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The Protein Tyrosine Kinases of the Sea Urchin *Anthocidaris crassispina*

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ABSTRACT—In order to know the function of protein tyrosine kinases (PTKs) in the development of sea urchin embryos, we performed reverse transcription-polymerase chain reaction (RT-PCR) to obtain partial cDNA fragments for PTK genes using primers to highly conserved regions of the PTK family. A total of seven PTK sequences were identified, two of which represented receptor PTK (RTK1 and RTK2), and five of which were non-receptor PTKs (NRTK1-5). RTK1 was highly similar to FGF receptor and Ret kinase, while RTK2 showed features of the insulin receptor family. NRTK1 and 2 belonged to the Src family and could be involved in egg activation at fertilization. NRTK3 showed the features of the Btk family kinases, while NRTK4 seemed to be a member of the Syk/ZAP70 family. NRTK5 is the Csk-type kinase of the sea urchin, which is known to negatively regulate the Src family kinases. RTK1 was not detected in unfertilized eggs and was activated after blastula stage. All the other PTK genes were expressed both maternally in unfertilized eggs and zygotically after fertilization, though each gene showed distinct temporal patterns.

INTRODUCTION

Protein tyrosine kinases (PTKs), first identified as transforming gene products, have been implicated in various cellular function such as cell growth, movement, and differentiation (Hunter, 1987). Many growth factors regulating embryogenesis and organogenesis are now known to mediate the signal by specifically activating their own receptor PTKs. Various animal systems and the genetic approaches have revealed the roles of some receptor PTKs, such as Let-23 in vulval formation of nematodes, DER/faint little ball/torpedo in oogenesis/embryogenesis, and Sevenless in the formation of eyes in *Drosophila* (Govind and Steward, 1991; Plyte, 1994). In mammalian systems, c-Kit turned out to be the PTK-type receptor for Steel factor and is required for the normal development of germ cells, pigment cells and hematopoietic cells (Morrison-Graham and Takahashi, 1993). Non-receptor PTKs are well understood with regard to the differentiation/activation of lymphocytes and neurogenesis, which have been confirmed by gene targeting experiments recently (Lowell and Soriano, 1996). The role of D-Abl in the development of the *Drosophila* nervous system was also suggested using genetic approaches (Hoffmann, 1991).

In higher vertebrates, however, it is a challenge to understand the roles of PTKs because of the complexity of morphology and the redundancy of the genes. Sea urchins and other echinoderms are deuterostomes, together with chordates and hemichordates, so that elucidation of the PTK functions in sea urchin development may greatly help understand the evolution of deuterostomes. The presence and the developmental changes of some PTK activities in sea urchin embryos were reported before (Dasgupta and Garbers, 1983). Furthermore, several groups have suggested a role for PTKs in the activation of sea urchin eggs at fertilization (Satoh and Garbers, 1985; Peaucellier *et al.*, 1988; Ciapa and Epel, 1991; Abassi and Foltz, 1994; Kinsey, 1996). To gain more insight into the role of PTKs in sea urchin embryogenesis, we tried to obtain cDNA fragments for various PTKs employing the RT-PCR method and cloned PCR fragments for two receptor PTKs and five non-receptor PTKs. All of them were shown to be expressed in temporally specific manners, suggesting their involvement in the early development of sea urchin embryos.

MATERIALS AND METHODS

Preparation of embryos

Eggs and sperm of the sea urchin *Anthocidaris crassispina* were obtained and embryos were cultured as described before (Yamasu *et al.*, 1995).

Preparation of RNA

Total RNA was prepared from embryos of various developmental stages by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987).

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Primers for the RT-PCR

Primers specific to PTKs were synthesized according to Wilks (Wilks, 1989) with slight modifications as follows: CGCTGCAGTGC-AC(A/C)GNGA(C/T)(C/T)T (PTK I; sense strand for VHRDL plus *Pst*I linker) and CGGAATTCC(G/A)TA(A/G)GACCANAC(A/G)TC (PTKII; antisense strand for DVWSYG plus *Eco*RI linker).

