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A novel member of the dynamin family of GTP-binding proteins is expressed specifically in the testis

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SUMMARY

Dynamin is a member of a new GTPase family, which includes the mouse Mx protein, the yeast VPS1 and the *Drosophila shibire* gene product. A high homology with the shibire product suggests a role for dynamin in the endocytotic process, but it is expressed only in mature neurons. We identified two additional dynamin-like proteins in rats, by using the polymerase chain reaction with degenerate primers corresponding to the GTP-binding areas conserved between dynamin and VPS1. The full coding sequence of one of them, dynamin-2, revealed that it has 848 amino acids and has great similarity with brain dynamin and the shibire product. Northern blot analysis and in situ hybridization revealed its expression to be specific to the seminiferous tubules in the testis. Dynamin-2 (testis type dynamin) was expressed in germ-cell-depleted testis as well, indicating its expression in Sertoli cells. Our data imply that a number of dynamin family proteins, which are products of distinct genes, may play different roles specific to each cell type in the same rat.

Key words: dynamin, GTP-binding protein, PCR, testis, Sertoli cell

INTRODUCTION

Dynamin is a member of a new GTPase family (Obar et al., 1990; Nakata et al., 1991) that includes mouse antiviral Mx protein, yeast VPS1 product, which is essential for vacuolar protein sorting, and *Drosophila shibire* gene product (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). In the *shibire* mutant, synaptic membrane recycling is blocked because of a disorder of endocytosis in the presynaptic terminals (Kosaka and Ikeda, 1983a). The blocking of endocytosis is not restricted to neural cells but is also found in other cells such as oocytes (Kessell et al., 1989) and Garland cells (Kosaka and Ikeda, 1983b). The extensive similarity in primary structure between dynamin and shibire suggests that the *shibire* gene encodes the *Drosophila* equivalent of dynamin, thereby indicating a role for dynamin in endocytosis (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). However, dynamin is expressed almost exclusively in neurons after maturation, and is rarely expressed in other tissues such as liver and kidney, where endocytosis is very active (Nakata et al., 1991; Scaife and Margolis, 1990). Therefore, it must be supposed that there are other members of the dynamin family of proteins in non-neuronal tissues. We observed weak hybridization of dynamin cDNA probe with RNAs in non-neuronal tissues of different size from the mRNA of brain dynamin (Nakata et al., 1991).

To search for new members of the dynamin family in mammals, we used the polymerase chain reaction (PCR), using degenerative primers corresponding to the GTP-binding areas conserved between dynamin and VPS1. In this report we describe the identification of two additional members of the rat dynamin family. Molecular cloning and characterization of one of them revealed that this new member is also expressed very specifically in certain well-differentiated cells, suggesting that a number of dynamin family proteins may have functions specific to each cell type in the same rat.

MATERIALS AND METHODS

PCR

Degenerate oligonucleotide primers corresponding to the amino acid sequences were synthesized. The sequence of the positive strand primer 1 was 5′-GA(TC)CA(AG)CC(CTAG)CC-(CTAG)CC(CTAG)GA(TC)AT(CAT)GA-3′, and the sequence of the negative strand primer 2 was a mixture of 5′-TT(CTAG)GT(AGT)AT(AGCT)AC(AGCT)CC(GTA)AT-(TCAG)GT-3′ and 5′-TT(GTAC)GT(GTAC)AG(AGCT)AC (AGCT)CC(GTA)AT(TCAG)G-3′. First-strand cDNA was synthesized using 10 μg of total RNA from brain, lung, liver, spleen, kidney and testis and primer 2 (Nunberg et al., 1989). Subsequently, the polymerase chain reaction was performed with primers 1 and 2 at 94°C for 1 min, 46°C for 1 min and 72°C for 2 min, for 40 cycles (Nunberg et al., 1989). Fragments of 180 bp were amplified only in the testis sample, except for that from brain.

The products were purified from agarose gels, treated with the Klenow fragment of DNA polymerase I, and phosphorylated with T4-polynucleotide kinase. The fragments were subcloned and sequenced as described previously (Nakata et al., 1991).

Cloning of dynamin-2

Rat testis λgt10 cDNA was constructed using oligo(dT) primer and primer 2. Clones were selected with probes generated from the PCR11 fragment. The cDNA inserts were subcloned in Bluescript. A number of ExoIII-MB nuclease deletions were made using the Takara kilodeletion kit. DNA sequences were determined by dideoxynucleotide sequencing with Sequenase (United States Biochemicals).

RNA preparation and northern blot analysis

Total RNAs from different tissues were prepared by the hot phenol method. RNA was quantified by measuring the absorbance at 260 nm, and the integrity of the RNA was checked by staining following agarose gel electrophoresis. RNA (15 μg) was denatured and fractionated on a formaldehyde-agarose gel, blotted onto a nitrocellurose filter, and baked at 80°C for 2 h. Probes for northern blotting were 180 bp PCR products subcloned into pBluescript. The inserts of PCR9 and dynamin-1 were cut out at *Xba*I and *Xho*I sites, and labelled using the hexanucleotide prime method. Hybridization was carried out at 65°C for 24 h in a solution containing 5×SSPE, 5×Denhardt's solution, 1% SDS and 100 μg/ml sheared denatured salmon sperm DNA. Blots were washed and exposed to X-ray film as described previously (Nakata et al., 1991), except for the more stringent final wash in 0.2×SSC, 0.1% SDS at 65°C for 1 h. Date-mated female rats were given a dose of 125 rad whole-body irradiation on day 19 of gestation. Parturition occurred on day 22. Newborn male rats were killed on postnatal day 24 for northern blot analysis and light microscopy of testis. For light microscopy, rat testis was fixed with 2% paraformaldehyde and 2.5% gultaraldehyde in 0.1 M sodium cacodylate. The tissue was postfixed with 1% OsO_4, dehydrated with ethanol, and embedded in Epon 812. The semi-thin sections were cut and stained with Toluidine Blue.

In situ hybridization

Rat testes were fixed by perfusion with 2% paraformaldehyde in PBS. They were processed for in situ hybridization as described previously (Nakata et al., 1991). ^{35}S-labeled cDNA probes covering the C-terminal 1.6 kb sequence of dynamin-2 (or Bluescript *Pvu*I fragments to control for spurious labeling) were generated using the hexanucleotide priming method (Feinberg and Vogelstein, 1983). The sections were counter-stained with hematoxylin-eosin and were photographed with bright-field optics (Zeiss Axiophoto).

RESULTS

PCR identification of dynamin-like proteins in mammals

We identified two dynamin-like proteins in non-neuronal tissues using the polymerase chain reaction (PCR). A pair of degenerate primers complementary to the conserved sequences in the N-terminal GTP-binding area between dynamin and VPS1 (Fig. 1) were used to amplify segments of genes encoding dynamin-like proteins from total RNA from various tissues. cDNA was synthesized from 10 μg of total RNA from brain, lung, liver, spleen, kidney and testis using primer 2. Subsequent amplification with primers 1 and 2 resulted in products of 180 bp, the size expected from the requirements for the spacing between the GTP-binding motifs, only in brain and testis, suggesting that the expression of the authentic brain dynamin is nil or below the level of detection by PCR amplification in the other tissues. The 180 bp products generated by amplification in the testis were purified and cloned. From a total of 16 PCR fragments that were cloned and sequenced, nine were found to encode dynamin-like proteins. These nine fragments fell into two classes, encoding, respectively, predicted proteins 73% (PCR9) and 83% (PCR11) identical to dynamin in the regions between the primers (Fig. 1). The homology in these amplified regions indicates that the proteins are closer to dynamin and shibire than to the other dynamin-related proteins, such as VPS1 and Mx. However, authentic brain dynamin was not detected in the clones sequenced in the testis.

Molecular cloning of dynamin isoform from testis

cDNA clones covering the full coding region were isolated from rat testis cDNA library by hybridization with a PCR11 fragment. The cDNAs contain an open reading frame of 848 amino acids, compared with 851 amino acids for brain dynamin (Fig. 2). The deduced amino acid sequence of the protein is identical to 89% of the rat brain dynamin sequence (65.7% identical at the nucleic acid level), suggesting that it is a testis counterpart of brain dynamin. To distinguish between these two dynamins, we call brain dynamin dynamin-1 and testicular dynamin dynamin-2. Alignment of the three dynamins revealed their homology not only in the GTP-binding domain but throughtout their length. Dynamin-2 also contains a C-terminal proline-rich region (770 amino acids) but this area is not well conserved among these proteins.

Expression of dynamin-2 in Sertoli cells

Northern blot analysis showed that dynamin-2 mRNA (3.5 kb in length) is expressed exclusively in the testis; brain dynamin is expressed only in the brain (Fig. 3a,b).

In situ hybridization analysis showed that dynamin-2 is expressed in seminiferous tubules, which contain only germ line cells and their nursing cells (Sertoli cells), and is not expressed in interstitial cells (Fig. 4a). Control β-actin is expressed in interstitial cells as well as in seminiferous tubules (Fig. 4c). To examine further the cells that express

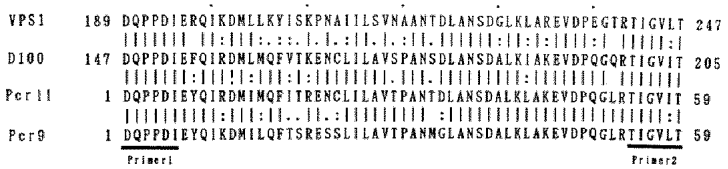

Fig. 1. Amino acid sequences of the clones amplified by the polymerase chain reaction from rat testis RNA. For comparison, the comparable dynamin and VPS1 sequences are shown on top. Location of primers used for PCR are underlined. Of 16 cloned fragments that were sequenced, 9 were found to encode Pcr9 or Pcr11 type.

Fig. 2. Alignment of deduced amino acid sequence of the dynamin-2 (T-dynamin) with rat brain dynamin (B-dynamin) and *Drosophila shibire* (Shibire). The sequences were aligned using the GAP program of the Genetics Computer Group with a GAP weight of 3.0 and a length weight of 0.1. The dynamin-2 sequence is identical to 81% of the brain dynamin and 69% of the *Drosophila shibire* gene product. Consensus GTP-binding motifs are overlined.

dynamin-2, we performed northern blot analysis of germ-cell-depleted testis. Germinal cell aplasia (Sertoli cell-only syndrome, SCO) was induced in male rats by fetal irradiation (125 rad) on day 19 of gestation (Tindall et al., 1975). While normal rats contain a large number of germ line cells in the seminiferous tubules (Fig. 5b), the testis of irradiated rats contains virtually only Sertoli cells (Fig. 5c). Northern blot analysis of the testis of normal and irradiated rats showed that the level of the expression of dynamin-2 mRNA was the same as that of actin mRNA in both rats (Fig. 5a), indicating its expression in Sertoli cells.

DISCUSSION

Our findings imply that multiple members of the dynamin

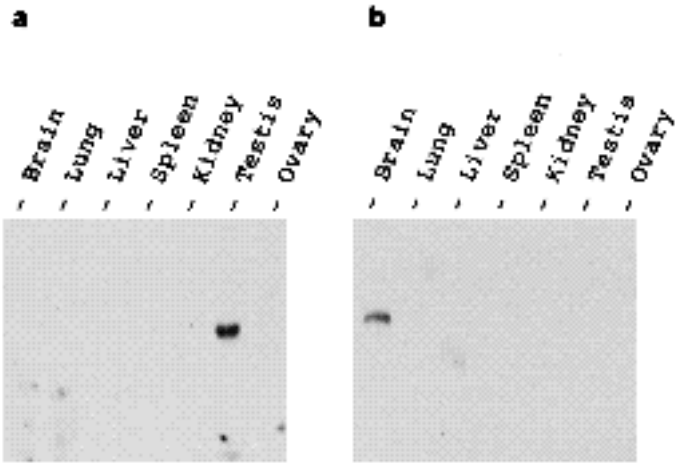

Fig. 3. Northern blot analysis of dynamin-2 (a) and rat brain dynamin-1 (b) in the various tissues. Total RNA (15 μg) was fractionated and blotted on nylon membrane. Dynamin-2 is expressed as specific for testis.

