CALEB/NGC Interacts with the Golgi-associated Protein PIST*

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CALEB/NGC is a novel member of the epidermal growth factor protein family expressed in axon and synapse-rich areas of the nervous system and shown to be important for neurite formation. It can bind to the extracellular matrix proteins tenasin-R and tenasin-C. Here we show that CALEB/NGC interacts with the Golgi-associated protein PIST. PIST was originally described as an interaction partner of the small GTPase TC10 and was then found to be Golgi-associated by binding to syntaxin-6 and to be important for the transport of frizzled proteins and the cystic fibrosis transmembrane conductance regulator to the plasma membrane. In addition, PIST was demonstrated to be involved in autophagy and linked to processes of neurodegeneration. CALEB/NGC interacts with PIST in the yeast two-hybrid system. This interaction can be confirmed by co-immunoprecipitations and co-localization studies. The juxtamembrane cytoplasmic peptide segment of CALEB/NGC, highly conserved during evolution, mediates the binding to PIST. CALEB/NGC co-localizes with PIST in the Golgi apparatus of transfected COS7 cells and in Golgi-derived vesicles after brefeldin A or nocodazole treatment. Co-localization studies in primary hippocampal cells and analysis of Purkinje cells of colchicine-treated rats, serving as an in vivo model system to block microtubule-dependent transport processes, support the view that PIST is an interaction partner of CALEB/NGC and implicate that this interaction may play a role in the intracellular transport of CALEB/NGC.

CALEB/NGC (chicken acidic leucine-rich EGF-like domain-containing brain protein/neuroglycan C) is a neural transmembrane protein, which was originally discovered in a screen for novel molecules implicated in cell differentiation processes of the nervous system (1–3). It is expressed in axon and synapse-rich areas of the nervous system and was shown to be important for neurite formation (1, 4). CALEB/NGC is a type I transmembrane protein, the extracellular part of which contains the following domains. A highly acidic peptide segment is able to mediate binding to the extracellular matrix proteins tenasin-R and tenasin-C (5). An EGF-like domain located near the transmembrane region is structurally very similar to the corresponding domains of members of the EGF family of transmembrane growth and differentiation factors like EGF itself, neuregulin, and TGF-α (6, 7). Many functions can be attributed to the members of this protein family, and it is also well established that these proteins serve as ligands for ErbB receptor tyrosine kinases. Currently, it is unclear whether CALEB/NGC may also be able to stimulate ErbB receptors. Whereas the functions of the members of the EGF family of transmembrane growth and differentiation factors as ligands for ErbB receptor tyrosine kinases had been examined in detail, several studies in the last years focused on the functions of the cytoplasmic domains of these transmembrane proteins. For example, it was found that proteolytic cleavage of the extracellular domain of transmembrane neuregulins is regulated by their cytoplasmic tails (8). Activated release of membrane-anchored TGF-α was also shown to be dependent on the intracellular domain of this protein, and the carboxyl-terminal valine residue of TGF-α was recognized as important for this processing (9, 10). Moreover, mutants of TGF-α lacking the carboxyl-terminal valine were present at substantially reduced levels at the cell surface, indicating that the C terminus of TGF-α might be important for intracellular trafficking of the transmembrane precursor. Indeed, two different PDZ domain proteins, TACIP18/syntenin-1 and p59/GRASP55, were discovered, which bind to the C terminus of pro-TGF-α and affect its targeting to the cell surface (11, 12). GRASP55 was formerly shown to play a role in the stacking of the Golgi apparatus, and syntenin-1 had been published as an interaction partner for the C termini of several transmembrane proteins including syndecans, class B ephrins, Eph A7, neurexins, the anion exchanger AE2, neurofascin, different glutamate receptor subtypes, schwannomin, and the protein-tyrosine phosphatase η (13–21).