Amplification of PTK cDNAs by nested RT-PCR

Five μg of total RNA was reversed-transcribed with 2.5 pmole $(dT)_{17}$-Ri-Ro (Frohman, 1990), 200 units MMLV reverse transcriptase (Gibco BRL, Gaithersburg, USA), and 10 units of RNase inhibitor (Toyobo, Osaka, Japan) at 37°C for 2 hr. Aliquot of the cDNA obtained was first amplified using 25 pmole each of PTKI and Ri adapter primers; then an aliquot from the first PCR was further amplified in a nested manner using 100 pmoles each of PTKI and PTKII primers. The amplification was performed by 30 thermal cycles as follows; 94°C for 45 sec, 40°C for 30 sec, 72°C for 3 min (first PCR) or 1 min (second PCR). After digestion with *Pst*I and *Eco*RI, the products were purified by gel electrophoresis and cloned into pUC19 vector.

DNA sequencing

The nucleotide sequence was determined by the dideoxy sequencing method with 7-deaza Sequenase™ DNA sequencing Kit (version 2.0; USB, Cleveland, USA). In order to efficiently screen different PTK cDNAs among PCR product clones obtained, a dideoxy reaction of a single base was conducted and run on sequence gels first, and the clones were classified based on the ladder pattern for the single base. Then the representative clone of each group was fully sequenced from both ends of the insert. Search for genes or proteins similar to the PCR products was conducted by BLAST (GenBank, Release 94.0; Swiss-Prot, Release 32.0).

RNA-blot analysis

Total RNA from embryos of various stages was subjected to electrophoresis on formaldehyde/agarose gels (10 μg/lane), blotted and fixed onto nylon membranes (HyBond N; Amersham, Little Chalfont, UK). Blotted membranes were hybridized with probes which had been labeled with [α-^{32}P]dCTP and washed as follows: twice with 2 × SSC, 0.1% SDS and once with 1 × SSC, 0.1% SDS, each for 10 min at room temperature, and finally twice with 0.2 × SSC, 0.1% SDS at 68°C, each for 15 min. The expression was analyzed by autoradiography or a Molecular Imager (Bio-Rad, Hercules, USA).

RESULTS

Amplification of cDNA for PTK genes by RT-PCR

Catalytic domains of protein kinases can be further divided into eleven subdomains which are especially highly conserved among various kinase species and during the course of evolution (Hanks *et al.*, 1988). The region between subdomain IV and IX was amplified by RT-PCR using degenerate primers (PTKI and PTKII) and total RNA from unfertilized eggs, cleavage-stage embryos, and late gastrulae as templates. PCR products of about 200 bp, which was expected from the PTK consensus sequence, were cloned into pUC19 plasmid. One hundred and one recombinant plasmids were selected at random in total, and were subjected to sequence analysis.

Sequence analysis of the PCR products

The sequences of the PCR products were compared with those in the database (BLAST search), and it turned out that 42 clones were from seven PTK genes (Fig. 1), 26 clones from seven serine/threonine kinase (STK) genes (unpublished data), and the rest were not from kinase genes. Among the seven PTK gene fragments, two appeared to encode receptor PTKs and five non-receptor PTKs, as will be described below, so that they were designated as *RTK1*, *RTK2*, *NRTK1*, *NRTK2*, *NRTK3*, *NRTK4*, and *NRTK5*, respectively (RTK for receptor tyrosine kinase, and NRTK for non-receptor tyrosine kinase).

