Vol. 189, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1992 Pages 371-377

EXPRESSION OF DRG DURING MURINE EMBRYONIC DEVELOPMENT

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Received October 12, 1992

SUMMARY: We had previously characterised a cDNA which encodes a novel GTP-binding protein DRG. The expression of *drg* gene is down-regulated during the embryonic development of murine central nervous system. Further analysis of *drg* mRNA and protein in adult mouse tissues and various cell lines of different origins indicated that it is expressed widely, albeit at low and variable levels. *In situ* hybridisation analysis of mRNA expression in sections of mouse embryos indicated that *drg* is expressed strongly in various embryonic tissues. The expression of *drg* mRNA is greatly reduced in newborn animals. At cellular level, DRG protein can be detected in the cytoplasm. These observations suggest that DRG may play multiple roles in development and normal cell metabolism.

In an attempt to understand the molecular mechanisms controling the developmental program of the central nervous system (CNS) of higher organisms, we carried out a screening for mouse genes whose expression is most prominent in developing brain and down-regulated after birth. The approach is based on the assumption that the genes having important functions during certain stage of development must be under tight regulation both temporally and spatially. The experiment led to the isolation of ten independent cDNA clones for novel genes with interesting structures and expression patterns (1). One of these cDNA encodes a novel GTP-binding protein DRG (2). The GTP-binding proteins are a growing family of proteins involved in a large number of diversed cellular functions such as growth regulation, protein synthesis, secretion, etc. (3,4). These proteins share a striking conservation of structure and the common biochemical activity of GTP hydrolysis. These proteins can be classified into three sub-families : (a) the small GTP-binding proteins consisting of the product of *ras* (p21*ras*) and the related proteins; (b) GTPases functioning in protein synthesis, such as bacterial elongation factor (EF-Tu) and related proteins ; and (c) the alpha subunit of the heterotrimeric, signal transducing G proteins such as transducin

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Abbreviations: GST, glutathione S-transferase; MBP, maltose-binding protein; CNS, central nervous system.

0006-291X/92 $4.00

(3,4). The predicted sequence of DRG protein contains all the conserved residues of the GTP-interacting domains, G1- G5. Outside these regions however, DRG protein does not share any homology with the known members of the GTPase family (2). This makes this protein a unique and novel GTP-binding protein . We have shown that the GST-DRG fusion protein expressed in bacteria and the endogenous DRG protein immunologically detected in embryonic brain, can bind GTP (2). In the present communication we have analysed the expression of *drg* gene in developing mouse embryos and adult tissues. The expression patterns of the *drg* gene and protein indicates that it may play an essential role in various types of cells.

EXPERIMENTAL PROCEDURES

Isolation of RNA and Northern Analysis

Poly A^+ RNA was isolated by one cycle of oligo-dT cellulose-absorption using a kit (Fast Track, Invitrogen) according to the instructions provided by the manufacturer. Two to 5 μg of poly A^+ RNA was electrophoresed on 1.2% agarose, 2.2M formaldehyde gels, transferred to nylon membrane (Biodyne A, Pall), and hybridised to nick-translated radiolabelled probes at 42° C in 50% formamide, 5x SSPE, 0.1% SDS, 5x Denhardt's solution and 100μg/ ml denatured salmon sperm DNA (5). The final wash was in 0.2x SSC, 0.1% SDS at 60° C.

In Situ *Hybridisations*

The procedure was adapted from reference 6. In brief, post-coital stages E8 (with uterine tissue), E10, E14 and newborn (N1) animals and brain tissue from adult animals (at least 3 months old) were cryostat sectioned at a thickness of 20μm and mounted on glass slides treated with Vectabond (Vector laboratories). After pretreatment, sections were soaked in hybridisation buffer (50% formamide, 2x SSPE, 10mM dithiothreitol, 2mg/ ml yeast RNA, 0.25 mg/ ml bovine serum albumin, 0.5 mg/ ml salmon sperm DNA, and 0.5 mg/ ml polyadenylic acid), and hybridised at 65° C for 12 hours with 10^5 cpm/ slide of either a sense or an antisense RNA probe derived from the 426 bp of the 3'-region of the DRG cDNA (base no. 1093-1518 in figure 1 of reference 2). After the hybridisation, sections were treated with 40 μg / ml of RNase at 37° C for 30-60 miunutes, washed and dehydrated (6). Autoradiograhy was performed at 4° C with Hyperfilm ß- max (Amersham) for 1-2 weeks.

