A cDNA clone, predicted to encode a variant form of the type 1 fibroblast growth factor receptor (FGFR1) containing a dipeptide Val-Thr (VT) deletion at amino acid positions 423 and 424 located within the juxtamembrane region, was isolated from a Xenopus embryo (stage 8 blastula) library. Sequence analysis of genomic DNA encoding a portion of the FGFR1 juxtamembrane region demonstrated that this variant form arises from use of an alternative 5' splice donor site. RNAse protection analysis revealed that both VT- and VT+ forms of the FGFR1 were expressed throughout embryonic development, the VT+ being the major form. Amino acid position 424 is located within a consensus sequence for phosphorylation by a number of Ser/Thr kinases. We demonstrate that a VT+ peptide was specifically phosphorylated by protein kinase C (PKC) in vitro, but not by protein kinase A (PKA). A VT- peptide, on the other hand, was not a substrate for either enzyme. Phosphorylation levels of in vitro synthesized FGFR-VT+ protein by PKC were twice that of FGFR-VT- protein. In a functional assay, Xenopus oocytes expressing FGFR-VT+ or FGFR-VT- protein were equally able to mobilize intracellular Ca2+ in response to basic fibroblast growth factor (bFGF). However, pretreatment with phorbol 12-myristate 13-acetate significantly reduced this mobilization in oocytes expressing FGFR-VT+ while having little effect on oocytes expressing FGFR-VT-. These findings demonstrate that alternative splicing of Val423-Thr424 generates isoforms which differ in their ability to be regulated by phosphorylation and thus represents an important mechanism for regulating FGFR activity.

Fibroblast growth factors (FGFs) play a role in a number of cellular responses, including mitogenesis, differentiation, angiogenesis, and transformation (reviewed in Ref. 1). The family of FGFs consists of nine distinct members (2), related by amino acid sequence and their ability to bind heparin, that mediate the interactions of FGFs with extracellular matrix, cell-cell, and cell-matrix interactions. FGF binding to the extracellular domain of FGFR results in receptor activation through dimerization and autophosphorylation. The activated receptor can then bind and phosphorylate a number of intracellular substrates, thus altering their catalytic activity and initiating intracellular signal transduction cascades (reviewed in Ref. 3).

FGFRs are encoded by four genes whose transcripts are alternatively spliced to produce a number of variant forms (reviewed in Ref. 3). Each of the four FGFR types is capable of binding more than one member of the FGF family, the ligand binding specificity being determined not only by the receptor type but by the splicing form. For example, alternative splicing of exons encoding the COOH-terminal half of the third Ig domain of FGFR2 leads to production of FGFRs that no longer recognize FGF-7 (4). In addition, Shi et al. (5) has described an alternatively spliced FGFR isoform that encodes a truncated, kinase-defective receptor which can heterodimerize with full-length FGFRs and reduce tyrosine kinase activity. Clearly, alternative splicing represents an important mechanism by which FGFR activity can be regulated.

FGFs induce differentiation of mesoderm in Xenopus embryonic tissue (6–8), and FGFR signaling has been shown to be required for this developmental event (9). Mesoderm induction during embryonic development is precisely regulated in time and space to produce a distinct pattern of mesodermal tissues. In order to investigate the molecular mechanisms involved in regulating this complex developmental process, it is important initially to determine which FGFR genes are involved and how FGFR signaling is regulated. Evidence to date suggests that FGFR1 is likely to be important, since both mRNA (10, 11) and protein (12) for FGFR1 are present in Xenopus blastulae, the stage during which mesoderm induction takes place in the embryo. In addition, we have demonstrated that FGFR1 was activated during GGF-induced mesoderm differentiation in Xenopus (12). Consequently, we decided to focus on the FGFR1 gene and determine which FGFR1 isoforms may be important for mesoderm induction.

Two reports have described FGFR1s cloned from Xenopus, however, neither isolated cDNA from embryos. Musci et al. (10) cloned a three-Ig domain FGFR1 from an oocyte library, whereas Friesel and Dawid (11) cloned both two- and three-Ig forms from a Xenopus cell line (XTC). Accordingly, we prepared and screened a cDNA library from Xenopus blastulae for FGFR1 species. This paper describes a Xenopus FGFR1 isoform which differs in its ability to be regulated by protein kinase C (PKC).
EXPERIMENTAL PROCEDURES

Materials—Xenopus laevis were purchased from Nasco and maintained as described in Ref. 13. Eggs were artificially inseminated, the jelly coats removed, and the embryos cultured as described in Godsave et al. (14). Synthetic peptides corresponding to FGFR-VT + (IPLR-RQTVTGSDSS) and FGFR-VT - (IPLRRQVSGDSS) were purchased from the Alberta Peptide Institute (Edmonton, Alberta). Recombinant Xenopus βGF was expressed and purified according to Kimelman et al. (15). Oocytes were harvested at 2-3°C at 20°C. The anti-FGFR1 used for immunoprecipitation in this study was a polyclonal antibody raised against a synthetic COOH-terminal peptide (12).

cDNA Cloning and Sequencing of an FGFR1 Isoform from Xenopus Embryos—A cDNA library was constructed from mRNA isolated from stage 8 Xenopus blastulæ using the λ ZAP II kit (Stratagene) as directed. The library was screened using a 400-bp fragment of the Xenopus FGFR1 cDNA previously cloned by PCR.2 This 400-bp fragment was amplified from Xenopus stage 17 first strand cDNA using oligonucleotide primers within the FGFR1 tyrosine kinase domain. The 400-bp amplification product was then cloned in the EcoRI site of Bluescript KS+ and sequenced on both strands, verifying its identity as a 1.2-kb genomic fragment containing the VT region (see Fig. 1 for location of primers), for 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. PCR products were separated on 1% agarose, the 1.2-kb band cut out and the DNA extracted from the agarose gel with Qiagen (Qiagen) according to the manufacturer’s directions. Sequencing was performed as above, using the PCR primers.

RNase Protection Analysis—RNA was extracted and purified from whole embryos using the LiCl/urea protocol described in Goldin (18). RNase protections were performed as in Paterno et al. (7). The RNA antisense probe was prepared from a BstEII-HgiI cDNA fragment of XFGFR-A2 (gift from Dr. Robert Friesel, American Red Cross) cloned into the EcoRI site of Bluescript KS+ plasmid (Stratagene). A transcription from the T7 promoter yielded a 261-base probe which protected a fragment of 162 bases for the VT region (Fig. 2). By comparing the genomic DNA by alternative splicing, we sequenced a genomic fragment containing the VT region (Fig. 1), which encodes most of the open reading frame (amino acids 3–789) of the FGFR-VT- cDNA, into the same sites of a pcDNAIneoplasmid mammalian expression vector containing the FGFR-A2 cDNA. The FGFR-A2pcDNAIneoplasmid plasmid contains the coding region of an FGFR1 isoform lacking the first Ig domain (I1) inserted into the BamHI site of pcDNAIneoplasmid (Invitrogen). The FGFR-VT receptor construct was generated by subcloning a BstEII-Ral cDNA fragment (Fig. 1) of the FGFR-A2pcDNAIneoplasmid encoding the transmembrane and intracellular domains, into the same sites of FGFR-VT- pcDNAIneoplasmid. The FGFRSP64T constructs used for expression in Xenopus oocytes were generated by subcloning a BamHI fragment, containing the entire FGFR coding region, from the FGFR-VT- pcDNAIneoplasmid or FGFR-VT+ pcDNAIneoplasmid constructs in the BstEII site of the SP6T vector (20).

Oocyte Injections and Protein Analysis—cDNA was transcribed using the SP6 Ribomax system (Promega) from the FGFRSP64T constructs (described above) that had been linearized with XbaI. 4.6 nl containing 500 pg of cRNA was microinjected into stage VI Xenopus oocytes prepared and cultured as described in Amaya et al. (9). Injected oocytes were metabolically labeled by culturing for 24 h at 22 °C in medium containing 1 mc/ml [35S]methionine (1000 Ci/mmol; DuPont NEN). After extensive washing, the oocytes were solubilized and the FGFR immunoprecipitated as described in Ryan and Gillespie (12). The immunoprecipitates were analyzed by 8% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS

Mesoderm induction takes place during blastula stages of Xenopus development. In our efforts to understand the role of the FGFR in this induction event, we set out to identify FGFR isoforms that are expressed during blastula stages. We prepared a cDNA library from mid-blastula (stage 8) Xenopus embryos and screened it for FGFR1. A positive plaque containing a 3.8-kb insert was purified and sequenced. The cDNA consisted of a open reading frame of 2.4 kb bracketed by an HhaI site of pcDNAIneoplasmid (Invitrogen). The FGFR-A2pcDNAIneoplasmid contains the same sites of a pcDNAIneoplasmid mammalian expression vector containing the FGFR-A2 cDNA. The FGFR-A2pcDNAIneoplasmid plasmid contains the coding region of an FGFR1 isoform lacking the first Ig domain (I1) inserted into the BamHI site of pcDNAIneoplasmid (Invitrogen). The FGFR-VT receptor construct was generated by subcloning a BstEII-Ral cDNA fragment (Fig. 1) of the FGFR-A2pcDNAIneoplasmid encoding the transmembrane and intracellular domains, into the same sites of FGFR-VT- pcDNAIneoplasmid. The FGFRSP64T constructs used for expression in Xenopus oocytes were generated by subcloning a BamHI fragment, containing the entire FGFR coding region, from the FGFR-VT- pcDNAIneoplasmid or FGFR-VT+ pcDNAIneoplasmid constructs in the BstEII site of the SP6T vector (20).

