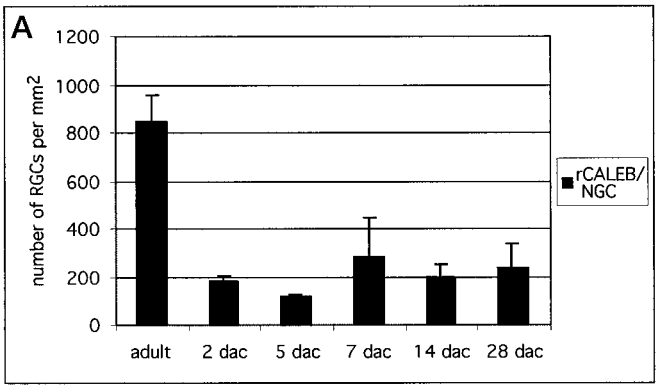


FIG. 6. **Detection of rCALEB/NGC mRNA in the developing and adult retina by *in situ* hybridization.** *A*, cross-section through the eye of E17 rat embryos, with the lens at the top and the optic nerve showing down. The *in situ* hybridization signals (see *arrowhead*) in the RGC layer indicate the presence of rCALEB/NGC mRNA. *In situ* hybridization signals were detected in RGCs in segments of retina whole mounts from P0 rats (*B*), P15 rats (*C*), and adult rats (*D*) with antisense cRNA probes of CALEB. RGCs containing rCALEB/NGC mRNA appear with dark somata and bright nuclei (see *arrows* in *D*). The intensity of the *in situ* hybridization signal varies over the RGC population. *Bar* in *A*, 120 μm; *bar* in *D*, 30 μm.



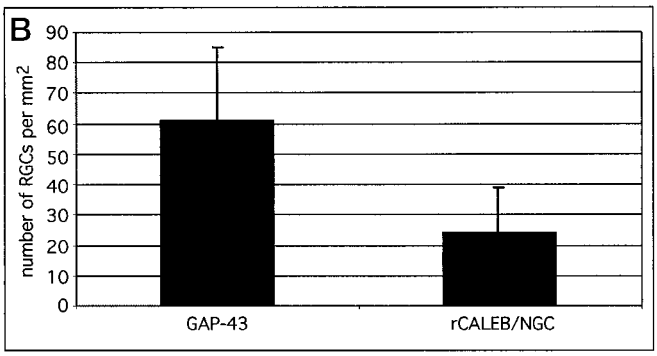


FIG. 7. **Quantification of RGCs displaying *in situ* hybridization signals with antisense cRNA probe of rCALEB/NGC in the normal adult retina, in retinae after ONL, and in grafted retinae.** The height of the *bars* indicates the average number of rCALEB/NGC cRNA-stained RGCs and the S.D. *A*, the number of RGCs containing rCALEB/NGC mRNA changes after optic nerve crush. Approximately 800 RGCs/mm^2 in the adult retina (*adult*) are positive for rCALEB/NGC mRNA. That is 30–50% of all RGCs present (compared with data from Refs. 39, 43, and 49–51). This number decreases 2 and 5 days after optic nerve crush (*dac*) to values below 10% when compared with the total number of surviving RGCs at these time points. On day 14 and 28 after crush ~200 RGCs/mm^2 are positive for rCALEB/NGC mRNA. Compared with the total number of RGCs present at these time points (data from Refs. 39, 43, and 49–51), more than 60% of the surviving cells express mCALEB/NGC mRNA. *B*, very few RGCs in grafted rats contain rCALEB/NGC mRNA. In these rats, a small percentage of RGCs is able to regenerate an axon. Most of them express GAP-43. Only one-third of the number of RGCs, which are positive for GAP-43 mRNA (regenerating axons), also express rCALEB/NGC mRNA.

were guided by the following characteristics of the extracellular portion of CALEB. 1) Our previously published binding studies were performed with material from embryonic chicken retina in which the 80-kDa polypeptide dominates. 2) The amino-terminal segment, which is highly enriched in the regularly spaced amino acids leucine and proline (LP motif), appears to be less conserved, and in the chick is only found in the 140- and 200-kDa components but not in the 80-kDa components. 3) The acidic segment and the EGF-like domain are highly conserved between chick, rodents, and humans and are expressed in the three CALEB forms (80, 140, and 200 kDa) identified to date. The amino acid sequence of the EGF-like module that is closely located to the transmembrane segment is similar to the corresponding sequences of members of the EGF family of transmembrane growth and differentiation factors (9) that are known to bind to ErbB receptor tyrosine kinases (10). Although it is currently not known whether the EGF-like domain of CALEB might interact with the ErbB proteins, our studies demonstrate that the EGF-like domain is not sufficient to bind to TN-C or TN-R. In contrast the acidic peptide segment located amino-terminally to the EGF-like domain is important for TN-C or TN-R binding in chick and mouse. Similar acidic peptide segments have been described only in a few other transmembrane proteins; two of these, the β-amyloid precursor protein and the fibroblast growth factor receptor 1 (53, 54), are expressed in the nervous system. Our finding that the EGF-like domain of CALEB, which shows similar sequence characteristics to those described for the EGF-like domains of members of the EGF family of growth and differentiation factors, does not bind to TN-C or TN-R implies that it may interact with another currently unknown protein distinct from these ECM glycoproteins. In this context it is of interest that the EGF-like domain

of CALEB and of its putative species homolog mCALEB/NGC is encoded by two exons.[2] The first encodes the part of the EGF-like domain containing the first four cysteine residues and the second encodes the remaining part of this domain. This genomic structure points to an evolutionary relationship between CALEB and several other members of the EGF family of growth and differentiation factors (55–58).

