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Acknowledgements

We thank J. S. R. Dunlop for suggesting that we should examine the isotopic systematics of microscopic sulphur species in the North Pole barite; K.-U. Hinrichs, K. Londry, R. Summons, B. Thamdrup and K. Habicht for discussions; I. O'Brien, O. Thomas and L. Salling for technical assistance; and D. Des Marais for comments and suggestions. This work was supported by the Danish Grundforkningsfond (Basic Research Foundation) and by the Australian Research Council (R.B.).

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A primitive sarcopterygian fish with an eyestalk

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The discovery of two Early Devonian osteichthyan (bony fish) fossils[1–4] has challenged established ideas about the origin of osteichthyans and their divergence into actinopterygians (teleosts and their relatives) and sarcopterygians (tetrapods, coelacanths, lungfishes and related groups)[5–7]. *Psarolepis* from China[1,2,8,9] and an unnamed braincase from Australia[3] combine derived sarcopterygian and actinopterygian characters with primitive features previously restricted to non-osteichthyans, suggesting that early osteichthyan evolution may have involved substantial parallellism between sarcopterygians and actinopterygians. But interpretation of these fossils has been hampered by poor phylogenetic resolution[1,3]. Here we describe a basal sarcopterygian fish, *Achoania* gen, et sp. nov., that fills the morphological gap between *Psarolepis* and higher sarcoptergyians. We also report the presence of eyestalk attachments in both *Achoania* and *Psarolepis*, showing that this supposedly non-osteichthyan feature occurs in basal sarcopterygians as well as the actinoptergyian-like Australian braincase[3].

Sarcopterygii (Romer, 1955)

Achoania gen. nov.

Diagnosis. A sarcopterygian with an anteroventrally sloping intracranial joint; robust and rod-like premaxilla that lacks a posterodorsal process and does not contribute to the orbital margin; toothed median rostral that does not separate the premaxillae; large internasal cavities occupying about 33% of the length of the ethmosphenoid floor; small drop-shaped parasphenoid flanked by flat roughened surface; robust postorbital pila; large descending processes on ventral surface of sphenoidal region; very wide suborbital ledge; and prominent eyestalk scar. The external bones are covered by large-pore cosmine similar to that in *Psarolepis*.

Type species. *Achoania jarvikii* sp. nov.

Diagnosis. As for genus.

Etymology. Generic name referring to the absence of choana, from Greek *a* (not) and *choane* (funnel). Specific name in honour of the late Erik Jarvik.

Holotype. V6235, a fairly complete anterior cranial portion, IVPP, Beijing.

Locality and horizon. Qujing, E. Yunnan, China. Xitun Formation (late Lochkovian, Early Devonian)

Remarks. The new genus is represented by one anterior cranial portion (Fig. 1), which differs substantially from *Psarolepis* in the shape of the premaxilla, the parasphenoid, the internasal cavity (which occupies more than 70% of the length of the ethmosphenoid floor in *Psarolepis*), the postorbital pila and structures on the ventral side of the sphenoid. All the anterior cranial portions of *Psarolepis* are uniformly distinct from *Achoania* with no intermediate variations, and the posterior cranial specimens and lower jaws of *Psarolepis* fail to match the morphology of *Achoania*.

Description. The most impressive feature of *Achoania* gen. et sp. nov., is a heart-shaped unfinished area (Fig. 1c, d) in the interorbital wall, which lies immediately behind the optic canal and has outward-facing edges. Two small cup-shaped recesses lie posterodorsal and ventral to this area, the posterodorsal recess supported by a posteriorly running horizontal ridge. By comparison with placoderms[7,10], primitive chondrichthyans[7,11] and the Australian braincase (AMF101607)[3,4], the unfinished area represents the eyestalk attachment area, and the two small recesses represent sites for eye muscle attachment.