To investigate the possibility that this deletion is generated by alternative splicing, we sequenced a genomic fragment containing the VT region (Fig. 2). By comparing the genomic DNA sequence to the cDNA sequence, the amino acid sequence and 5’ and 3’ consensus splice sequences (5’: (C/A)AG/GU (G/A)AG; 3’: (U/C)U,NCA/G) reviewed in Refs. 21 and 22, we were able to examine a number of possible origins for these two isoforms, including alternative exons and alternative 5’ or 3’ splice sites. We concluded that the most likely mechanism for the production of the two receptor forms is the use of alternative 5’ splice donor sites (Fig. 2). Splicing to produce FGFR-VT – would make use of an excellent consensus 5’ splice donor site, whereas the splice site to produce FGFR-A2 or XFGFR lacks three of the eight consensus nucleotides. Therefore, one would predict that the major splicing product would be FGFR-VT –.
Interestingly, RNase protection of total RNA from embryos at various developmental stages revealed that in fact VT\textsubscript{1} mRNA was the major form (Fig. 3). In addition, there appeared to be little change in ratio of the VT\textsubscript{1}/VT\textsubscript{2} isoforms at the developmental stages examined. 

A similar deletion of Thr-Val was reported for a FGFR1 cDNA cloned from a human hepatoma cell line (23). These authors suggested that this location may represent a possible site for phosphorylation by a Ser/Thr kinase. Comparison with consensus sequences for various Ser/Thr kinases (24) revealed that amino acid position 424 was located within a consensus sequence for phosphorylation by PKC and PKA; in FGFR-VT\textsubscript{2}, a Ser is in this position, whereas in the VT\textsubscript{1} isoform, a Thr is in this location. We decided to examine whether this Ser or Thr could be phosphorylated by PKC or PKA. Two peptides, corresponding to amino acids 417–428 of FGFR-VT\textsubscript{2} or amino acids 417–428 of FGFR-VT\textsubscript{1}, were phosphorylated in vitro by PKC and PKA.

**Fig. 1.** Amino acid comparison of Xenopus FGFR\textsubscript{1}s. The amino acid sequence of our clone, FGFR-VT\textsubscript{2}, was aligned with the FGFR1 (XFGFR) clone by Musci et al. (10) and that reported by Friesel and Dawid (11) (XFGFR-A2). Only amino acid changes are listed for XFGFR and XFGFR-A2, and dashes indicate amino acid deletions. The transmembrane domain is underlined and the position of the two PCR primers used to amplify the genomic fragment in Fig. 2 are indicated by half-arrows. Restriction enzyme sites used for plasmid construction are indicated by arrows on the corresponding amino acid sequence.

**Fig. 2.** Genomic fragment spanning the VT region. Partial sequence of the genomic fragment, amplified by PCR using primers (shown in Fig. 1) that bracket the VT region, is shown with the predicted amino acid sequence listed underneath. Predicted exon and intron sequences are shown in uppercase- and lowercase, respectively, with the sequence encoding Val\textsuperscript{423} and Thr\textsuperscript{424} shown in bold. Alternative 5′ splice donor sites used to generate the VT\textsubscript{2} or VT\textsubscript{1} isoforms are indicated by arrows.
domains and Val423-Thr424, thus differing from FGFR-VT. To this purpose, we constructed an FGFR1 that contains 3 Ig domains and only by the presence of Val 423-Thr424. We refer to this construct as FGFR-VT+. The substrates in this PKC assay were FGFR-VT or FGFR-VT+ protein isolated by immunoprecipitation from in vitro transcription/translation reactions. Both proteins were phosphorylated by PKC (Fig. 4B); however, twice as much [32P]PO₄ was incorporated into FGFR-VT+. This demonstrates that the full-length proteins were substrates for PKC and that presence of the VT increased the degree of phosphorylation. The fact that FGFR-VT+ protein, but not the peptide, was phosphorylated by PKC suggests that there are additional phosphorylation sites in the protein.

