The VT+ and VT− Isoforms of the Fibroblast Growth Factor Receptor Type 1 Are Differentially Expressed in the Presumptive Mesoderm of Xenopus Embryos and Differ in Their Ability to Mediate Mesoderm Formation*

(Received for publication, August 20, 1999, and in revised form, December 16, 1999)

Gary D. Paterno, Paula J. Ryan, Kenneth R. Kao, and Laura L. Gillespie‡

From the Terry Fox Cancer Research Laboratories, Division of Basic Medical Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3V6, Canada

Previously, we cloned a variant form of the type 1 fibroblast growth factor receptor (FGFR1), FGFR-VT−, from Xenopus embryos (Gillespie, L. L., Chen, G., and Paterno, G. D. (1995) J. Biol. Chem. 270, 22758–22763). This isoform differed from the reported FGFR1 sequence (FGFR-VT+) by a 2-amino acid deletion, Val423-Thr424, in the juxtamembrane region. This deletion arises from the use of an alternate 5′ splice donor site, and the activity of the VT+ and VT− forms of the FGFR1 was regulated by phosphorylation at this site. We have now investigated the expression pattern and function of these two isoforms in mesoderm formation in Xenopus embryos. Cells within the marginal zone are induced to form mesoderm during blastula stages. RNase protection analysis of blastula stage embryos revealed that the VT+ isoform was expressed throughout the embryo but that the VT− isoform was expressed almost exclusively in the marginal zone. The ratio of VT+ : VT− transcripts in the marginal zone indicated that the VT+ form was predominant throughout blastula stages except for a brief interval, coinciding with the start of zygotic transcription, when a dramatic increase in VT− expression levels was detected. This increase could be mimicked in part by treatment of animal cap explants with FGF-2. Overexpression of the VT+ isoform in Xenopus embryos resulted in development of tadpoles with severe reductions in trunk and tail structures, while embryos overexpressing the VT− isoform developed normally. A standard mesoderm induction assay revealed that a 10-fold higher concentration of FGF-2 was required to reach 50% induction in VT+ -overexpressing animal cap explants compared with those overexpressing the VT− isoform. Furthermore, little or no expression of the pan-mesodermal marker Brachyury (Xbra) was detected in VT+ -overexpressing embryos, while VT− -overexpressing embryos showed normal staining. This demonstrates that VT+ overexpression had a negative effect on mesoderm formation in vivo. These data are consistent with a model in which mesoderm formation in vivo is regulated, at least in part, by the relative expression levels of the VT+ and VT− isoforms.

* This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (to L. L. G. and G. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 709-737-6293; Fax: 709-737-7010; E-mail: lgillesp@morgan.ucs.mun.ca.

Fibroblast growth factors (FGFs) represent a family of related polypeptides known to stimulate a variety of cellular activities (reviewed in Ref. 1), including mesoderm differentiation in the Xenopus embryo (2). Their effects are mediated by high affinity transmembrane FGF receptors (FGFRs) containing intrinsic tyrosine kinase activity (reviewed in Ref. 3). Like other receptor tyrosine kinases, FGF signal transduction is initiated by ligand binding and results in activation of several well characterized intracellular signaling pathways, including the protein kinase C (PKC) and Ras/mitogen-activated protein kinase pathways (4, 5).

Four FGFR genes have been described to date, FGFR1–FGFR4, along with a number of alternately spliced variants (reviewed in Refs. 3 and 6). Previously, we cloned from Xenopus embryos an alternately spliced isoform of the FGFR1 that contains a deletion of Val423-Thr424 in the juxtamembrane region (7). We demonstrated that this site could be phosphorylated by PKC. Furthermore, in a functional assay, activation of PKC by the phorbol ester phorbol 12-myristate 13-acetate significantly reduced the activity of the VT-containing isoform (VT+) in Xenopus oocytes while having little effect on the deletion isoform (VT−). We speculated that differential expression of these two isoforms might represent an important mechanism for regulating FGF activity in the Xenopus embryo (7).