Little is known about the transport of other members of the EGF family of transmembrane proteins to the cell surface. We wanted to address this issue to the transmembrane protein CALEB/NGC. Large parts of the cytoplasmic domain of this protein are highly conserved between chicks, mice, rats, and humans (5). So far, neither any interaction partner nor any function can be attributed to the intracellular part of CALEB/NGC. In this study, we identified the Golgi-associated protein PIST (PDZ domain protein interacting specifically with TC10; also called FIG (fused in glioblastoma), GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein), or CAL (CFTR-associated ligand)) (22–25) as an interaction partner for CALEB/NGC. PIST was originally described as a PDZ domain protein, which binds to the small GTPase TC10 (22). Then it was shown to be Golgi-associated via binding to the Q-SNARE (Q-soluble NSF attachment protein receptor) protein syntxin-6 (23). Further studies reported that PIST binds to cell surface proteins frizzled 5 and frizzled 8, to the cystic fibrosis transmembrane conductance regulator (CFTR), and to the chloride channel ClC-3B and regulates the expression of these proteins at the plasma membrane (24–26). Recent work demonstrated a novel isoform of PIST to be involved in auto-

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The abbreviations used are: EGF, epidermal growth factor; CFTR, cystic fibrosis transmembrane conductance regulator; PDZ, PSD-95-Disc Large-ZO-1; TGF-α, transforming growth factor-α.
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phagy and neurodegeneration by linking the δ glutamate receptor to the autophagy regulator protein Beclin 1 (27).

In contrast to the transmembrane proteins frizzled 8, CFTR, and CIC-3B, where the corresponding C termini bind to the PDZ domain of PIST, in the case of CALEB/NGC, the juxtamembrane cytoplasmic peptide segment mediates the interaction with this Golgi-associated protein. Co-immunoprecipitation of transfected HEK293 cells confirmed the results obtained by yeast two-hybrid analysis. We present data about a co-localization of CALEB/NGC and PIST in the Golgi apparatus of transfected COS7 cells and in Golgi-derived vesicles after brefedrin A or nocodazole treatment. Analysis of primary and transfected hippocampal cells and the results of our experiments with an in vivo model system to block microtubule-dependent transport in neurons support the view that the interaction of CALEB/NGC and PIST may play a role for the intracellular transport of CALEB/NGC from the soma of neurons into the processes.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Mapping of the Interaction of CALEB/NGC and PIST—A PCR fragment encoding the total cytoplasmic domain of CALEB/NGC was amplified from human cDNA (Marathon-Ready, Clontech) with the oligonucleotides 5'-CAGCATTTCCAA-GAAGCTCTCTGTTGAGAAGGAGAAGATGT-3' (hCyt1) and 5'-CAGCATTTCCAG-CCTCTGTTGTTGAGAAGGAGAAGATGT-3' (hCyt2). The PCR fragment was cloned into the bait vector pAS2–1 (Clontech) using EcoRI and BamHI restriction sites, and the resulting construct was transformed into the yeast strain AH109 (Clontech) (28). The yeast strain Y87 pretransformed with a cDNA library from mouse brain cloned into the vector pACT2 (Clontech) was then mated with the AH109 strain that contains the bait construct according to the manufacturer’s protocol. Considering mating efficiency and titer of the library, 10,7 × 10^6 colonies were screened. 23 His+ colonies were isolated from selective medium lacking leucine, tryptophan, and histidine supplemented with 5 μg/ml 1,2,4-triazole. 19 of these colonies developed a blue color when tested that expression of the MEL1 gene. Two clones were found to encode the full-length sequence of PIST as described (22).

