Developmental Expression of the \textit{RET} Protooncogene\(^1\)

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Abstract

The \textit{RET} protooncogene encodes a transmembrane protein of the receptor-type tyrosine kinase family whose ligand has not yet been identified. Its activation \textit{in vivo} is restricted to human carcinomas of the thyroid. In order to learn more about the possible role played by \textit{RET} during normal development, we have examined its expression by performing \textit{in situ} hybridization experiments on mouse embryos. Here, we show that the \textit{RET} protooncogene is expressed during mouse embryogenesis in an unusual temporal and spatial manner. In fact, its expression was first detected around day 10 of gestation in the basal plate of the neural tube and in the developing encephalic ganglia, and later its pattern of expression was definitely established in neural structures, mostly in neural crest derivatives (spinal and encephalic ganglia). As far as the central nervous system is concerned, \textit{RET} expression was confined to the ventral part of the midbrain from 12.5 days postcoitum (dpc) until birth. \textit{RET} was also found to be expressed within structures of sensory organs such as the ganglial layer of the retina and the olfactory epithelium. A peculiar pattern of \textit{RET} expression was clearly observed in the wall of the gut and in the nephrogenic zone of the developing kidney cortex, specifically in the metanephrogenic vesicles. Finally, \textit{RET} was found to be expressed in the liver mostly between 12.5 dpc and 14.5 dpc. In conclusion, its expression in the early stages of embryogenesis suggests that \textit{RET} may play a role in the differentiation of specific neural structures and the excretory system.

Introduction

An activated form of the \textit{RET} protooncogene was first isolated by the transfection of NIH 3T3 cells with DNA from a human T-cell lymphoma (1). Other activated forms of \textit{RET} were subsequently isolated from colonic and gastric carcinomas (2, 3). In all cases, the transforming activity was acquired \textit{in vitro} by fusion of the tyrosine kinase domain of \textit{RET} to the NH\(_2\) terminus of heterologous genes occurring during transfection. Conversely, \textit{in vivo}, \textit{RET} is activated in the neoplastic tissue of human thyroid papillary carcinomas (4–10). In these tumors, activation is mostly due to the fusion of the tyrosine kinase domain of \textit{RET} to a gene named \textit{H4} or \textit{D10S170}, giving rise to the chimeric transforming oncogene \textit{RET/PTC} (6). In conclusion, the activation of \textit{RET} by rearrangement seems to be restricted to thyroid carcinomas (10), and more specifically to their papillary subtype (7). In addition, recently, germline mutations of \textit{RET} have been described in patients afflicted by the MEN2A syndrome, thus strongly suggesting an involvement of \textit{RET} in the pathogenesis of medullary thyroid carcinomas and pheochromocytomas, tumors which characterize this syndrome (11).

The \textit{RET} protooncogene encodes a protein structurally related to transmembrane receptors with a cytoplasmic tyrosine kinase domain (12, 13). As in the case of other receptors with tyrosine kinase activity, the protein coded for by the \textit{RET} protooncogene has an amino-terminal signal sequence, a cysteine-rich extracellular domain, a transmembrane domain, and a tyrosine kinase domain, interrupted by an insertion sequence of 27 amino acids (13). These properties of the \textit{RET} protein strongly imply its role as a receptor for an unknown growth factor. Recently, it was reported that a cadherin-related sequence is present in the extracellular domain of the \textit{RET} protein. In fact, the \textit{RET} protein shows the presence of a sequence about 110 amino acids long, which is tandemly repeated 3–4 times in the extracellular domains of all vertebrate cadherins. These observations suggest that \textit{RET} expression may mediate a homophilic interaction between different cells (14).

The involvement in specific subsets of tumors suggests that \textit{RET} may play a role in the proliferation of specific types of cells. This is also suggested by the peculiar distribution of its tissue expression. Some information is already available about the expression of \textit{proto-RET} in human, rat, and mouse normal and tumor tissues. \textit{RET} has been found to be expressed in many of the human neuroectodermal tumor cells, like neuroblastoma cell lines (15), medullary thyroid carcinomas, and pheochromocytomas (16). Moreover, in both medullary thyroid carcinoma (16) and neuroblastoma cell lines (17), \textit{RET} expression increases upon induction of differentiation by several agents, examples suggesting that \textit{RET} might have a role in the differentiation and/or proliferation of neural cells. In addition, its expression has been observed in some human neoplastic cell lines such as HL-60 promyelocytic leukemia and THP-1 monocyctic leukemia (13). In normal adult rat tissues, very low levels of \textit{RET} expression

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have been observed in lung, heart, spleen, and small intestine; conversely brain, thymus, and testis showed significantly higher expression of RET (18). High levels of RET transcripts were detected in the cytotoxophoblasts of rat placenta during the midgestational period (days 10 and 11), but not in the trophoblasts on day 14 of gestation, when the placenta was undergoing morphological changes (19). Finally, in the adult mouse, RET expression has been reported only in the spinal cord and in lymph nodes of C3H/HeJ-gld-gld mice (13, 20).