family of GTPases exist in mammals. In this work, we have identified two dynamin-like proteins in rats on the basis of their sequence similarity to conserved regions of the GTP-binding area between VPS1 and dynamin. These two, in addition to brain type dynamin and interferon-induced Mx proteins, increase the total number of dynamin-like GTPases, which establishes that several members of the dynamin-like GTPase family may play roles in any single mammal. However, several arguments suggest that our PCR screen is incomplete and that the number of dynamin family GTPases in rats is larger. (i) No segments were recovered from tissues such as liver and kidney, where receptor-mediated endocytosis is very active. In fact, we observed a weak hybridization of the dynamin cDNA probe with RNAs from these tissues with different sizes from the mRNA of the brain dynamin (Nakata et al., 1991). (ii) Yeast VPS1 products play a role in vacuolar protein sorting rather than endocytosis (Rothman et al., 1989), suggesting that the VPS1 gene does not encode the yeast equivalent of dynamin. Because VPS1 product is an essential protein for yeast (Rothman et al., 1989), there may be members of this GTPase family in mammals corresponding to yeast VPS1.

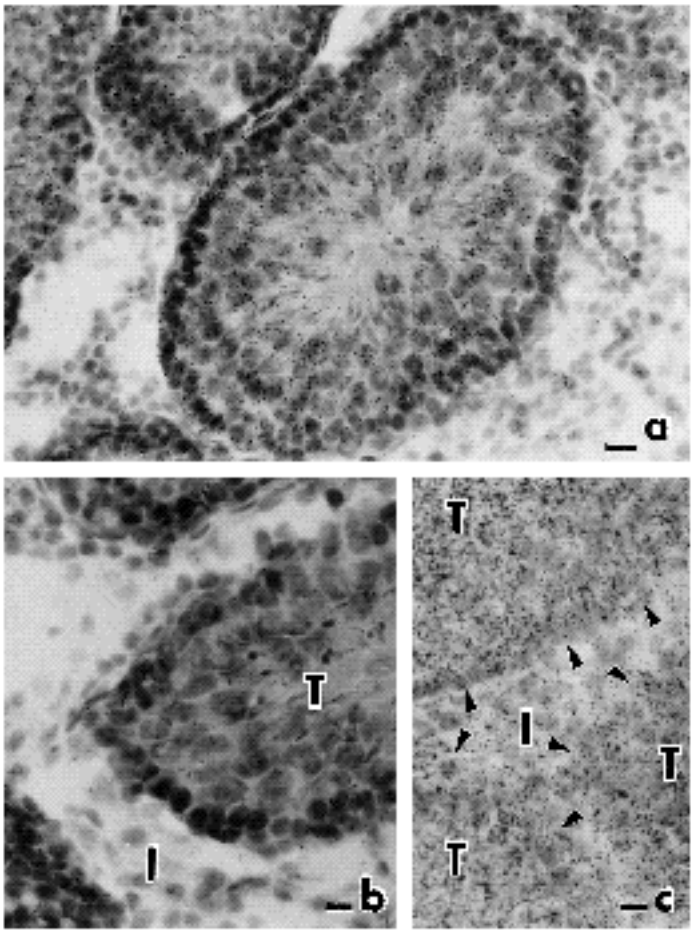

Fig. 4. In situ hybridization analysis of rat testis probed with rat dynamin-2 (a), and β-actin (c) and Blues cript (b) as controls. Signal for dynamin-2 was detected exclusively in the seminiferous tubules, not in the interstitial cells. β-Actin was expressed in both tubules (T) and interstitial cells (I). In c, the boundary of the seminiferous tubules is indicated by arrowheads. Bars, 10 μm.