We demonstrated here that the fibrinogen-like module at the carboxyl terminus of TN-C or TN-R, which is the most highly conserved domain within all tenascin family members, is important for binding to CALEB. Whether or not the cysteine-rich segments of TN-C/R are necessary for binding, in addition to the fibrinogen-like domain, cannot be deduced from these experiments. However, CALEB was eluted by EDTA from an affinity column containing immobilized EFn^- suggesting that the interaction between CALEB and TN-C/R is divalent ion-dependent or that the structural integrity of either CALEB or EFn^- is dependent on divalent ions. In this context it should be noted that the fibrinogen-like globes of TN-C and TN-R contain a segment that is related to EF-hand calcium-binding sites identified in the γ chain of fibrinogen, in thrombospondin, and in calmodulin (59–61) and therefore might require divalent ions to fold appropriately for binding. CALEB is not the only protein known to bind to the fibrinogen-like domain of TN-C. For example, this domain also mediates the interaction with the ECM proteins neurocan and phosphacan/RPTP-β/ζ which is enhanced by the presence of calcium ions (19). Furthermore, this domain allowed lymphocyte rolling on TN-C substrates (62), an effect mediated by a yet unknown receptor. In addition, several integrins have been shown to interact with the fibrinogen-like domain of TN-C including $\alpha_2\beta_1$ on endothelial and $\alpha_V\beta_3$ on Chinese hamster ovary cells (24, 25).

At present the function of the interaction between CALEB and TN-C or TN-R has not been defined. By analogy to the EGF-like domain of ARIA, a member of the EGF family of growth and differentiation factors, which might become tethered to the ECM via its Ig-like domain after proteolytic liberation from its transmembrane precursor (6), it is conceivable that TN-C or TN-R also immobilize a putative soluble fragment of CALEB containing the EGF-like domain and the acidic peptide segment in the ECM. However, so far such a proteolysis product of CALEB has not been detected. Alternatively, CALEB might be a cellular receptor for TN-C and TN-R.

We have shown previously that in an *in vitro* test system Fab fragments of antibodies to CALEB inhibit neurite formation (11). To begin to understand whether CALEB might be important for axonal growth *in vivo*, we analyzed the expression of the putative rat homolog of CALEB, rCALEB/NGC, in the rat retina which serves as a model system to study axon regeneration in the mammalian central nervous system (43, 45, 49). This system has been frequently used to follow the temporal expression pattern of a number of different mRNAs encoding cell surface proteins implicated in axon growth after ONL (39, 46). In rats receiving a sciatic nerve transplant a fraction of RGCs (not more than 5%) are capable of regenerating an axon into the graft. In the developing rat retina, rCALEB/NGC mRNA is generated by most RGCs which is in agreement with previous observations that CALEB is present in the embryonic chick optic fiber layer (11). In the adult rat, however, only ~30–50% of the RGCs produce rCALEB/NGC mRNA. It is currently not known whether these cells represent a subpopulation of RGCs or whether this expression pattern is a reflection of a dynamic and transient synthesis of rCALEB/NGC mRNA. After ONL, the number of RGCs synthesizing rCALEB/NGC mRNA declines until 5 dac. During this period, most of the RGCs survive indicating that after loss of target innervation rCALEB/NGC synthesizing RGCs down-regulate their rCALEB/NGC-encoding mRNA. However, 5 days after ONL, when the quantity of RGCs begin to decline due to cell death, the number of RGCs producing rCALEB/NGC mRNA did not decline further suggesting that at 14–28 dac most of the surviving RGCs continue to synthesize rCALEB/NGC mRNA. One possible interpretation for this temporal pattern of rCALEB/NGC mRNA production by RGCs might be that those RGCs, which express rCALEB/NGC encoding mRNA during the period after ONL, are somehow more resistant to cell death. This question awaits further investigations. The described time course for rCALEB/NGC mRNA synthesis after ONL is different from the temporal expression pattern described for other mRNAs examined so far. For example, TAG-1, an axon-associated cell adhesion molecule, as well as the netrin receptors are expressed by adult rat RGCs, but their mRNA is down-regulated and lost after ONL (39, 46). Almost all RGCs synthesize L1 mRNA before ONL, and the number of RGCs producing this type of mRNA decreased in parallel with the loss of RGCs due to cell death (39). GAP-43 mRNA is not generated by adult RGCs, but the expression of its mRNA is up-regulated in the first 5 days after ONL and then subsequently declines (47, 48). GAP-43 is thought to be important for axon extension and sprouting (63), and in grafted rats most RGCs, retrogradely labeled with horseradish peroxidase used to identify RGCs that regenerate an axon, synthesize GAP-43 mRNA. In contrast, only one-third of those RGCs in grafted rats, which generate GAP-43 mRNA, also synthesize rCALEB/NGC mRNA. Expression of rCALEB/NGC mRNA in developing and in a fraction of axon-regenerating RGCs in adults suggests a role for CALEB in the differentiation process of RGCs including axon formation. Additional indications on the *in vivo* function of CALEB might result from the analysis of mCALEB/NGC-deficient mice generated by homologous recombination.

In summary, the data presented in this report increase our understanding of the molecular function of CALEB. We established that the acidic peptide segment of CALEB is necessary for binding TN-C and TN-R and that the fibrinogen-like module of TN-C and TN-R is responsible for mediating the interaction with CALEB. Furthermore, CALEB-like proteins that are binding partners for TN-C and TN-R are present in mouse and rat, and we have shown that the same segment in mCALEB/NGC and CALEB is involved in binding the fibrinogen-like domain of TN-R. We analyzed the expression of rCALEB/NGC following optic nerve lesion and during graft-assisted axon regeneration, and we found that RGCs dynamically regulate the synthesis of rCALEB/NGC mRNA in response to lesion and that a subfraction of RGCs express CALEB mRNA when regenerating an axon.

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