In the blastula stage Xenopus embryo, cells located in the equatorial region (marginal zone) are induced to differentiate into mesoderm in response to signal(s) from neighboring vegetal cells (reviewed in Refs. 8 and 9). Use of a dominant negative form of the FGFR1 to block FGF activity has provided evidence that FGF/FGFR signaling is required for normal development of the mesoderm (10). The current view is that FGF does not act as the initial inducing signal(s) but rather as a competence factor in the responding cells and that its activity is required for the full range of responses leading to mesoderm formation (9, 11). In this report, we examine the role of the VT+ and VT− isoforms in mesoderm formation in Xenopus and show that the two isoforms are differentially expressed in the presumptive mesoderm and that they differ in their ability to mediate mesoderm formation in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Embryos and Microinjections—Xenopus laevis were purchased from Nasco. Eggs were artificially inseminated, and embryos were cultured as described under Godsave et al. (12); embryonic stages were determined according to Nieuwkoop and Faber (13). Stage 8 blastulae were dissected into animal, vegetal, and marginal zone regions as described (14). cRNA was transcribed from FGFRSP64T constructs (7) using the

1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; PCR, polymerase chain reaction; RT-PCR, RT, reverse transcription-PCR; PKC, protein kinase C; nt, nucleotide(s).
FIG. 1. FGFR-VT– expression is spatially and temporally restricted during blastula stage. A, schematic illustrating the 261-nt probe that corresponds to the sequence of the VT+ isoform as well as the VT+ and VT– protected fragments produced by RNase digestion for the analysis shown in B. Digestion of probe:VT+ hybrids results in a 162-nt protected fragment, while digestion of probe:VT– hybrids results in digestion of the 6-nt single strand loop encoding the VT (black box), producing two protected fragments of 107 and 49 nt. The size in nt is listed below each fragment. B, RNase protection analysis of FGFR-VT+ and FGFR-VT– mRNA in Xenopus blastulae. Stage 8 blastulae were dissected into animal, vegetal, and marginal zone regions (as illustrated in the schematic diagram shown above lanes 3–7), as described (14). Total RNA was isolated from each region, and RNase protection analysis was performed using a [32P]-labeled 261-nt probe, as in Ref. 7. A representative experiment is shown. Lane 1, probe; lane 2, digested probe; lanes 3–7, protected fragments from in vitro transcribed FGFR-VT+ cRNA, in vitro transcribed FGFR-VT– cRNA, marginal zone cells (M), animal cells (A), and vegetal cells (V), respectively. The positions of the undigested probe (arrow), the VT+ protected fragments (square brackets), and VT– 107-nt protected fragment (arrow) are indicated; the 49-nt VT– protected fragment is not shown. C, RT-PCR analysis of the VT+ and VT– temporal expression pattern in marginal zone cells. Blastula stage embryos were collected at the following postfertilization times: 4.5 h (stage 7), 5.0 h (stage 8), 5.5 h (stage 8), and 6.0 h (stage 8). Marginal zones were dissected, and total RNA was isolated as in B. RT-PCR was performed as described under "Experimental Procedures," and the VT+ and VT– products, which differ in size by 6 nt, were analyzed by autoradiography on a 6% polyacrylamide, 6 M urea sequencing gel. A representative experiment is shown. Amplification products of VT+ cDNA (lane 1) and VT– cDNA (lane 2) mark the position of the VT+ and VT– products (arrows) representing the two isoforms in the marginal zone cells (lanes 3–6). Each marginal zone sample was also amplified using primers for histone (H4), as an input control, and for elongation factor 1-α, as a measure of zygotic transcription. Dev Time, development time.

FIG. 2. FGF can stimulate an increase in VT– expression. Animal cap explants were cultured with (+) or without (−) 100 ng/ml FGF-2, and at the time indicated above each lane, RNA was extracted. RT-PCR analysis was performed as in Fig. 1C. The experiment was performed on four separate occasions, and the increase in VT– expression ranged from 2- to 3-fold. A representative experiment is shown. The positions of the VT+ and VT– isoforms as well as the H4 input control are indicated.

Sp6 Ribomax system (Promega). 4.6 ml containing diethyl pyrocarbonate-treated H2O or 650 pg/ml cRNA was microinjected into stage 1 embryos. Embryos were cultured at room temperature until they reached the stage required for the assays described below.