The alignment of the products of these seven PCR fragments with the consensus sequence of tyrosine kinases (Fig. 1; Wilks, 1989) showed that all of these actually represent

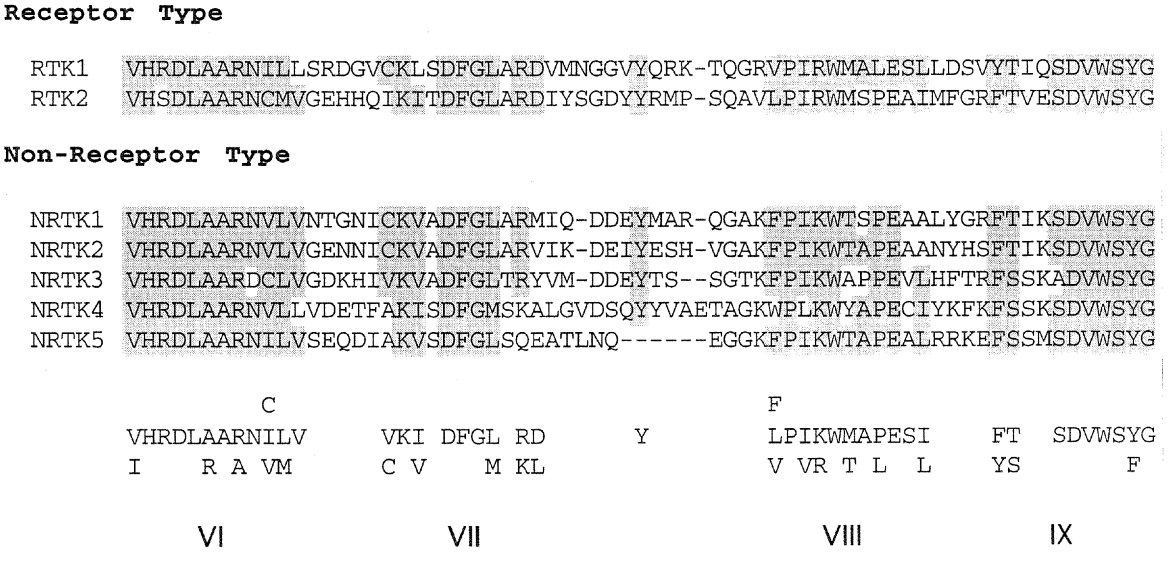

Fig. 1. Alignment of the amino acid sequences of sea urchin protein tyrosine kinases deduced from the PCR fragment. Shaded residues represent the amino acids which is consistent with the consensus sequence of the protein tyrosine kinases shown in the bottom (Hanks *et al.*, 1988). Roman numerals show the highly conserved subdomains in the kinase domain.

PTK genes. DFG in subdomain VII, which is highly conserved in the PTK family, is actually seen in the seven gene products obtained here. In subdomain VI, DLKPEN is the strong indicator of STKs, whereas the PTK consensus is either DLRAAN or DLAARN (Superti-Furga and Courtneidge, 1995). Basically all the seven fragments satisfy this requirement (Fig. 1). Another indicator region is in subdomain VIII, where the PTK consensus is P(I/V)(K/R)W(T/M)APE while the STK consensus is G(T/S)XX(Y/F)XAPE (Hanks *et al*., 1988). In the former case, the amino acid following tryptophan (W) is usually threonine (T) in non-receptor PTKs while it is methionine (M) in receptor PTKs. Here, again, the seven fragments clearly showed the feature of PTKs. Furthermore, RTK1 and RTK2 were regarded as receptor-PTKs, whereas NRTK1-5 seemed to be non-receptor PTKs.

For identification, we compared each of the deduced partial amino acid sequences of the seven PTKs to those of four representative receptor-type PTKs as well as eight human PTKs each of which represents one of the eight subfamilies of non-receptor PTKs (Table 1). As was expected, RTK1 and 2 showed higher identities to receptor-type PTKs, while NRTK1-5 were closer to non-receptor PTKs.

RTK1 showed the highest homology to FGF receptor-type PTK, and in fact, BLAST search indicated 61% identity to human FGF receptor 3, 60% identity to human c-Ret, and 58% identity to CSF-1. