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Molecular Brain Research 48 (1997) 140–144

MOLECULAR BRAIN RESEARCH

Short communication

Ras-GRF, the activator of Ras, is expressed preferentially in mature neurons of the central nervous system

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Accepted 11 March 1997

Abstract

In rodents, the Ras-specific guanine-nucleotide exchange factor (Ras-GRF) is expressed in different areas of the brain and, at a reduced level, also in the spinal cord. No expression of the 140 kDa Ras-GRF was detected in dorsal root ganglia and all other tissues tested. Analysis of primary cultures derived from brain reveals that this exchange factor is only present in neurons of the central nervous system. In primary hippocampal cultures, the expression of Ras-GRF increases in parallel with the onset of a neuronal network and in the whole brain it increases sharply after birth. © 1997 Elsevier Science B.V.

Keywords: CDC25Mm; Ras-GRF; Ras; Hippocampal culture

The Ras genes code for ubiquitously expressed 21 kDa guanine-nucleotide-binding proteins that play a pivotal role in cell proliferation [2]. p21 Ras is highly expressed both in developing and adult brain [10,20] and recent data suggest a possible involvement of Ras not only in neuronal differentiation but also in the neuronal survival and in synaptic activity [9]. p21 Ras act as molecular switches that cycle between active GTP-bound and inactive GDP-bound state [12]. Activation of Ras proteins is believed to be mediated by the action of guanine nucleotide exchange factors (GEFs) that stimulate the formation of the GTP-bound state. In mammalian cells, two distinct families of Ras-specific GEF have been so far isolated: SOS (homologue of *Drosophila* Son of Sevenless) [3,6] and CDC25Mm/Ras-GRF proteins [5,13,17]. While the former is ubiquitously expressed and is activated by a variety of extracellular stimuli, much less is known about the function of CDC25Mm/Ras-GRF and the signalling pathway in which it is involved. It has been reported to play a role in Ras activation induced by calcium [7], lysophosphatidic acid [22] and muscarinic receptor agonist [14] but not by activated tyrosine kinase receptors [18,22].

Northern analysis [13,17] indicate that CDC25Mm/Ras-GRF is expressed only in the brain, although it has been reported that the human GRF is present, at a reduced level, also in other tissues as well as in several tumor cell lines [11]. Subcellular fractionation of the mouse brain has shown that it is localized in synaptosomes and enriched in post-synaptic densities [19]. No clear information, however, is still available on the cell populations in which CDC25Mm/Ras-GRF is expressed.

In order to address these questions, in situ hybridization has been carried out on sections of brain and spinal cord of Wistar rats at 28 days of age. Fig. 1 shows a section of hippocampal formation probed with sense (A) and antisense (B) dig-labeled CDC25Mm RNA. As expected from the data reported by Wei et al. [21], the staining clearly shows high level of expression of Ras-GRF in the hippocampal formation mainly in the cellular layers of the CA1 to CA3 regions and in the dentate gyrus. In addition, in situ hybridization of spinal cord reveals significant expression of Ras-GRF (Fig. 1C). The labeling in the spinal cord is localized only in the gray matter which includes the cell bodies of motor and sensory neurons.

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PII S0169-328X(97)00120-4

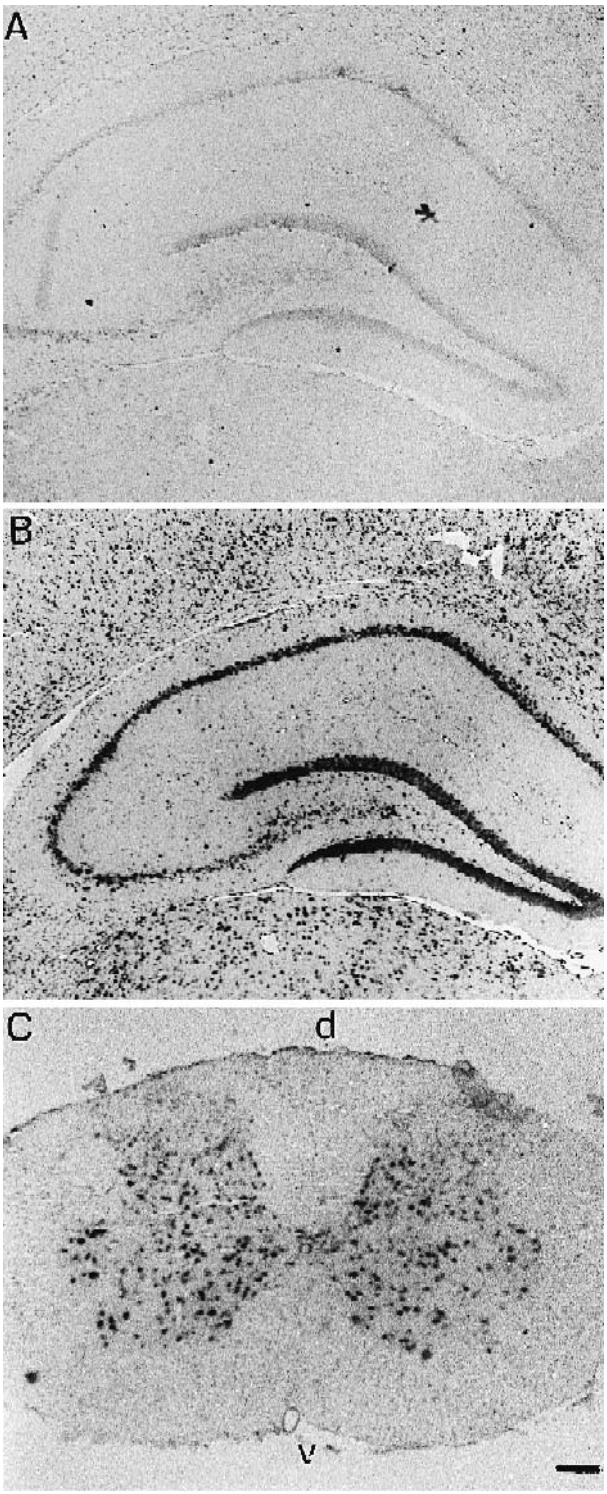

Fig. 1. In situ hybridization of hippocampal formation and spinal cord with CDC25Mm/Ras-GRF probes. Coronal sections of hippocampal formation (A,B) and spinal cord (C) [15] were hybridized with digoxigenin-labeled sense (A) or antisense (B,C) cRNA probes for CDC25Mm. The CDC25Mm probes were derived from a pGEM plasmid containing the 5 kb cDNA of CDC25Mm. For preparation of antisense and sense probes, the plasmid was cut with *Pst*I and *Nde*I, respectively. cRNAs were transcribed in vitro in the presence of digoxigen in UTP and in situ hibridization was performed as described [15]. Bar = 250 μm. v, ventral; d, dorsal.

Higher magnification (data not shown) revealed that the staining is found in different cell types originating from both the sensory and the motor system. No labeling was detected in the white matter.

Different areas of the brain and the spinal cord were dissected from Wistar rats at 28 days of age and equal amounts of total protein extracts were subjected to Western blot analysis with antibodies against Ras-GRF. As shown in Fig. 2A, the p140 Ras-GRF is expressed at a high level in all the brain regions examined, viz. thalamus, hippocampus, cortex and cerebellum and, although at a lower level, in the spinal cord also. Instead, the 140 kDa band was not detected in extracts of dorsal root ganglia from juvenile rats (PN9 and PN23) (Fig. 2B). A large pannel of mouse tissues were also analyzed and expression of CDC25Mm was found in none of the non-neuronal tissues (Fig. 2C). The fact that expression of CDC25Mm/Ras-GRF was detected in the spinal cord (Fig. 1C and Fig. 2A) allows us to conclude that this factor is expressed in the central nervous system (CNS) rather than exclusively in the brain.

In order to determine the cell population expressing this exchange factor, we examined brain-derived cultures of neuronal and glial cells. Primary cultures of hippocampal neurons were obtained from hippocampus from E18 rats while granular cells were prepared from cerebellum of PN7 rats. In both cases, growth of non-neuronal cells was prevented by the addition of antimitotic agents 2 days after seeding. Cultures of glial cells were prepared from the cortex of PN1 rats and astrocytes from cerebellum of PN7 rats. After 12 days in culture, cellular extracts were prepared and immunoprecipitation followed by Western blot analysis with anti Ras-GRF antibodies was carried out. Fig. 3 shows that a clear band of 140 kDa is present in the immunoprecipitates obtained from both granular and hippocampal cells while such band is not detected in astrocytes and glial cells. Interestingly, in different neuroblastoma cell lines (SK-N-BE, N18) (data not shown), as in the dorsal root ganglia (Fig. 2B), which all derive from the neural crest, the 140 kDa was absent. Taken together, these data indicate that in rodents the Ras-GRF protein is expressed at a detectable level only in neurons of the CNS and not in the peripheral nervous system.

Cultures of hippocampal cells were analyzed at different times after seeding, viz. at days 0, 7 and 15 (Fig. 4A). At the time of seeding, both neuronal and non-neuronal cells are present in the culture and the immunoreactive 140 kDa band is barely detectable. Its intensity largely increases at day 7 when, due to the presence of antimitotic agents, essentially only neuronal cells are present in the culture. Between days 7 and 15, the observation of the cultures reveals the onset of a well defined neuronal network (Fig. 5). During this period, a further increase of Ras-GRF takes place (Fig. 4A), showing that expression of Ras-GRF in hippocampal cultures parallels neuronal differentiation.

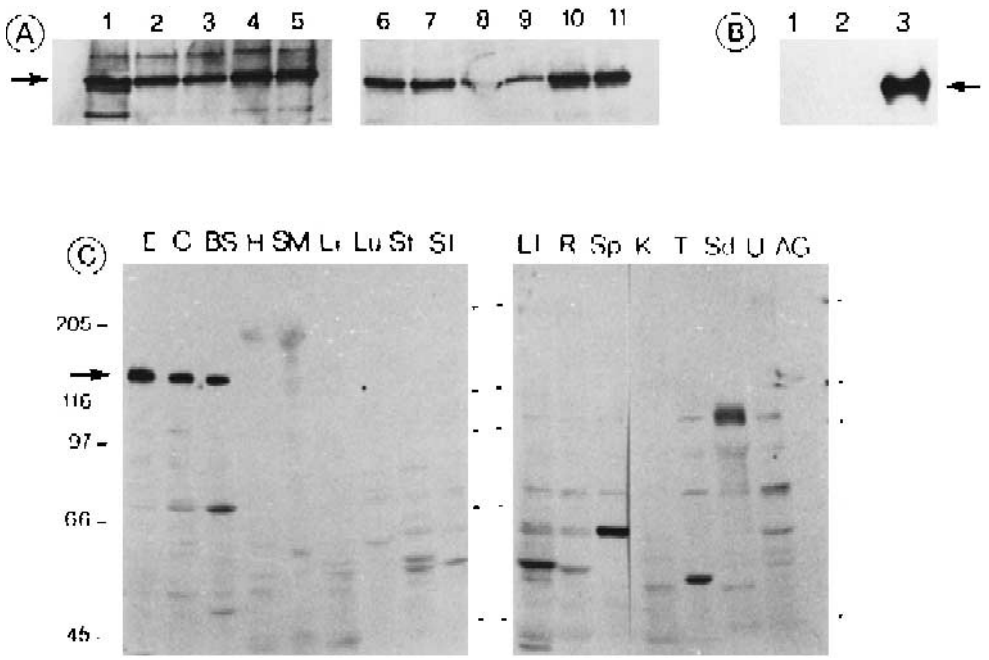

Fig. 2. Western blot analysis of different areas of the central nervous system and of different non-neuronal tissues. A: different regions of rat brain and spinal cord (28 day PN) were dissected, solubilized in 1% SDS. 100 μg proteins for each area were analyzed by Western blotting with anti Ras-GRF antibodies (Santa Cruz) using the ECL system (Amersham). Lane 1, whole brain; lanes 2–3, cerebellum; lanes 4–5, thalamus; lanes 6–7, cortex; lanes 8–9, spinal cord; lanes 10–11, hippocampus. B: dorsal root ganglia from PN9 (lane 1) and PN23 (lane 2) rats were analyzed as above together with extracts of total brain (PN23) (lane 3). C: different tissues and organs of the mouse were analyzed as above. Encefalum (E), cerebellum (C), brain stem (BS), heart (H), skeletal muscle (SM), liver (Li), lung (Lu), stomach (St), small intestine (SI), long intestine (LI), rectum (R), spleen (Sp), kidney (K), testis (T), spermiduct (Sd), uterus (U) and adrenal gland (AG).

This finding is in accordance with the observation that in the rat, as previously reported for the mouse [8], the expression of Ras-GRF in the whole brain increases very sharply in the first few days after birth (Fig. 4B), a timing that correlates with the establishment of most mature functional connections.

Metabolic labeling of hippocampal cultures with [^{32}P]orthophosphate followed by immunoprecipitation with Ras-GRF antibodies shows that Ras-GRF is phosphorylated (Fig. 6A). In addition, in hippocampal cells, Ras-GRF is associated with calmodulin: in fact, immunoprecipitation with antibodies against Ras-GRF and Western blotting with antibodies against calmodulin allows to detect a doublet of 15–20 kDa corresponding to different isoforms of calmodulin [16] (Fig. 6B, lane 3). This association is specific and calcium-dependent. In fact, calmodulin is not present in the immunoprecipitate when a pre-immune serum is used (Fig. 6B, lane 1) and is completely released if the immunoprecipitate is washed with EGTA (Fig. 6B, lane 2).

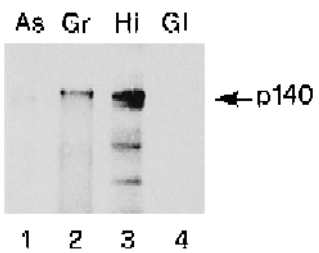

Fig. 3. Ras-GRF is expressed in neuronal cells. Cultures of hippocampal cells (Hi), granular cells (Gr), astrocytes (As) and glial cells (Gl) were prepared as described in the text and in the legend of Fig 5. After 12 days in culture, the cells were solubilized in hot 1% SDS and diluted to a final concentration of 0.1% SDS in Ripa buffer. Immunoprecipitation with anti Ras-GRF antibodies was carried out from 500 μg total proteins and the immunoprecipitates were separated by SDS-PAGE and immunodecorated with anti Ras-GRF antibodies.

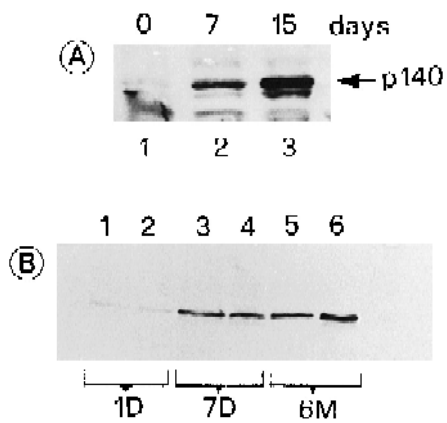

Fig. 4. Time course of the expression of Ras-GRF in cultures of hippocampal cells and in whole brain. A: total extracts of hippocampal cultures (100 μg) at days 0, 7 and 15 after seeding were analysed by Western blotting with antibodies against Ras-GRF. B: brains of neonatal (1D), 7 days (7D) and 6 months (6M) rats were extracted and 100 μg protein were analyzed as above.