**RNAse Protection and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Probe preparation, RNA extraction, and RNase protection analysis were performed as described (7). RT-PCR was performed as described in Ref. 15 using forward (5′-GGGCTGCTTTTGGTCCGCAAT-3′) and reverse (5′-CATTGATGAGCTGGAGTCCCC-3′) primers that bracket the VT region and generate 156- and 162-bp fragments for the FGFR-VT– gene products, respectively. Histone H4 was used as an input control with forward and reverse primers as described (16). EF1α was amplified using 5′-CGGTATCGAGGAAAGCTCCCAG-3′, as forward and reverse primers, respectively. The [32P]CTP-labeled PCR products were analyzed in the linear range for amplification, determined empirically (16) to be 19 cycles for histone H4, 22 cycles for EF1α, and 25 cycles for FGF, and visualized on a 6% polyacrylamide/6 M urea gel by autoradiography. Quantitation by densitometry was performed as in Ref. 17.

**Protein Analysis**—For expression analysis of injected FGFR cRNA, 0.5 μCi of [35S]methionine was co-injected into each embryo. Protein extraction, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis analysis were performed as described. The anti-Xenopus FGFR1 used for immunoprecipitation was a polyclonal antibody raised against a synthetic C-terminal peptide (18).

**Mesoderm Induction Assays**—Recombinant Xenopus FGF-2 was expressed and purified according to Kimelman et al. (19). Animal cap explants were excised from injected embryos and treated with FGF-2 as described (18). Explants were cultured for various times then extracted for RNA analysis or scored for mesoderm induction as in Ref. 2.

**Whole Mount in Situ Hybridization**—Whole mount in situ hybridiza-
FIG. 3. Overexpression of the FGFR-VT1 but not the VT− isoform leads to abnormal development in Xenopus. FGFR-VT1 and VT− cRNA was prepared and microinjected into fertilized eggs as described under “Experimental Procedures.” Control embryos (Con) were injected with the same volume of diethyl pyrocarbonate-treated H2O. A, embryos were left to develop for 72 h at room temperature until they reached tadpole stage and then scored for normal development (n); the percentage is based on the total number injected. A total of 150 embryos were used for each experiment, and the averages and S.D. values of 14 individual experiments are shown. B, total RNA (five embryos per treatment) was extracted at 24 h after injection and analyzed by RT-PCR (as described in the legend to Fig. 1) for VT+, VT−, and histone H4 (input control) expression levels. The positions of the VT+, VT−, and H4 PCR products are indicated. C, embryo proteins were labeled by injection of [35S]methionine, and after 24 h, total protein was extracted (100 embryos per sample), immunoprecipitated, and analyzed as described under “Experimental Procedures.” The position of FGFR1 protein is indicated.

RESULTS AND DISCUSSION

Previously, we demonstrated that the activity of the VT+ and VT− isoforms could be differentially regulated by phosphorylation (7). One obvious question is whether these two isoforms function differently in mesoderm formation in Xenopus embryos. Induction to form mesoderm takes place in the marginal zone cells of the blastula stage embryo, so we began by examining the spatial expression pattern of the VT+ and VT− isoforms during this stage of development.

Embryos were dissected into three regions representing the three germ layers: animal (presumptive ectoderm), marginal zone (presumptive mesoderm), and vegetal (presumptive endoderm). RNA protection assays of these three regions revealed that the VT− isoform was expressed predominantly in the marginal zone with very little detectable message in the animal and vegetal regions (Fig. 1B). In contrast, only small differences in VT+ expression were observed in the three regions (Fig. 1B).

Although we had previously reported that the VT+ isoform was the major form expressed throughout development and that no change in the ratio of VT+ to VT− isoforms was detected (7), the time intervals used in that study were large in order to cover a broad range of developmental stages. In light of the results in Fig. 1B, we decided to reexamine the temporal VT+ and VT− expression patterns in the marginal zone, using shorter time intervals and focusing on the stages when mesoderm induction is known to take place. Blastula stage embryos were collected at 0.5- to 5-h time intervals, and the VT+ and VT− expression levels in the marginal zone were analyzed by RT-PCR. For this purpose, we employed primers that bracket the VT region and generate VT+ and VT− products that can be distinguished on a sequencing gel. Our analysis revealed that in early blastulae (4.5 h postfertilization; late stage 7), the VT+ isoform was the major form in marginal zone cells (Fig. 1C, lane 3), consistent with our previous findings (7). However, 30 min later (stage 8), a dramatic increase in the level of VT− relative to VT+ was observed, such that the VT− isoform became predominant (Fig. 1C, lane 4). This was quickly followed by a decrease in VT− expression to initial levels, with the VT+ isoform remaining predominant at all subsequent time points examined (Fig. 1C, lanes 5 and 6).

Our previous work demonstrated that these two FGFR1 isoforms arise by alternate use of a 5′ splice donor site during transcription (7). In Xenopus embryos, however, zygotic transcription does not begin until midblastula transition (21). This occurs during stage 8, but the precise timing of this developmental event cannot be determined by either the number of cell divisions or the time after fertilization (Ref. 21; reviewed in Ref. 22). Instead, an increase in elongation factor 1-α expression, one of the earliest transcripts to be expressed by the embryonic genome (23), has been frequently used to indicate that zygotic transcription has begun. We measured elongation factor 1-α levels in our marginal zone samples and determined that expression levels began to increase as early as 5 h (Fig. 1C, lane 4). This demonstrates that the increase in VT− expression takes place concurrent with the onset of zygotic transcription.

We investigated the possibility that FGF itself was involved in this switch in expression pattern, since Musci et al. (24) and Friesel and Dawid (25) have reported that FGFR1 mRNA levels in animal cap explants were regulated by FGF. We cultured
blasts, the large increase in VT expression is clear, however, that FGF induction alone cannot account for this. While embryos overexpressing the VT isoform were examined for their ability to develop into normal tadpoles. VT-injected embryos began to develop abnormally at 11–12 h postinjection: gastrulation was incomplete, leaving an enlarged blastopore with protruding yolk plug (not shown). Despite this, embryos continued to develop, albeit abnormally. The obvious effect was a reduction in trunk and tail structures in the resulting tadpoles (Fig. 4B). This differential effect was not due to differential stability or translation of the cRNAs, since equivalent levels of VT and VT RNA (Fig. 3B) and protein (Fig. 3C) were detectable at 24 h, long past the stage when abnormalities first became apparent in the VT-overexpressing embryos.

The VT+ abnormalities were similar to those reported by Amaya et al. (10) in embryos overexpressing a dominant negative FGFR1 (XFD). These authors showed that XFD inhibited endogenous FGFR signaling and that overexpression in embryos caused severe posterior truncations resulting from a reduction in posterior mesoderm development, as well as deficiencies in gastrulation movements. This similarity suggested that the VT+ phenotype may result from a deficiency in mesoderm formation. To test this, we investigated the effect of VT+ and VT- overexpression on mesoderm formation in vitro and in vivo.

First, we measured the FGF-2 dose-response curve in explants from embryos microinjected with either WT+ or VT-cRNA and compared it with the curve for explants from H2O-injected embryos. Our results demonstrate that overexpression of the VT+ isoform reduced the level of mesoderm induction by FGF-2: overexpressing explants required a 2-fold higher concentration of FGF than control explants to achieve 50% induction (Fig. 5A). Explants overexpressing the VT- isoform, on the other hand, reached 50% induction at a 5-fold lower concentration than control explants (Fig. 5A). Thus, overexpression of VT+ decreased sensitivity to FGF, while overexpression of VT- dramatically increased sensitivity. Examination of the VT+ and VT- expression levels in these explants revealed that the sensitivity to FGF was directly correlated with the relative expression levels of the two isoforms (Fig. 5B). The ratio of VT- to VT+ in explants from VT+-injected embryos was half that of control explants (Fig. 5B, lanes 1 and 3), as was the proportion of induced VT+ explants at virtually every concentration of FGF tested (Fig. 5A). Explants from VT+-injected embryos, on the other hand, had the highest ratio of VT- to VT+ (Fig. 5B, lane 5) and the highest sensitivity to FGF (Fig. 5A). In fact, 30% of the latter were induced in the absence of added FGF-2 (Fig. 5A). This autoinduction may result from interaction of overexpressed VT- with maternally derived FGF present in animal cap explants (9).

From the results of the dose-response curves, one would predict that overexpressing the VT+ isoform would result in a decreased level of mesoderm formation in vitro. To test this hypothesis, we examined the expression of an early panmesodermal marker Xbra, which is normally expressed throughout the presumptive mesoderm of the early gastrula stage embryo (26). Furthermore, FGFR signaling is required for Xbra expression (reviewed in Ref. 27). Staining for Xbra was not detectable or was very faint in VT+-injected embryos (Fig. 6A). VT- injected embryos, on the other hand, expressed levels similar to those of H2O-injected controls (Fig. 6A). Expression of chordin, a dorsal lip marker involved in neural development (28, 29), was unaffected in VT+ embryos (Fig. 6B), demonstrating that lack of Xbra expression in VT+-injected embryos was not the result of a nonspecific inhibition of transcription. Thus, the VT+ isoform can function to negatively regulate mesoderm formation in Xenopus embryos.

In this study, we have shown that while the VT+ isoform was predominant in the presumptive mesoderm during most of

![Fig. 4. FGFR-VT+ overexpression effects on embryonic development.](http://www.jbc.org/ Downloaded from http://www.jbc.org/ on July 26, 2018)
blastula stages, a brief but dramatic increase in VT− mRNA expression occurred during this time period (Fig. 1C), coinciding temporally with mesoderm induction in vivo (9). It would be important to determine how this transient burst in VT− expression affects known FGF signaling pathways in the embryo, such as mitogen-activated protein kinase and phospholipase Cγ. Labonne and Whitman (30) reported that mitogen-activated protein kinase activity was first detectable during midblastula and peaked by midgastrula. Phosphorylation of phospholipase Cγ, on the other hand, occurred over a period of 1.5 h during early blastula to midblastula stages (18). While activation of these pathways persisted over a longer time interval than that reported in this study, it is possible that the VT− protein has a longer half-life than its cognate mRNA. We are attempting to generate antibodies that can distinguish between the VT+ and VT− isoforms and would enable investigation of isoform-specific signaling pathways.

We have also shown that the VT+ and VT− isoforms differ significantly in their ability to mediate mesoderm induction. This difference in isoform function could be due to PKC activity. PKC is known to be activated during mesoderm induction by FGF (4), and activated PKC caused a substantial reduction in FGF signaling through the VT1 isoform (7). The differential activity of these two FGFR isoforms means that two tissues in the embryo, one expressing predominantly VT2 and the other predominantly VT1, could be exposed to the same, low concentration of FGF, and only the tissue expressing high levels of VT− would be induced. Our data suggest then that mesoderm formation in vivo is dependent not only on the local concentration of FGF but also on the relative expression levels of the VT+ and VT− isoforms in the responding tissue. This hypothesis provides a possible mechanism for

**Fig. 5.** The VT+ and VT− isoforms have differential effects on mesoderm induction in vitro. **A**, animal cap explants (30 per sample) from stage 8 blastulae of H2O-injected (○), VT−-injected (■), or VT+ -injected (▲) embryos were cultured in the presence of the indicated concentration of FGF-2 for 72 h. Mesoderm induction was scored by morphological criteria as described in Ref. 2. Values from 10 individual experiments were plotted; the bars represent S.E. **B**, total RNA was extracted from stage 8 animal cap explants (five per sample) from injected embryos and analyzed by RT-PCR for VT+, VT−, and histone H4 expression levels, as described in the legend to Fig. 1C. A representative experiment is shown. The positions of the VT+, VT−, and H4 PCR products are indicated. Quantitation by densitometry of the VT+ and VT− expression levels in each sample was performed as described in Ref. 17, and the ratio of VT− to VT+ obtained from these measurements is indicated below the appropriate lane.
restricting mesoderm induction to marginal zone cells, although all cells in the blastula stage embryo have been shown to express FGFRs (11, 14). While much of the work on mesoderm induction has focused on analysis of potential inducers, such as eFGF, activin, and Vg1 (reviewed in Refs. 9, 31, and 32), our results demonstrate that regulated expression of receptors and receptor isoforms also plays a critical role.