However, no member more homologous to yeast VPS1 has been identified yet in mammals. (iii) Because only a few members of the dynamin family have been identified to date, there is a possibility that our degenerate primers

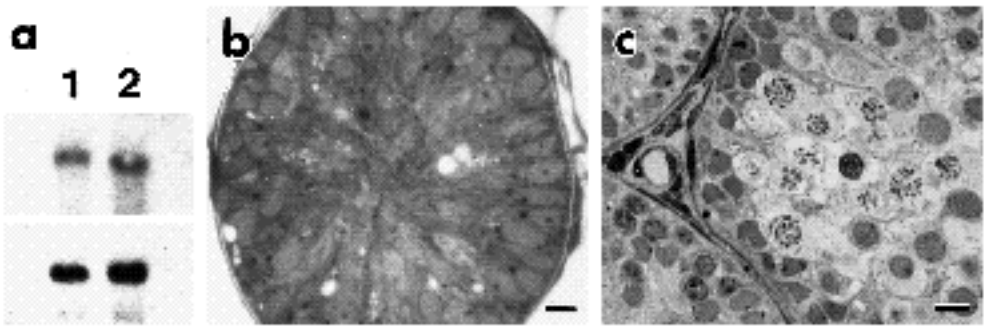

Fig. 5. Northern blot analysis of dynamin-2 in germ-cell-depleted testis. Whole body irradiation (125 rad) of pregnant rats at 19 days of gestation produced male offspring with seminiferous tubules remarkably free of germinal epithelium and containing essentially only Sertoli cells (b). Control normal testis (c). Bars, 10 μm. Poly(A^+) RNA (5 μg) from normal (lane 2) and irradiated rat testis (lane 1) was fractionated, blotted on nylon membrane, and probed with ^{32}P-labeled dynamin-2 cDNA (a). Dynamin-2 is expressed in germ-cell-depleted testis as well as in normal testis. The lower lanes in (a) show the same blot probed with the control β-actin cDNA.

cannot cover all the members of the dynamin family. In the case of *Drosophila*, although isoforms of the *shibire* gene products formed by alternative splicing have been reported (Chen et al., 1991), the distinct gene products have not been identified yet. In contrast to the cell type-specific expression of dynamins in rats, the *shibire* gene products play a role not only in neurons but also in other types of cells (Kosaka and Ikeda, 1983a,b). Hence the data suggest that the dynamin family in mammals is larger than that in *Drosophila* and contains a minimum of four members.

Our data imply that dynamin-2 is expressed in Sertoli cells. Their non-proliferating nature, and the abundance and uniform spacing of microtubules are reminiscent of nerve cells (Fawcett, 1975). In fact, dynamin-1 in the brain is expressed after the cessation of cell division (Nakata et al., 1991) and has a microtubule-activated GTPase activity (Sphetner and Vallee, 1992; Maeda et al., 1992). Numerous primary lysosomes (Fawcett, 1975) and phagocytotic activity have been reported in Sertoli cells (Clegg and MacMillan, 1965). Dynamin-2 may be involved in these endocytotic processes in Sertoli cells just like the *shibire* products in *Drosophila* cells.

Expression of dynamins is tissue specific. No segments of the brain type dynamin were recovered from non-neural tissues even though brain type dynamin sequences should have been amplified by our degenerate primers. Northern blotting analysis detected a band in brain only when probed with a short fragment of dynamin-1 cDNA and washed under stringent condition (Fig. 3). Dynamin-1 was not detected in non-neuronal tissues by western blotting (Scaife et al., 1990). Dynamin-2 mRNA was detected only in the testis. In a previous report, we described a weak hybridization of rat brain dynamin-1 cDNA probe (2 kb in length) with non-neural tissues that was different in length from the mRNAs of brain dynamin (Nakata et al., 1991) under mild washing conditions. Our present results suggest strongly that these bands are derived from cross-hybridization with mRNAs for non-neuronal types of dynamin family proteins. The tissue-specific expression of dynamin family members suggests that the members may play different roles, according to cell type, in mammals. When we think of other dynamin family proteins such as Mx proteins and VPS1 products, we expect a very restricted role for each member in a wide range of processes involved in vesicular trafficking (Krug et al., 1985; Staeheli and Haller, 1987; Rothman et al., 1990). Previously known GTPase families, such as low molecular weight GTPases, consist of many member proteins, each member is expressed in different tissues, and plays a similar, but different, role in cells (Bourne et al., 1990). Identification of the members of the dynamin family of proteins using PCR, and information about the conserved regions of dynamin family proteins obtained from the expanding number of members would reveal the dimensions of this novel family of GTPases in mammals.

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