For the mapping of the interaction between CALEB/NGC and PIST, different CALEB/NGC and PIST constructs were cloned into the vectors pAS2–1 and pACT2, respectively. Primers for the PIST constructs were as follows: 5'-GGGGATCCCATGTCGGCGGGTGGCCCA-3' (PIST1) and 5'-GTCGAGCTCTTTAAGTCTTGGCA-3' (PIST2); used with primer PIST1 for full-length PIST; 5'-GAAGAGCTCTTCAG-GTCGACCTGAGACTACCTGCAA-3' (PIST3-PDZ; used together with primer PIST1 for construct Pi-1P); 5'-GAAGAGCTCTTCAATTGGACCG-GACTCCCTGTT-3' (PIST5; used together with primer PIST1 for construct Pi-5); 5'-CAGCAGACCTCTGAGCTTACTTTCCTCTTTTCC-3' (PIST6; used together with primer PIST1 for construct Pi-6); 5'-GCGGGACCTTCCAAACCTTGTGGCAGCTTTGGA-3' (PIST7; used together with primer PIST1 for construct Pi-7); 5'-AGCGGATCCCAAGAGCTGCTCAAGGTGACT-3' (PIST9; used together with primer PIST1 for construct Pi-9); 5'-CTGGAGATCATATGAAAGTTTGAAGCCTTCTGTCCTCTCAAAG-3' (PIST10; used together with primer PIST1 for construct Pi-10); 5'-CCGGATCCGCAAGAGCTGCTCAAGGTGACT-3' (PIST11; used together with primer PIST1 for construct Pi-11). Primers PIST11 and PIST-PDZ were used for construct Pi-11 and PIST5 and PIST1 and PIST3 for construct Pi-1P, respectively.

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PIST binds to CALEB/NGC in the yeast two-hybrid system. A, full-length PIST containing the two coiled-coil domains (CC) and the PDZ domain binds to the cytoplasmic peptide segment of CALEB/NGC in the yeast two-hybrid system. Yeast colony growth was determined in tryptophan/leucine-deficient medium (WL; transformation control) or medium that additionally lacks histidine (WLH) or histidine and adenine (WL/H). The ranges of yeast colony numbers in the corresponding selection medium after 4–7 days of incubation are shown. Data are obtained from one representative out of two to four independent experiments. Yeast colony growth indicating binding of PIST constructs and CALEB/NGC constructs could be detected in the presence of a weak (histidine synthesis; H) or even a strong (adenine synthesis; A) selection marker. No colony growth on these selective media could be detected in the negative controls with bait or prey vectors only (data not shown). Of all constructs examined, only full-length PIST, the construct m11f2, and construct Pi-29 bind to CALEB/NGC, which indicates that the integrity of the PIST protein seems to be important for binding to CALEB/NGC. B, as a bait for the yeast two-hybrid screen, the cytoplasmic domain of the longer isoform of human CALEB/NGC (bCALBEb); the additional peptide is indicated by a black box) was used. However, not only this domain is able to bind to PIST, but also the cytoplasmic peptide segment of the shorter isoform of human CALEB/NGC (bCALBeb) and a construct that contains only the juxtamembrane cytoplasmic peptide segment of CALEB/NGC (CCmx). In contrast, two constructs that lack this peptide segment (CC1 and CCex), do not bind to PIST. TM, transmembrane domain; bg, background, indicating growth of very small (<0.5-mm) yeast colonies after a long (longer than 7–10-day) incubation time.

FIG. 1. PIST binds to CALEB/NGC in the yeast two-hybrid system.
Taken together, we found that the intracellular peptide segment of CALEB/NGC, which is located near the transmembrane region and which is highly conserved between all species examined so far, binds to the coiled-coil and PDZ domains containing PIST protein in the yeast two-hybrid system.

PIST Associates with CALEB/NGC in HEK293 Cells and Can Be Precipitated by the CALEB/NGC-derived Juxtamembrane PIST-binding Peptide from Transfected HEK Cells and from Brain Tissue—To further analyze the interaction between CALEB/NGC and PIST, we co-transfected HEK293 cells with expression plasmids encoding tagged versions of CALEB/NGC and PIST, respectively. We used for these experiments four different CALEB/NGC constructs. In one case, the full-length isoform of CALEB/NGC from mouse (designated mACALEBb in Fig. 3D) was co-expressed with PIST. PIST expression can be detected in intracellular structures surrounding the nucleus (Fig. 3B). When being co-expressed together with PIST, a significant portion of CALEB/NGC co-localizes with PIST near the nucleus in intracellular structures reminiscent of the Golgi apparatus (Fig. 3, A and C, arrowheads). Our biochemical data indicate that the juxtamembrane cytoplasmic peptide segment of CALEB/NGC is necessary and sufficient for binding PIST. One isoform of chicken CALEB/NGC (designated CALEBa-80 in Fig. 3H) has, in contrast to all other isoforms in all species known so far, a very short intracellular domain, which, nevertheless, contains the PIST-binding peptide segment. We expressed this isoform of CALEB/NGC together with PIST and found it to co-localize (Fig. 3G) in similar intracellular areas as described above. To demonstrate that the extracellular region of CALEB/NGC is not important for the interaction with PIST, we co-expressed PIST together with a construct of CALEB/NGC, which lacks the extracellular part but contains the transmembrane region and the intracellular domain (designated 449 in Fig. 3L). Once again a strong co-localization can be seen (Fig. 3K). However, when co-expressing PIST together with a CALEB/NGC-derived construct that comprises large parts of the intracellular domain but lacks the PIST-binding juxtamembrane peptide segment (designated CCEX in Fig. 3P), no co-localization can be detected (Fig. 3O). The results of these co-localization studies are in strong accordance with the data of the biochemical interaction assays.
Fig. 3. CALEB/NGC co-localizes with PIST in COS7 cells. COS7 cells were co-transfected with plasmids encoding FLAG-tagged constructs of CALEB/NGC and VSV-tagged PIST. The CALEB/NGC-derived constructs were visualized by indirect immunofluorescence with antibodies directed to the FLAG epitope and Alexa488-conjugated secondary antibodies (A, E, I, and M). Indirect PIST staining was performed with antibodies to the VSV tag and Cy3-conjugated secondary antibodies (B, F, J, N). The overlays of the CALEB/NGC and PIST stainings are shown in C, G, K, and O. Schematic drawings of the CALEB/NGC-derived constructs, which were co-expressed together with full-length PIST, are depicted in D, H, L, and P. PIST strongly co-localizes with the longer isoform of mouse CALEB/NGC (denoted mCALEB in D), with the shorter isoform of chicken CALEB/NGC (denoted CALEB-80 in H), and with the CALEB/NGC-derived construct 449 (denoted 449 in L), which contains only the transmembrane region and the cytoplasmic domain of mouse CALEB/NGC. The arrowheads in C, G, and K indicate these co-localizations. No co-localization can be detected (O) when PIST is co-expressed together with the CALEB/NGC-derived construct CCEX (denoted CCEX in P), which contains most of the intracellular part of mouse CALEB/NGC but lacks the juxtamembrane peptide segment. Bar, 20 μm.

cells for endogenously expressed PIST with an affinity-purified polyclonal antibody and found it to be localized near the nucleus in a region that is similar or identical to the Golgi apparatus (Fig. 4H). After expression of FLAG-tagged CALEB/NGC (Fig. 4G), a co-localization of CALEB/NGC with the endogenous PIST can be observed in or near the Golgi apparatus (Fig. 4I).

It is well known that the integrity of the Golgi apparatus depends on the microtubule network (36, 37). Therefore, after destroying the microtubule network, the Golgi apparatus disperses into small Golgi-derived vesicles. We examined the interaction between CALEB/NGC and PIST after disruption of the Golgi apparatus due to depolymerization of the microtubules by nocodazole. We found that these two proteins co-localize in vesicular structures even after the destruction of the Golgi apparatus (Fig. 5C). A similar co-localization of CALEB/NGC and endogenously expressed PIST in Golgi-derived vesicles after destruction of the Golgi apparatus by nocodazole can be observed (data not shown). To substantiate that these vesicular structures indeed are Golgi-derived vesicles, we performed a double staining of CALEB/NGC and the Golgi marker Golgin-97 after disruption of the Golgi apparatus by nocodazole. A strong co-localization of CALEB/NGC with Golgin-97 can be detected (Fig. 5F). In addition to nocodazole, we used another drug, brefeldin A, to reversibly destroy the Golgi apparatus (38, 39). As was seen with nocodazole, CALEB/NGC co-localizes with the Golgi marker GM130 in Golgi-derived vesicles after dispersal of the Golgi apparatus (Fig. 5J). In summary, our results support the view that CALEB/NGC interacts with PIST in the Golgi apparatus and in Golgi-derived vesicles after destruction of the Golgi apparatus with nocodazole or brefeldin A.