Indirect Immunofluorescence Analysis

Cells were grown in Lab-Tek chamber slides, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde for 30 min. Free aldehyde groups were quenched in 50 mM NH_4Cl for 15 min and cells permeabilised by 0.1% Triton X100 treatment for 5 min. Subsequent blocking, antibody dilutions, and washings were done in 0.2% BSA in PBS. Anti MBP-DRG antiserum (2), precleared with *E. coli* acetone extract (Sigma), was used at 1:500 dilution. Binding of DRG specific antibodies was detected with fluorescein-conjugated anti rabbit IgG (Dako). Analyses of fluorescence-labeled cells were performed using an Olympus microscope linked to Meridian ACAS 570 Interactive Laser Cytometer and Hewlett Packard Paint Jet printer.

Immuno Blot Analysis

Tissues were dissected, homogenized on ice and lysed in 2x sample buffer (7). Twenty to fifty μg of each sample was electrophoresed on SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore) using standard protocols (8). Western blot analysis was carried out with 1: 250 dilution of rabbit anti-MBP-DRG serum as primary antibody and alkaline phohphatase conjugated goat anti-rabbit IgG (Dako) as the second antibody.

RESULTS

From the data presented earlier (1) and inFigure 1A, it is clear that the expression of *drg* mRNA is down regulated in the developing brain. We further studied the expression of the *drg* mRNA and protein in adult mouse tissues and several cultured cell lines . Analysis of the RNA isolated

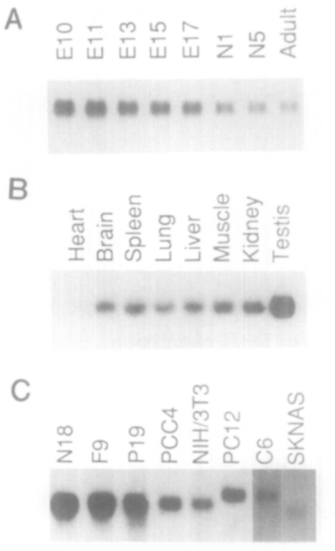

Figure 1. Northern blot analysis of *drg* mRNA expression.
In all cases 2.5 μg poly A^+ RNA was applied to each lane. (A) RNA isolated from embryonic head (E10, E11) or brain (E13-adult) at the indicated developmental stages were analysed. E, embryonic; N, postnatal. Exposure time was approximately 12 h. (B) RNA isolated from adult tissues were analysed. Approximate exposure time was 24 h. (C) RNA isolated from several cell lines were analysed. N18 (mouse neuroblastoma); F9, P19 and PCC4 (mouse embryonal carcinoma); NIH/3T3 (mouse fibroblast); PC12 (rat pheochromocytoma); C6 (rat glioma), SKNAS (human neuroblastoma). The exposure time for C6 and SKNAS was 48 h and for the rest of the lanes 24 h.

from various mouse tissues and several cell lines indicated that *drg* gene is expressed widely, albeit at variable levels (Figure 1B,C). Among adult mouse tissues, testis expressed the *drg* mRNA at relatively high levels (Figure 1B). All cell lines studied expressed *drg* transcripts. Under the stringent condition of hybridization, the mouse *drg* probe also detected a slightly larger rat mRNA species and a somewhat smaller human trancript (Figure 1C). The immuno blot analysis of protein extracts prepared from the brain at different stages of development showed that, unlike the gradual decrease in the steady state levels of mRNA (Figure 1A) the levels of the DRG protein remains roughly constant during embryonic stages of the brain followed by a rapid decline in the brain of post-natal animals (Figure 2A). This may indicate the existance of a post-transcriptional mechanism which keeps the level of this protein constant during embryogenesis. Expression of DRG protein was also detected in all the tissues from the adult mouse examined (Figure 2B). To establish the cellular localisation of the DRG protein, we carried out indirect immunofluorescence analysis of cells using anti-DRG serum. DRG seems to be localised predominantly in the cytoplasmic portion of the NIH/3T3 cells (Figure 3). This finding is consistent with that predicted DRG protein lacks any motifs for membrane anchoring and any possible transmembrane or nuclear localisation signals.

The distribution of the *drg* mRNA in the developing mouse embryos and newborn animals was analyzed by *in situ* hybridisation using antisense riboprobes generated from subcloned fragments of the *drg* cDNA. The earliest embryonic stage we used was approximately E7, and the results

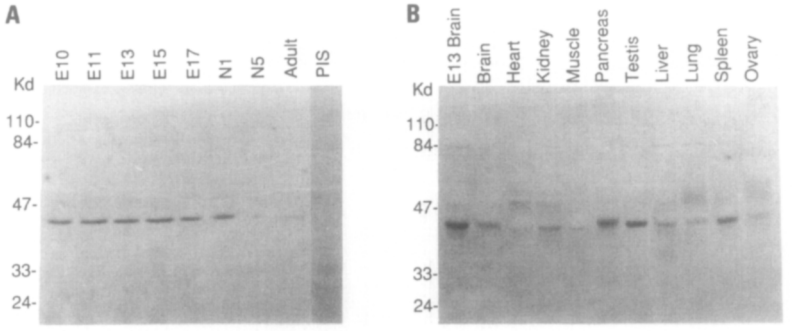

Figure 2. Immuno blot analysis of DRG expression.
(A) Total heads (E10, E11) or brain (E13- adult) were isolated at the indicated developmental stages. E, embryonic; N, postnatal. 20 μg of each protein sample was analysed. PIS, preimmune serum reacted with E13 sample. (B) Fifty μg of protein extracted from various adult mouse tissues was analysed.

indicate strong signals in the embryonic body and much weaker signals in the surrounding uterus tissue derived from the mother, however at this early stage of development it is difficult to discern the exact location of expression within the embryo (Figure 4A,B). In the E10 sections strong signals could be seen in the neural tube and weaker ones in the rest of the body (Figure 4C, D). In the E14 sections *drg* expression can be seen in several tissues, but most prominent signals are found in the brain, spinal cord, liver, gut, thymus and cervico-thoracic ganglia (Figure 4E-G). In newborn pups (post-natal day 1), the overall expression of *drg* was dramatically reduced and although the expression could be detected in a number of different tissues at low levels, stronger signals were seen only in the brain, submaxillary gland and the

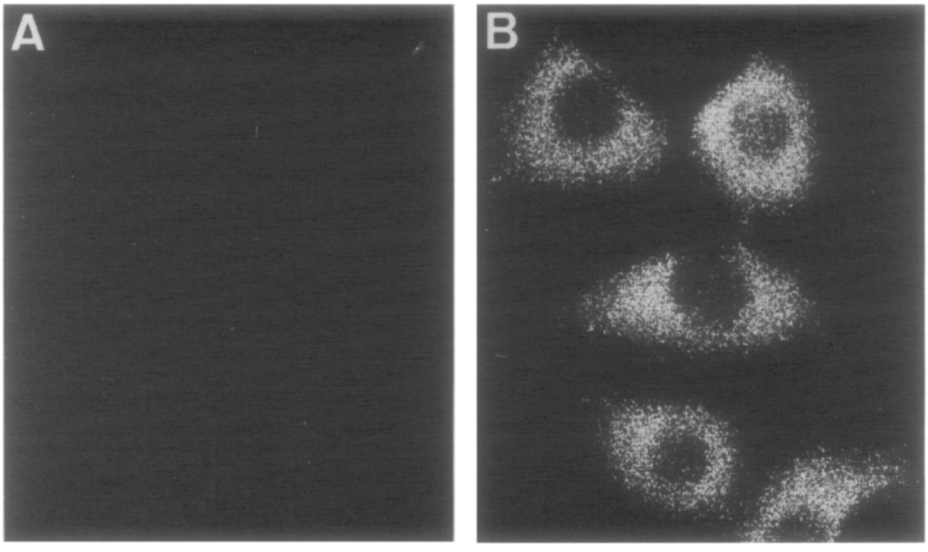

Figure 3. Indirect immunofluorescence analysis of DRG expression in NIH/3T3 cells.
(A) preimmune serum or (B) anti MBP-DRG serum. In both A and B, the number of cells in the field is approximately same. Figure shows the printouts from Hewlett Packard Print Jet printer.