Acknowledgments—We thank Corinne Mercer for expert technical assistance.

REFERENCES
1. Szebenyi, G., and Fallon, J. F. (1999) Int. Rev. Cytol. 185, 45–106
2. Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987) Nature 236, 197–200
3. Klint, P., and Claesson-Welsh, L. (1999) Int. Rev. Cytol. 185, 45–106
4. Gillespie, L. L., Paterno, G. D., Mahadevan, L. C., and Slack, J. M. W. (1992) Mech. Dev. 38, 99–108
5. Huang, J., Mohammadi, M., Rodrigues, G. A., and Schlessinger, J. (1995) J. Biol. Chem. 270, 5065–5072
6. Friesel, R. E., and Maciag, T. (1995) FASEB J. 9, 919–925
7. Gillespie, L. L., Chen, G., and Paterno, G. D. (1995) J. Biol. Chem. 270, 22758–22763
8. Kimelman, D., Christian, J. L., and Moon, R. T. (1992) Development 116, 1–9
9. Isaacs, H. V. (1997) Cell. Mol. Life Sci. 53, 350–361
10. Amaya, E., Musci, T. J., and Kirschner, M. W. (1995) Cell 66, 101–111
11. Newport, J., and Kirschner, M. (1982) Cell 30, 675–686
12. Masui, Y., and Wang, P. (1998) Biol. Cell 90, 537–548
13. Krieg, P. A., Varnum, S. M., Wormington, W. M., and Melton, D. A. (1989) Dev. Biol. 133, 93–100
14. Musci, T. J., Amaya, E., and Kirschner, M. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8365–8369
15. Friesel, R., and Dawid, I. (1991) Mol. Cell. Biol. 11, 2481–2488
16. Smith, J. C., Price, B. M., Green, J. B., Weigel, D., and Herrmann, B. G. (1991) Cell 67, 79–87
17. McClung, C., and Smith, J. C. (1995) Curr. Biol. 5, 62–67
18. Sasai, Y., Lu B, Steinbeisser, H., Geissert, D., Gont, L. K., De Robertis, E. M. (1994) Cell 79, 779–790
19. Sasai, Y., Lu B, Steinbeisser, H., and De Robertis, E. M. (1995) Nature 376, 333–336
20. Labonne, C., and Whitman, M. (1997) Dev. Biol. 183, 9–20
21. McDowell, N., and Gurdon, J. B. (1999) Semin. Cell Dev. Biol. 10, 311–317
22. Joseph, E. M., and Melton, D. A. (1998) Development 125, 2677–2685

FIG. 6. Overexpression of the VT+ isoform reduces mesoderm formation in vivo. Fertilized eggs were injected as described in the legend to Fig. 2 and cultured until they reached gastrula stage (stage 10.5). Whole mount in situ hybridization was performed as described under “Experimental Procedures,” using a probe for either Xbra (A) or chordin (B). The white arrows indicate regions of expression in the control embryos. Scale bar, 0.25 mm.
The VT+ and VT− Isoforms of the Fibroblast Growth Factor Receptor Type 1 Are Differentially Expressed in the Presumptive Mesoderm of *Xenopus* Embryos and Differ in Their Ability to Mediate Mesoderm Formation

Gary D. Paterno, Paula J. Ryan, Kenneth R. Kao and Laura L. Gillespie

*J. Biol. Chem.* 2000, 275:9581-9586.
doi: 10.1074/jbc.275.13.9581

Access the most updated version of this article at [http://www.jbc.org/content/275/13/9581](http://www.jbc.org/content/275/13/9581)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/275/13/9581.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 31 references, 13 of which can be accessed free at [http://www.jbc.org/content/275/13/9581.full.html#ref-list-1](http://www.jbc.org/content/275/13/9581.full.html#ref-list-1)