Except for its recently reported presence in AMF101607 (ref. 3), an eyestalk had been considered to exist only in two non-osteichthyan groups, chondrichthyans and the extinct placoderms. The presence of an eyestalk area in *Achoania* is corrobrated by the discovery of a similar structure in a newly collected *Psarolepis* specimen (Fig. 2), which shows, on both sides of the interorbital wall, a large unfinished area with well-defined margins between the optic nerve canal and pituitary vein canal. In addition, re-examination of a previously described specimen (V8136)[9] reveals a similarly positioned, although distorted, unfinished area on the exposed side of the interorbital wall. The well-preserved eyestalk area on both sides of the new *Psarolepis* specimen and the similar arrangement of foramina, pits and ridges in the interorbital wall of *Psarolepis* and *Achoania* strongly indicate that the eyestalk area in these two forms is a natural structure rather than an artefact of preservation or preparation. The eyestalk should now be considered a general gnathostome feature retained by early osteichthyans on both the actinopterygian and sarcopterygian lineages.

In other cranial features, *Achoania* resembles *Psarolepis* and differs from previously known sarcopterygians in having large, closely spaced pores on the cosmine surface, an anterodorsally facing anterior nostril, a toothed median rostral, a straight rather than lyre-shaped trajectory of the supraorbital sensory canal and a

short parasphenoid not pierced by internal carotid canals. Like *Psarolepis*[9], *Achoania* has a postorbital pila which forms a bridge connecting the top of the basipterygoid process and the side of the braincase wall, and laterally flanks the canal for the jugular vein.

Achoania differs from *Psarolepis* and agrees with typical sarcoptergyians in having a premaxilla without a posterodorsal process. Well-preserved sutural surfaces indicate that the posterior nostril was enclosed posteriorly by a separate bone, presumably the lacrimal as in other sarcopterygians. The sphenoidal region of *Achoania* is longer than in *Psarolepis*, but shorter than in other osteichthyans (except *Onychodus*). *Achoania* thus bridges the morphological gap between *Psarolepis* and other sarcopterygians, and helps to resolve their relationships.

A phylogenetic analysis, based on a new data matrix of 158 morphological characters, places *Achoania* and *Psarolepis* as successive sister groups to all other sarcopterygians (Fig. 3a). Clade decay values for surrounding nodes are relatively high, indicating that their position is well supported. The position of the Australian braincase AMF101607 is less clear, but it is probably a very primitive actinopterygian. This topology matches one of the alternative topologies that were put forward (but could not be resolved) in previous analyses[1–3].

The new phylogeny has major evolutionary and biogeographical implications. Past analyses of osteichthyans[5,6,12,13] have generated inferred ancestral character complements for the clade based on known 'primitive' genera such as *Mimia* and *Porolepis*, but these differ substantially from the character suite revealed by *Achoania*, *Psarolepis* and AMF101607. Our well-supported resolution of *Achoania* and *Psarolepis* as stem-group sarcopterygians shows that their seemingly non-osteichthyan characters, such as an eyestalk, a placoderm-like shoulder girdle with a separate spinal plate, and paired and median fin spines[1,8], are present in the base of the osteichthyan crown group; in all likelihood these characters are primitive for the Osteichthyes as a whole. This implies that many apparent synapomorphies between previously known actinopterygians and sarcopterygians (undivided cleithrum, lack of eyestalk, lack of fin spines) are convergent between the two lineages. We can no longer assume that direct comparisons between derived sarcopterygians and actinopterygians will be informative about primitive conditions for the Osteichthyes as a whole. Instead, attention should focus on basal taxa, such as the Early Devonian ?actinopterygian

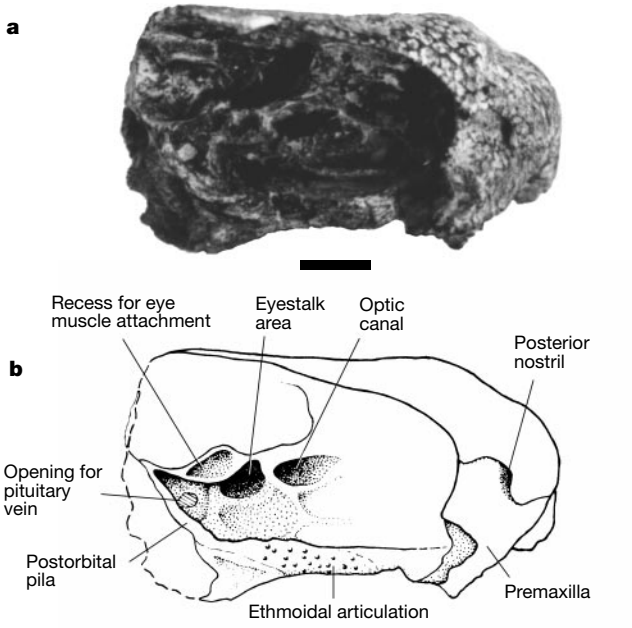

Figure 2 *Psarolepis* specimen (IVPP V11490.1) in posterolateral view, showing the eyestalk attachment area. **a**, Photograph; **b**, drawing. Scale bar, 2 mm.

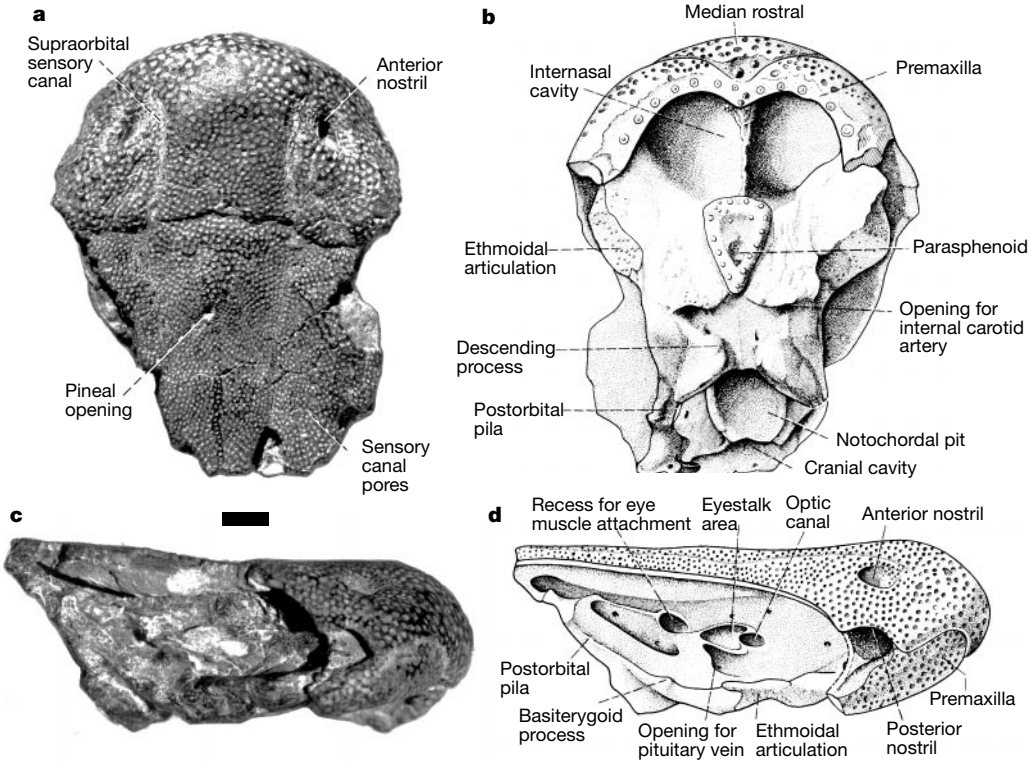

Figure 1 *Achoania jarviki* gen. et sp. nov., holotype, IVPP V6835. An isolated anterior cranial portion. **a**, Dorsal view (photograph), showing large-pore cosmine similar to that in *Psarolepis*. **b**, Ventral view (drawing), showing toothed median rostral, large internasal cavities, small drop-shaped parasphenoid. **c**, **d**, Right lateral views of same specimen showing the eyestalk attachment area. Scale bar, 2 mm.

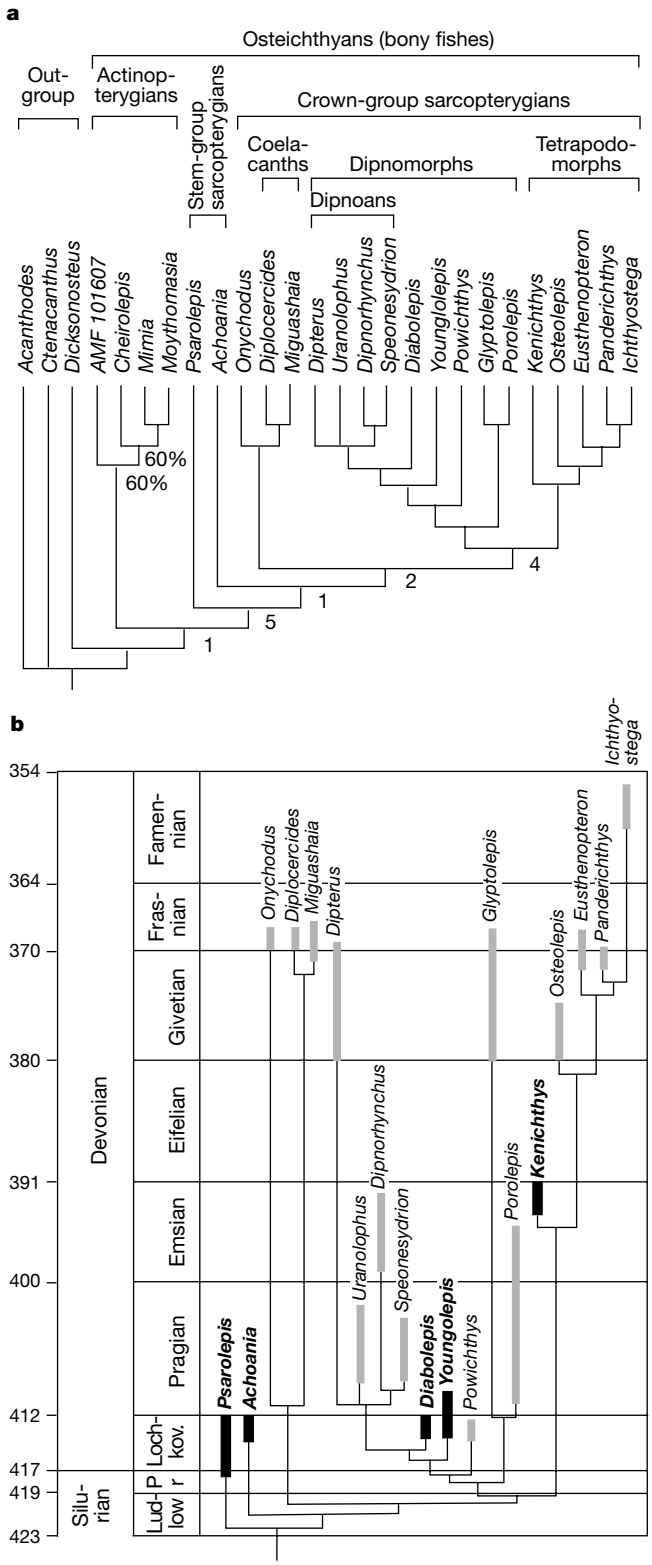

Figure 3 Phylogenetic analysis. **a**, Fifty per cent majority rule consensus tree calculated from 15 equally parsimonious trees of length 317 steps; consistency index 0.568, homoplasy index 0.432, retention index 0.765, and rescaled consistency index 0.434. All branches have 100% support except those that define the position of AMF101607, which have only 60% support. The topology is the same as the strict consensus tree except that the nodes with 60% support collapse in the latter. Numbers below the tree indicate the Bremer Support/clade decay values for the adjacent nodes. **b**, The sarcopterygian part of the tree projected against stratigraphy. Lochkov., Lochkovian; Pr, Pridoli. Taxa from Yunnan are shown as broad black range bars with names in bold print. Dates along left margin in millions of years.

Dialipina[14] which has a shoulder girdle comparable in structure to that of *Psarolepis*. We must also reassess some supposed autapomorphies of different osteichthyan groups, such as the 'extracleithrum' of coelacanths which, in the primitive genus *Miguashaia*[15,16], closely resembles a spinal plate.