In order to learn more about the expression of proto-RET and consequently about its possible role during embryogenesis, we have performed in situ hybridization studies on mouse embryos. Here, we demonstrate that the proto-RET gene is expressed in both neural structures and the kidney of the developing mouse.

Results

In order to obtain a RET-specific probe, we have cloned a portion of the mouse RET cDNA from total embryo poly(A)+ RNA by reverse transcriptase-PCR using as amplifiers the oligodeoxynucleotides m-ret1 and m-ret2, as described in "Materials and Methods." Sequence analysis showed that the 270-bp cDNA fragment obtained and named ret-270 corresponded to the sequence 2293-2562 of the mouse proto-RET cDNA (20); moreover, Northern blot experiments confirmed the specificity of the ret-270 probe (data not shown). Ret-270 was used to generate an antisense riboprobe in order to analyze the spatial and temporal distribution of RET expression during mouse embryogenesis by in situ hybridization. This antisense probe was able to detect RET protoconogene transcripts of 7.0, 6.0, 4.5, and 3.9 kilobases by Northern blot using poly(A)+ RNA from total mouse embryo 12.5 dpc. As a control of the specificity of the hybridizations, we have used a sense probe obtained by cloning the ret-270 fragment in the opposite orientation in the plasmid vector pGem3Z (data not shown). The structures that we have considered positive were those hybridizing only to the antisense probe. In addition, perfectly corresponding results were obtained using as probe a 51-bp-long synthetic antisense oligodeoxynucleotide (KD-probe of Ref. 19), corresponding to the tyrosine kinase domain of the RET protoconogene; the specificity of this probe has been reported previously (19).

Neural Structures. RET expression was detectable from around day 10 of gestation and, at this time and thereafter, was restricted mostly to neural tissues. In fact, the earliest signal that we detected was in sagittal sections at 9.5 dpc, at the level of the spinal cord (Fig. 1a); transverse and frontal sections show a clear expression of RET, at 10.2 dpc, in the spinal cord at the level of the basal plate (Fig. 2d) and in cephalic ganglia (Fig. 2a). The expression in the basal plate of the spinal cord was retained throughout mouse development. In particular, RET expression at 10.2 dpc was localized in the ventral mantle layer (Fig. 3a) and subsequently became progressively restricted to neurons confined in the basal plate in a region of the ventral horn where somatic motoneurons are located (Fig. 3, b, c, and d, respectively 12.5, 14.5, and 17.5 dpc). At 10.2 dpc, spinal ganglia appeared to be negative (Fig. 2a); subsequently, they were found to express RET but not homogeneously. In fact, at 12.5 dpc, a characteristic signal was clearly present only in subsets of cells situated in the spinal ganglia, as shown in sagittal (Fig. 1e) and frontal (Fig. 2e) sections. In the sections depicted at high magnification in Fig. 3, it is clearly shown that RET expression seemed to be confined only to small groups of ganglial cells (Fig. 3, b and c); this expression was retained until birth, as shown in Figs. 1f and 3d. Cephalic ganglia also expressed RET from 10.2 dpc (Fig. 2a) until birth; the distribution of the RET-positive cells within these structures, similarly to that described in the spinal ganglia, was localized to subsets of cells (Figs. 1, d and f; and 2, a and c).

As far as the brain is concerned, RET expression was clearly present only in the ventral part of the midbrain; this expression was first detected around 12 dpc (high magnification, in Fig. 4a), and was maintained through day 14.5 dpc (Figs. 1c and 2c) and further, until birth (data not shown). RET was found to be clearly expressed in the external layers of the intestinal wall, where parasympathetic plexi (neural crest-derived cells) are localized (Figs. 1, b, c, e, and f; and 2, b, c, and f). Fig. 4, f and g, also shows this characteristic signal at high magnification.

Sense Organs. A faint hybridization restricted to a few cells surrounding the olfactory epithelium was observed at 17.5 dpc (Fig. 4c). The retina also expressed RET; the high magnification reported in Fig. 4b shows that a clear signal was restricted to the ganglion layer.

Excretory System. The nonneural structures which were found to express RET were the developing kidney, the liver, and the genital eminence. In fact, RET expression was clearly observed in the developing metanephros from 12.5 dpc (Fig. 4d) to 14.5 dpc (Figs. 1, c, e, and f; and 2f) and became faintly detectable at 17.5 dpc, as shown in sagittal sections (Fig. 1d); no expression was ever detected in mouse adult kidney. High magnifications (Fig. 4, d and e) demonstrated that the signal was mainly restricted to an area corresponding to the nephrogenic zone of the developing kidney cortex (21); in this area, the metanephric differentiation is characterized by a reciprocal induction between the ureteric bud, which stimulates nephrogenic mesenchymal cells to cluster and undergo conversion to a polarized epithelium (22-24), and the condensed mesenchymal cells, which cause the branching of the ureteric bud (25-27). Observations, at higher magnification, of sequential stages of nephric tubulogenesis (according to Lehtonen (28)), indicated that RET expression was mainly located in the metanephrogenic vesicles (Fig. 4, d and e).

Other Organs. We have already reported that follicular cells of the human adult thyroid do not express the RET protoconogene (29). Recently, we have been able to detect the presence of RET transcripts in the parafollicular thyroid C-cells that are considered to be of neural crest origin. Since RET is characteristically activated in human thyroid carcinomas (see "Introduction"), we have investigated its expression in the thyroid of the developing mouse: no expression was ever detected (data not shown).

The medullary region of the suprarenal gland (another tissue of neural origin) also did not hybridize to RET (Fig. 4e).

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1 The abbreviations used are: cDNA, complementary DNA; poly(A)+, polyadenylated; dpc, days postcoitum; bp, base pair(s); PCR, polymerase chain reaction.

2 N. Fabien, unpublished data.
Finally, a scattered signal was detected in the developing liver. This expression was evident at 12.5 dpc (Figs. 1, b and e; and 2b) and at 14.5 dpc (Figs. 1, c and f; and 2, c and f), and it became faintly detectable at 17.5 dpc (Fig. 1d). This result was confirmed by Northern blot analysis (data not shown). Mouse adult liver was reported to be negative for RET expression by *in situ* hybridization technique (data not shown) and by Northern blot analysis (20).

**Discussion**

**RET** encodes a receptor-type tyrosine kinase protein, whose ligand has not yet been identified. The scarce amount of information available to date seems to indicate that **RET** may exert some function in the differentiation of neural crest-derived cells. In fact, the **RET** gene has been found to be expressed in neuroendocrine neoplasias, and its mRNA levels increase during the differentiation of medullary thyroid carcinoma (16) or neuroblastoma (17) cell lines.

We have investigated the expression of the **RET** gene during mouse embryogenesis: **RET**-specific transcripts were present in several neural structures and were observable from day 9.5 of gestation. In particular, we have found **RET** expression in the mantle layer of the spinal cord, in the ventral part of the midbrain, and in cephalic ganglia from 10.2 dpc. Subsequently (12.5 dpc), spinal ganglia also showed a positive signal. It is worthwhile to mention that, in all cases,
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Fig. 2. Proto-RET expression in frontal and transverse sections (d and d') of mouse embryos of various ages (a, a' and d, d', 10.2 dpc; b, b' and c, c' and f, f', 12.5 dpc; c, c' and d, d', 14.5 dpc). Prime superscript, bright field. In a, arrowhead, cephalic ganglia. In b, arrowheads, the V cephalic ganglion. In d, arrowheads, expression in the basal plate. In e, arrowhead, spinal ganglia. In f, arrowheads, kidney and spinal ganglia. Sc, spinal cord; v, V cephalic ganglion; g, gut; k, kidney; ov, otic vesicle; sg, spinal ganglia; hb, hindbrain; bp, basal plate.
expression was restricted to small clusters of presumably specific cells. This expression was retained throughout mouse embryonic development and possibly remains throughout adult life; in fact, the spinal cord of the adult mouse has been reported to contain RET transcripts (20). RET expression was also detected in the external layer of the intestinal wall. Since the parasympathetic intestinal plexi (autonomic nervous structures) are located in this position, we strongly suggest that this positive signal has to be ascribed to these structures. Interestingly, the medullary region of the suprarenal gland, another neural crest-derived organ, appeared to be negative for RET expression. Human pheochromocytomas, which originate from the adrenal medulla, are, in contrast, consistently positive for RET expression (16). It will be interesting to investigate whether its expression in human pheochromocytomas depends on the transformed state of the tissue. Finally, we describe RET expression in the sensory organs; RET transcripts were present in the ganglion layer of the retina and in cells surrounding the olfactory epithelium.

Expression of the RET gene in cells of neural origin is very interesting considering the recent finding of an involvement of RET in the MEN2A syndrome (11), which is characterized by two types of tumors, medullary thyroid carcinomas and pheochromocytomas, both of which are considered to be of neural crest origin.

We have also detected the presence of RET transcripts in the developing liver. From 12.5 to 16.5 dpc, the liver is the major fetal hematopoietic organ. Since the level of RET expression observed diminishes from 14.5 dpc to 17.5 dpc, and furthermore, adult liver does not express RET (20), we would postulate that RET expression in the liver is restricted to cells of the hematopoietic lineage.

Apart from the liver, the only nonneural expression domain of RET was the developing kidney. In fact, we have found RET expression in the nephrogenic zone of the kidney cortex in a region which corresponds to the earliest stages of developing nephrons. It is interesting to note that recent studies described the expression of several other "neurogenic" molecules during kidney development, such as N-CAM (30), c-myc and N-myc (31), and a low affinity nerve growth factor receptor, on which metanephric tubule formation seems to be dependent (32). Moreover, expression of the nerve growth factor receptor and neurotrophin 3 in adult kidney suggests an autocrine-paracrine activity of these molecules (33).

In conclusion, RET is expressed in neural structures and in the developing kidney during a restricted time period of differentiation. According to recent theories of histospecification (34), we can postulate that this pattern of expression is possibly due to similar mechanisms of signal transduction involving RET, shared by different sets of local morphogenetic programs (34). Since RET codes for a receptor for a still unknown ligand, this information will probably be useful to direct the search for its ligand to those tissues expressing RET.

Materials and Methods

Probe Synthesis and Labeling. A portion of the mouse proto-RET cDNA has been isolated by reverse transcriptase-PCR, using as a template poly(A)+ RNA extracted from mouse total embryo and the following 5' and 3' primers: m-ret1: 5'-GCCTCCCAGAGTGTTACAGA-3' (proto-RET 2293–2313 (20))
m-ret2: 5'-GGAGATGAGGTCACCCATGGT-3' (proto-RET 2562–2542 20)

RNA amplification was performed following the method described by Kawasaki (35). Briefly, 5 μg of RNA were reverse transcribed using the antisense primer m-ret2 and were subsequently subjected to 35 cycles of PCR (94°C for 30 s, 55°C for 1 min, and 72°C for 2 min), with a thermal cycler (Perkin-Elmer-Cetus). Ten % of the reaction was analyzed on a 2% agarose gel. The amplified fragment (270 bp long) was designated ret-270, cloned in pGem3Z vector (Promega), and completely sequenced; as expected, it was found to correspond to the sequence 2293–2562 of the mouse proto-RET (20). Northern blot experiments confirmed the specificity of the probe ret-270 (data not shown). The plasmid was linearized, and transcription reactions were carried out in the presence of [35S]CTP (Amersham) and T7 polymerase (Riboprobe Kit, Promega Biotec) in order to obtain an antisense RNA probe. The same ret-270 fragment, cloned in the opposite orientation in pGem3Z, was used to obtain a sense probe, which we have used as a control of the specificity of the hybridization (data not shown). The template was then degraded with RNase-free DNase (Pharmacia), and the labeled RNA was purified over a Sephadex G-50 column. The transcripts were progressively degraded to an average length of 150 nucleotides by random alkaline hydrolysis to improve access to RNA in situ. The probe was dissolved at a working concentration of 1 × 10^6 cpm/μl in hybridization mix (36).

**Tissue Preparation.** CS7BL/6 mice were mated between 9 p.m. and 10 a.m. Day 0.5 postcoitum was assumed to begin at the middle of the day of vaginal plugging. Embryos of 10.2, 12, 14.5, and 17.5 dpc were collected and were classified according to Theiler staging (37). Pregnant female mice were killed by cervical dislocation, and embryos were collected in ice-cold phosphate-buffered saline under a dissection microscope (Zeiss SV11) and fixed in 4% paraformaldehyde in phosphate-buffered saline overnight.

**Hybridization and Washes.** Three independent embryos for each stage have been analyzed. In situ hybridization was carried out as described (36) with minor modifications. Thirty μl of the probe in hybridization mix were added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (65°C, 2× standard saline citrate-50% formamide) and treated with RNase to remove unhybridized and nonspecifically bound probe. Autoradiography was performed with Kodak NTB2 emulsion. Exposure times were between 10 and 15 days. After developing, sections were stained in 0.02% toluidine blue and mounted in DPX. Sections were
examined and photographed using a Zeiss SV11 microscope with both dark and bright field illumination.

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