One of the questions that remained was whether differential phosphorylation of these two isoforms by PKC affects receptor function. To examine this question, we measured mobilization of intracellular Ca²⁺ stimulated by FGF in oocytes expressing either form of the FGFR1. Mobilization of intracellular Ca²⁺, as measured by ⁴⁵Ca²⁺ efflux from oocytes, is commonly employed as a functional assay of FGFR activity (9, 10, 25). Xenopus oocytes were microinjected with H₂O (control) or mRNA encoding either FGFR-VT or FGFR-VT+. After a 24-h incubation period to allow for expression of FGFR protein, oocytes were loaded with ⁴⁵Ca²⁺ in calcium-free medium. ⁴⁵Ca²⁺ release into the medium was measured in response to addition of 100 ng/ml Xenopus bFGF (XbFGF) to oocytes; parallel samples were pretreated for 20 min with 250 nM PMA, a phorbol ester that activates PKC, before addition of XbFGF. H₂O-injected oocytes showed no response to XbFGF (Fig. 5A). Oocytes expressing either FGFR isoform exhibited a similar response to XbFGF treatment alone but not when stimulated with XbFGF in the presence of PMA (Fig. 5, B and C). Pretreatment with PMA resulted in a slight reduction in the magnitude of the ⁴⁵Ca²⁺ release by oocytes expressing FGFR-VT+ (Fig. 5B), whereas the ⁴⁵Ca²⁺ release by oocytes expressing FGFR-VT− was untreated.
FGFR1 Splice Variant

DISCUSSION

FGFs are known to mediate a number of diverse and complex cellular responses (reviewed in Ref. 1). The existence of nine different FGFR genes with a number of alternative spliced forms may in part explain the pleiotropic effects of the FGFR family. Thus, it will be important to investigate the biological activity of the different FGFR gene products, in response to different FGF members, in order to elucidate the signal transduction pathways leading to these varied responses. We have isolated an FGFR1 cDNA from Xenopus blastulae that differs from previously cloned Xenopus FGFRs by a Val-Thr deletion in the juxtamembrane region. Although similar isoforms have been cloned from a human hepatoma cell line (23) and from rat brain (26), their biological activity was not characterized. We show here that Thr

was significantly reduced (Fig. 5C). To verify that, in these experiments, the oocytes expressed equal amounts of FGFRVT- or VT+ protein, FGFRs were immunoprecipitated from oocytes labeled with [35S]methionine and the precipitates analyzed by SDS-polyacrylamide gel electrophoresis. The inset in Fig. 5C shows that there was no difference in the synthesis of VT- and VT+ FGFR proteins.

FIG. 5. FGF-stimulated 45Ca

release from oocytes expressing FGFR-VT- or FGFR-VT+ protein. Xenopus oocytes were microinjected with H2O or cDNA encoding either FGFR-VT- or FGFR-VT+ and labeled with [35S]methionine and the precipitates analyzed by SDS-polyacrylamide gel electrophoresis. The inset in Fig. 5C shows that there was no difference in the synthesis of VT- and VT+ FGFR proteins.
embryo. We are currently investigating which FGFR1 isoforms are expressed in different tissues of the Xenopus blastula and determining the biological role of these two isoforms in the developing embryo.

Acknowledgment—We thank Langtuo Deng for technical assistance.

REFERENCES
1. Baird, A., and Klagsburn, M. (1991) The Fibroblast Growth Factor Family, The New York Academy of Sciences, New York
2. Miyamoto, M., Naruo, K.-I., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993) Mol. Cell. Biol. 13, 4251–4259
3. Jaye, M., Schlessinger, J., and Dionne, C. A. (1992) Biochim. Biophys. Acta 1135, 185–199
4. Shi, E., Kan, M., Xu, J., Wang, F., Hou, J., and McKeehan, W. L. (1993) Mol. Cell. Biol. 13, 3907–3918
5. Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987) Nature 326, 197–200
6. Paterno, G. D., Gillespie, L. L., Dixon, M. S., Slack, J. M. W., and Heath, J. K. (1989) Development (Camb.) 114, 711–720
7. Amaya, E., Musci, T. J., and Kirschner, M. W. (1991) Cell 66, 257–270
8. Gillespie, L. L., Paterno, G. D., Mahadevan, L. C., and Slack, J. M. W. (1992) Development (Camb.) 106, 203–208
Cloning of a Fibroblast Growth Factor Receptor 1 Splice Variant from *Xenopus* Embryos That Lacks a Protein Kinase C Site Important for the Regulation of Receptor Activity
Laura L. Gillespie, Gang Chen and Gary D. Paterno

*J. Biol. Chem.* 1995, 270:22758-22763.
doi: 10.1074/jbc.270.39.22758

Access the most updated version of this article at [http://www.jbc.org/content/270/39/22758](http://www.jbc.org/content/270/39/22758)

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

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 16 of which can be accessed free at [http://www.jbc.org/content/270/39/22758.full.html#ref-list-1](http://www.jbc.org/content/270/39/22758.full.html#ref-list-1)