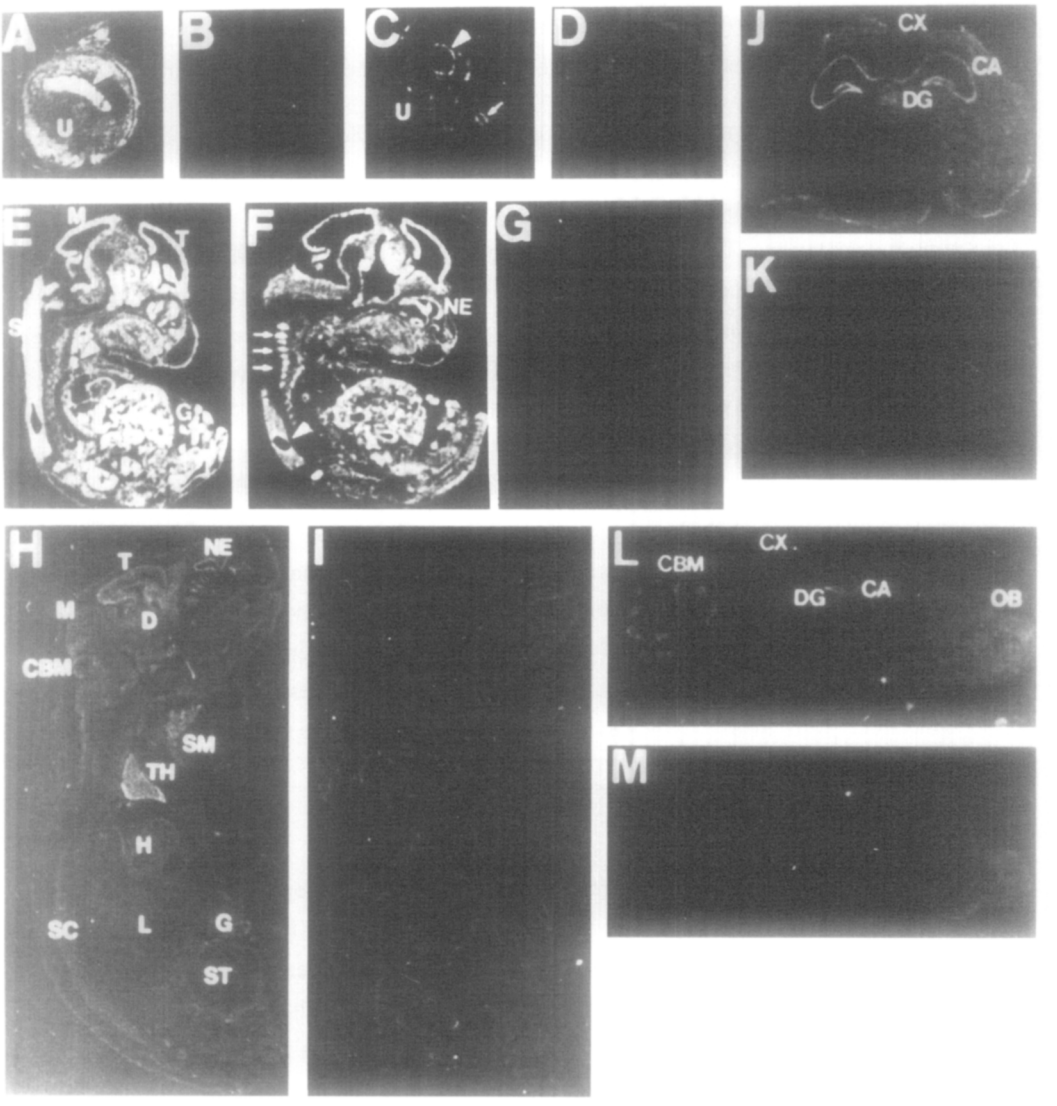

Figure 4. *In situ* mRNA analysis of *drg* expression.
The figure shows the negative images of the autoradiographs. (A,B) E8 embryo sections hybridised to antisense (A) and sense (B) probes. Strong signals can be seen in the embryonic body (arrowhead) and relatively weaker one in the surrounding uterus tissue (U). (C,D) E10 embryo sections hybridised to antisense (C) and sense (D) probes. Anterior (arrowhead) and posterior (arrow) regions of neural tube show relatively higher expression signals than rest of the embryo. (E-G) E14 embryo sagittal (E,G), and parasagittal (F) sections hybridised with antisense (E,F) and sense (G) probes. Varying levels of expression can be detected in all tissues. T, telencephalon; D, dicephalon; M, mesencephalon; SC, spinal cord; L, liver; G, gut; NE, nasal epithelia. In (E), the position of thymus is indicated by arrowhead. In (F), the location of cervico thoracic ganglia is indicated by small arrows and that of posterior neuropore by arrowhead. (H,I) Newborn (postnatal day 1) animal sectioned sagittaly and hybridised to antisense (H) and sense (I) probes. Overall expression of *drg* mRNA was much reduced at this stage compared to embryonic stages. Relatively stronger signals are visible in brain, thymus (TH) and submaxillary gland (SM). CBM, cerebellum; H, heart; ST, stomach. Other abbreviations are as in (E). (J-M) Coronal (J,K) and sagittal (L,M) sections of adult mouse brain hybridised to antisense (J,L) and sense (K,M) probes. Relatively stronger expression of *drg* can be seen in hippocampal formation. CA, cornu ammonis; DG, dentate gyrus; OB, olfactory bulb; and CX, cerebral cortex.

thymus (Figure 8H, I). In adult brain, the most prominent expression was seen in the cell fields of hippocampus formation (cornu ammonis and dentate gyrus). Weaker signals were observed in the regions of high cell density such as olfactory bulb and cerebeller cortex (Figure 4J-M).

In order to check if overexpression of *drg* resulted in phenotypic changes in the cells, we cloned the open reading frame derived from the DRG cDNA into the plasmid vector pSRα neo (9; Kitayama and Noda, unpublished data) and retroviral expression vectors LXSN (10) and pXT1 (11) in both orientations. The embryonic carcinoma P19, mouse fibroblast NIH/3T3 and rat pheochromocytoma cell line PC12 were transfected or infected with the expression vectors and cells carrying the exogenous DNA were selected for G418-resistance. No clearcut changes were observed in the growth properties of these cells (data not shown). We then checked the expression of transfected DRG by Northern and immuno blot analyses. To our surprise, in all cases, cells transfected with both the sense and antisense cDNA showed only marginal levels of expression over the endogenous levels of expression (data not shown). Repeated experiments with all three expression vectors showed similar results. We do not know the reason for these puzzling observations, however one likely explanation could be that the cells overexpressing both the sense and antisense cDNA fail to survive and we select out the cells expressing only low levels of mRNA for the introduced cDNA. In other words, small perturbations in the levels of DRG protein may be toxic to the cells. On the other hand, it is also possible that the subtle biological effects were masked, because the cell lines utilized in the study expressed relatively high levels of endogenous *drg* mRNA (Figure 1).

DISCUSSION

The expression of *drg* mRNA and protein is developmentally regulated in the CNS. It is interesting to note that the embryonic expression of this gene is not limited to the cells derived from the CNS alone, but includes the peripheral nervous system derived cervical ganglia, the thymus and the liver and perhaps all other tissues at lower levels. This general pattern of expression of *drg* might point to a wider physiological role for its protein. It may be argued however that since certain embryonic tissues express this gene at much higher level than others, it may play a more specific developmental role in certain types of cells. For example, at the E14 stage, *drg* mRNA is expressed much more intensely in the inner proliferating and differentiating layer of brain cells than other parts of the already differentiated brain, which may indicate a possible role for DRG protein in the proliferation and/or differentiation of neuronal cells. The strong expression of DRG both in embryonic and post-natal thymus is also intriguing. As thymus is the site where T cells mature, the higher expression of this gene may have implications for T cell differentiation and maturation. It may be possible that *drg* is another link between the CNS and the immune system as a member of the regulatory molecules like *PDGF* (12) and *RAG*-1 (6) which are expressed in both the brain and the lymphocytes.

The interspecies conservation of *drg*, both at the genomic and protein levels, indicates that this protein may serve some essential function in the physiology of the cell (2). Close homologues of *drg* are present in *Drosophila* and *Halobacterium*, and perhaps in other species as well (2). The

drg cDNA probe derived from the coding region, hybridises to DNA from a number of vertebrate and invertebrate species under relaxed hybridization conditions (S.K. and T.S., unpublished observations). The presence of *drg* in organisms as diverse as bacteria, is intriguing and it would be interesting to relate this evolutionary conservation to its biochemical and physiological roles. Some of the GTP-binding proteins are known to play different functions in different tissues. For instance, the Ras protein is involved in the transduction of growth signals in the fibroblasts, while it is involved in differentiation signals in some neuronal cells (13). The K*rev*-1 gene product, which induces reversion of *ras*-transformed fibroblasts to morphologically flat cells (14), also seems to play various roles in different tissues (15). Likewise, the DRG protein may play multiple roles in different tissues.

An attempt to directly uncover the biological function of DRG by overexpressing the *drg* gene in various cell lines failed to yield clear cut results in this study. More information on the function of this protein may be obtained by studying the phenotypes of the mice in which the *drg* gene has been disrupted through homologous recombination. Also, studies of the close homologs of *drg* found in *Halobacterium* and *Drosophila* may yield important insights into the physiological role of the mammalian counterpart.

ACKNOWLEDGMENTS

We would like to thank N. Mizuno for the help in the interpretation of *in situ* hybridisation data and T. Matsuzaki and Y. Yoshida for technical help. This project was supported by grants from the Science and Technology Agency (STA) of Japan and the internal funds from the Cancer Institute. S. K. was previously at RIKEN, Tsukuba Life Science Center, supported by a visiting scientist fellowship from STA .

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