The character distributions observed in *Achoania*, *Psarolepis* and AMF101607 make basal Osteichthyes appear more placoderm-like than recognized previously. Many workers place the placoderms as the most primitive jawed gnathostomes[7] and explicitly deny any homology between the placoderm and osteichthyan bone patterns, thus virtually guaranteeing wide phylogenetic separation. However, evidence is mounting for homology between the dermal shoulder girdles of osteichthyans and placoderms. Our analysis places the placoderm *Dicksonosteus* as the sister group of Osteichthyes, although the branching pattern within the Osteichthyes is unaffected by the removal of *Dicksonosteus* (or any other taxon) from the outgroup. Deep gnathostome phylogeny needs to be reviewed in the light of these findings.

The occurrence of two stem sarcopterygians (*Psarolepis* and *Achoania*) and three basal dipnomorphs (*Powichthys*[17], *Youngolepis*[18] and *Diabolepis*[19]) in the Lochkovian, but only one (*Psarolepis*) in the underlying Pridoli, suggests that the Sarcopterygii were diversifying rapidly around the Silurian/Devonian boundary. Stratigraphic mapping of the 50% majority rule tree (Fig. 3b) assigns a very brief duration to the internode between coelacanths and lungfishes plus tetrapods. This may help to explain the difficulty of resolving the coelacanth–lungfish–tetrapod three-taxon problem using molecular data[20,21].

The discovery of *Achoania* highlights the importance of Yunnan as a source of early sarcopterygians. Yunnan has yielded the whole sarcopterygian stem group (*Psarolepis* and *Achoania*), the earliest and most basal tetrapodomorph (*Kenichthys*), and two of the four most basal dipnomorphs (*Youngolepis* and *Diabolepis*). Five of the six described Lochkovian (basal Devonian) sarcopterygians come from the south China terrane—a palaeocontinent encompassing Yunnan and northern Vietnam which lay adjacent to eastern Gondwana during the Early Devonian—and four of these (*Psarolepis*, *Achoania*, *Youngolepis* and *Diabolepis*) occur in the Xitun Formation of Yunnan[9,18,19,22]. Further sarcopterygian material from the Xitun formation awaits description.

The Early Devonian vertebrate fauna of the south China terrane is highly endemic, and the reasonably abundant Lochkovian faunas from other areas almost always lack sarcopterygians and other south China elements[22]. This suggests that south China may be the place of origin of the Sarcopterygii. Future work on deep osteichthyan phylogeny should aim to test this hypothesis. □

Methods

Phylogenetic analysis

The analysis was performed by using the phylogenetic package PAUP 3.1 (ref. 24) with a data matrix of 26 taxa and 157 morphological characters (see Supplementary Information). Most parsimonious trees were obtained heuristically, using 500 replicates of random stepwise addition under the tree bisection–reconnection algorithm. All characters were unordered and unweighted.

Received 22 August; accepted 8 November 2000.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank M. M. Chang, P. Janvier and M. I. Coates for useful discussions, U. Samuelson and Zhang Jie for photographic work, Lu Xiufen for specimen preparation and Hu Huiqing for artwork. M.Z. & X.Y. acknowledge the support from the Chinese Foundation of Natural Sciences, Ministry of Science & Technology (China), and National Geographic Society (US). X.Y. thanks Kean University for faculty research and development support.

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Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18

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Excitatory amino-acid carrier 1 (EAAC1) is a high-affinity Na^+-dependent L-glutamate/D, L-aspartate cell-membrane transport protein[1]. It is expressed in brain as well as several non-nervous tissues. In brain, EAAC1 is the primary neuronal glutamate transporter[2,3]. It has a polarized distribution in cells and mainly functions perisynaptically to transport glutamate from the extracellular environment[2–4]. In the kidney it is involved in renal acidic amino-acid re-absorption and amino-acid metabolism[5–7]. Here we describe the identification and characterization of an EAAC1-associated protein, GTRAP3-18. Like EAAC1, GTRAP3-18 is expressed in numerous tissues[8,9]. It localizes to the cell membrane and cytoplasm, and specifically interacts with carboxy-terminal intracellular domain of EAAC1. Increasing the expression of GTRAP3-18 in cells reduces EAAC1-mediated glutamate transport by lowering substrate affinity. The expression of GTRAP3-18 can be upregulated by retinoic acid, which results in a specific reduction of EAAC1-mediated glutamate transport. These studies show that glutamate transport proteins can be regulated potently and that GTRAP can modulate the transport functions ascribed to EAAC1. GTRAP3-18 may be important in regulating the metabolic function of EAAC1.