CALEB/NGC Associates with PIST in Primary Hippocampal Cells—CALEB/NGC is a transmembrane protein, the expression of which had been postulated to be restricted to the brain (2). PIST, on the other hand, was demonstrated to be expressed in many cell types and tissues (22–24). In addition, it was published that PIST regulates the transport of several transmembrane proteins to the plasma membrane. We had shown that PIST and CALEB/NGC co-localize in the Golgi apparatus and in Golgi-derived vesicles of co-transfected heterologous cells. The Golgi apparatus is of very high importance for the maturation and the transport of proteins to the plasma membrane. We were interested in whether PIST could be involved in the intracellular transport of CALEB/NGC in neural cells. To explore this, we first examined whether CALEB/NGC associates with PIST in primary cells derived from brain. We established a primary hippocampal cell culture and co-transfected these cells with tagged versions of CALEB/NGC and PIST. The cells were fixed and analyzed by indirect immunofluorescence 24–48 h after transfection. We found a strong co-localization of CALEB/NGC with PIST in large (Fig. 6C, small arrowheads) or small (Fig. 6F, small arrowheads) vesicle-like or granule-like structures in the main processes of hippocampal neurons. The images in Fig. 6, A–C, give the impression of a street of vesicle-like structures that begins in the cell body and extends into the main neuronal processes. Next we examined hippocampal cells that had been transfected only with plasmids encoding CALEB/NGC. In this case we analyzed the cells 5 h after transfection and found CALEB/NGC to be located at the poles of the neurons and in the large diameter segments of the neurites emanating from the poles of the cells (Fig. 6G). When looking for endogenously expressed PIST, we found it to be present in the soma and the neurites (Fig. 6H). A co-localization can be detected at the poles of the cells and in the large diameter segments of the neurites (Fig. 6I). To examine the endogenous expression of both CALEB/NGC and PIST in hippocampal cells, we used antibodies directed to the cytoplasmic peptide segment of CALEB/NGC and to recombinant PIST (a gift from Wolf Wente and Hansju¨rgen Kreienkamp). To our surprise, we could not detect a significant co-localization of CALEB/NGC and PIST in many primary hippocampal cells at different times of development (data not shown). However, when analyzing hippocampal cells in a very early stage of differentiation (5 h in vitro), a few cells showed a strong co-localization of CALEB/NGC and PIST at one pole of the cells and in the major neurite (Fig. 6L). Taken together, in primary
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hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell culture, a co-localization that implicates an association of CALEB/NGC and PIST can be detected in native hippocampal cell 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**Fig. 6.** CALEB/NGC associate with PIST in primary hippocampal neurons. Primary hippocampal cells were prepared from rat embryos of embryonic day 19. These cells were either co-transfected with FLAG-tagged CALEB/NGC and VSV-tagged PIST (A–F), transfected with FLAG-tagged CALEB/NGC alone (G–I), or not transfected at all (J–L). The cells were transfected at 7 days in vitro, and fixed 36 h (A–F) or 5 h (G–I) after transfection. Staining for CALEB/NGC (A and D) and PIST (B and E) was performed as described in Fig. 3. A strong co-localization of CALEB/NGC and PIST can be detected in vesicle-like structures in the main neuronal processes as shown in two representative cells (arrowheads in C and F). CALEB/NGC was indirectly stained with anti-FLAG and Cy3-conjugated secondary antibodies (G), whereas endogenously expressed PIST was stained with polyclonal antibodies to recombinant PIST and Alexa488-conjugated secondary antibodies (H). When comparing the CALEB/NGC staining (G) with the staining of endogenous PIST (H), a co-localization at the poles and in the primary neurite of the presented neuron can be observed. When staining for endogenously expressed CALEB/NGC (J) and PIST (K) in primary untransfected hippocampal cells that had been fixed after 5 h in vitro, a co-localization at one cellular pole and in the main process was detected in a few cells (L). Bars, 15 µm (A–F), 10 µm (G–I), and 15 µm (J–L).