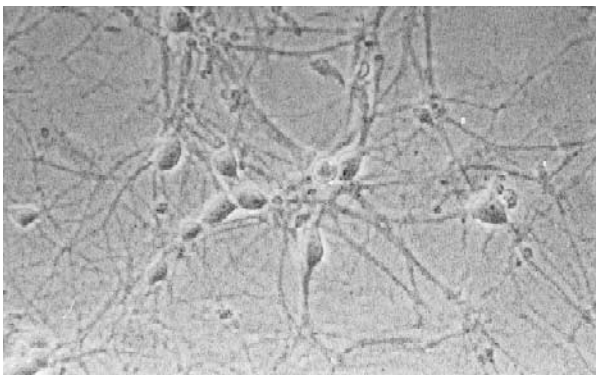

Fig. 5. Morphology of hippocampal cultures. Hippocampal cultures were obtained from E18 rats according to Banker et al. [1] with minor modifications. Cells were plated at high density (1×10^6 cells for 60-mm dishes) and 2 days after seeding 10 μM fluorodeoxyuridine and uridine were added. The photograph was taken with a phase-contrast microscope after 11 days in culture. Magnification 320×.

Phosphorylation of Ras-GRF and its association with calmodulin, have been previously reported in cells ectopically expressing Ras-GRF [4,7]. The finding that these events also occur in neurons gives a support to their physiological significance. Treatment with KCl to depolarize the cells or with the Ca^{2+} ionophore (A-23187) did not significantly alter either the phosphorylation of Ras-GRF or its association with calmodulin (data not shown), suggesting that an increase in calcium is not sufficient by itself to modify these parameters. In effect, the activation mechanisms of Ras-GRF may require other components in addition to calcium and calmodulin. Moreover, only in the case of stimulation with muscarinic agonists, an increase in the phosphorylation of Ras-GRF has been shown to correlate with an enhanced exchange activity [14].

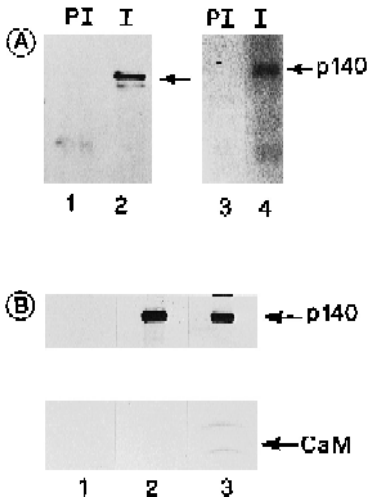

Fig. 6. In hippocampal cells, Ras-GRF is phosphorylated and associated with calmodulin. A: Ras-GRF was immunoprecipitated from hippocampal cells (15 days) either unlabeled (lanes 1–2) or metabolically labeled with [^{32}P]orthophosphate (lanes 3–4) using pre-immune serum (PI, lanes 1,3) or immune serum (I, lanes 2,4). Lanes 1–2: the immunoprecipitates were analyzed by Western blotting with Ras-GRF antibodies. Lanes 3–4: the autoradiography is shown. B: Ras-GRF was immunoprecipitated from hippocampal cells (15 days) with antibodies against Ras-GRF (lanes 2–3) or with pre-immune serum (lane 1). After separation by SDS-PAGE, the upper part of the blot was immunodecorated with antibodies against Ras-GRF and the lower part of the blot with antibodies to calmodulin. Lane 2: the immunoprecipitate was washed with EGTA before separation by SDS-PAGE.

The fact that CDC25Mm/Ras-GRF is expressed in neurons of the CNS only after birth, together with the finding that in hippocampal cultures its level increases in parallel with the onset of a neuronal network may support the view that both Ras and Ras-GRF may play a role in synaptic transmission [9]. Mice disrupted in the CDC25Mm gene have been obtained (R. Brambilla et al., submitted) and will tell us more about its function in vivo. Studies on neuronal cultures from control and knock-out mice will help us understanding the signal transduction pathway in which Ras-GRF is involved.

Acknowledgements

We thank E. Martegani and R. Brambilla for the helpful discussion. This investigation was partially supported by CNR ACRO Grant 95.00293.PF39 and by a grant from the Italian Association for Cancer Research (AIRC).

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