Using the C-terminal intracellular domain of EAAC1 (the last 87 amino acids) as bait in a yeast two-hybrid screen of an adult rat brain complementary DNA library, we isolated 78 clones displaying β-galactosidase activity. Restriction and sequencing analyses revealed that 10 of the clones with the strongest β-galactosidase activity were identical. This clone, designated E18, was completely sequenced and was found to be unique after GenBank analysis. JWA protein, (GenBank NP006398) a human, differentially displayed, vitamin-A-responsive gene, is 95% identical to E18, suggesting that E18 is a JWA protein homologue of rat.

E18 is a full-length complementary DNA containing an initiation methionine and a poly(A) tail. We named the protein glutamate transporter EAAC1-associated protein (GTRAP3-18). GTRAP3-18 encodes a protein of 188 amino acids (see Supplementary Information), with a calculated relative molecular mass of 22,500 (M_r 22.5K). Protein analysis indicated that it is a very hydrophobic protein with four possible transmembrane domains. Both the C-terminal and amino-terminal domains contain protein kinase C motifs and may be intracellular.

To confirm the yeast two-hybrid results, we examined the interaction of GTRAP3-18 with EAAC1 using *in vitro* and *in vivo* methods. For *in vitro* cell-free binding, EAAC1 was expressed as a fusion protein with glutathione *S*-transferase (GST), and GTRAP3-18 was produced and labelled with [^{35}S]methionine by *in vitro* transcription and translation. Purified GST or GST–EAAC1 fusion proteins immobilized on glutathione–sepharose were incubated with [^{35}S]labelled GTRAP3-18 protein. GTRAP3-18 bound specifically to immobilized GST–EAAC1 (lane 2) but not to GST alone (lane 3), indicating that they interact *in vitro* (Fig. 1a).

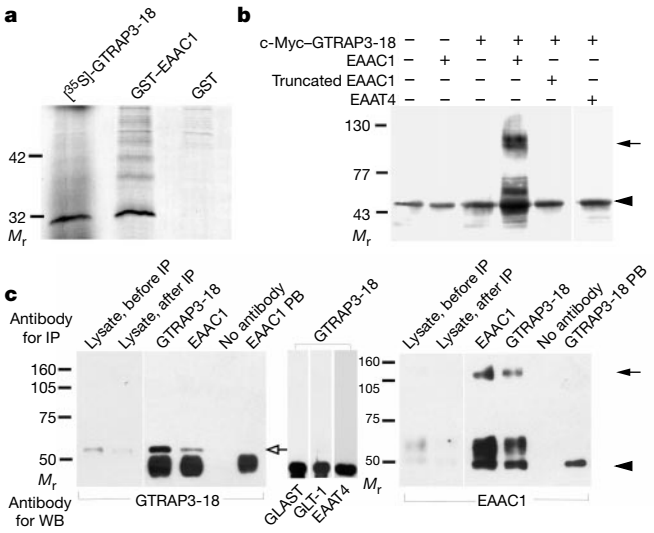

Figure 1 GTRAP3-18 interacts with EAAC1 *in vitro* and *in vivo*. **a**, SDS–PAGE analysis of cell-free *in vitro* binding. *In vitro* synthesized [^{35}S]-labelled GTRAP3-18 bound specifically to immobilized GST–EAAC1 and not to the negative control GST. **b**, Western blot analyses of immunoprecipitations from transfected HEK293 cells extracts, using anti-c-Myc antibodies for immunoprecipitation and anti-EAAC1 or EAAT4 antibodies for western blot. EAAC1 (monomer, arrowhead; dimer, arrow) was specifically co-immunoprecipitated with c-Myc–GTRAP3-18. An immunoprecipitation artifact is present in each lane at about 50–60K. **c**, Western blot (WB) analyses of immunoprecipitations (IP) from rat brain extracts. GTRAP3-18 (open arrowhead) was specifically co-immunoprecipitated with EAAC1. EAAC1 (monomer, arrowhead; dimer, arrow) was co-immunoprecipitated with GTRAP3-18. PB, peptide block.