![Image](image_url)

**Fig. 7.** CALEB/NGC associates with PIST in vesicle-like structures in the somata of Purkinje cell of colchicine-treated rats but not in the cell bodies of untreated Purkinje cells. Tissue sections of cerebella of colchicine-treated rats (A–C) and of untreated rats (D–F) were indirectly stained for CALEB/NGC (A and D) and PIST (B and E). A strong staining of CALEB/NGC can be seen in the granular cell layer (GCL) and in the molecular layer (ML), where the dendrites of the Purkinje cells reside (D). In contrast, the cell bodies of the Purkinje cells in the Purkinje cell layer (white arrowheads) are not decorated by antibodies to CALEB/NGC. PIST is expressed in the granular cell layer, in the molecular layer, and also in the Purkinje cell layer (E). No co-localization of CALEB/NGC and PIST can be observed in the cell bodies of the Purkinje cells (F). In the cell bodies of Purkinje cells of a colchicine-treated animal, however, a clear staining of CALEB/NGC is detected (A). In a close-up view, it can be seen that CALEB/NGC is present in vesicle-like structures (arrowheads, inset in A). Because PIST is also located in these vesicle-like structures (arrowheads, inset in B), a strong co-localization (arrowheads, inset in C) implicates an association of CALEB/NGC and PIST. WM, white matter. Bar, 80 µm.

CALEB/NGC. This is in contrast to several other PIST-binding transmembrane proteins as frizzled 5, frizzled 8, the CFTR, the chloride channel CIC-3B, and the α2 glutamate receptor. All of these proteins bind via their carboxyl-terminal PDZ interaction motifs to the PDZ domain of PIST. We could show that not the carboxyl-terminal but the juxtamembrane cytoplasmic peptide segment of CALEB/NGC is necessary and sufficient for binding PIST. This was demonstrated in the yeast two-hybrid system and was confirmed by affinity precipitation of PIST from transfected COS7 cells with the CALEB/NGC-derived PIST-binding peptide. Moreover, with this peptide it was possible to precipitate native PIST from detergent extracts of rat brain. The interaction between CALEB/NGC and PIST could also be corroborated by co-immunoprecipitations from transfected HEK293 cells. So far, it was not possible to perform co-immunoprecipitations from brain tissue. This may be due to the fact that the interaction between CALEB/NGC and PIST seems to be important for neural cells only in a narrow time window as explained below.

In heterologous cells expressing both CALEB/NGC and PIST, a strong co-localization of these two proteins were found in the Golgi apparatus and in Golgi-derived vesicles after brefeldin A or nocodazole treatment. This co-localization could also be detected when using different CALEB/NGC isoforms or other constructs that contain the juxtamembrane cytoplasmic peptide segment of CALEB/NGC but not with CALEB/NGC-derived constructs, which lack the CALEB/NGC-derived PIST-binding peptide. The other PIST-binding transmembrane proteins frizzled 5, frizzled 8, CFTR and CIC-3B were also shown to interact with PIST in the Golgi apparatus or in Golgi-derived vesicles. However, the consequences of these interactions were described as different for these proteins. Whereas PIST was described as favoring the retention of CFTR and CIC-3B within...
the cell (25, 26), which means to inhibit the transport to the plasma membrane, the same protein was suggested to support the transport of frizzled 5 and frizzled 8 to the plasma membrane, the same protein was suggested to support the cell (25, 26), which means to inhibit the transport to the likely Golgi-dependent, cellular processes. Future analysis of CALEB/NGC mediates this interaction. Our data obtained family member CALEB/NGC with the Golgi-associated protein treated animals.

be hardly detected in the cell bodies of Purkinje cells of un-
rats. Compared with colchicine-treated rats, CALEB/NGC can
structures in the somata of Purkinje cells of colchicine-treated
strong co-localization of CALEB/NGC and PIST in vesicle-like
this in an

In summary, we report an interaction of the neural EGF
model system for destroying microtubule-de-

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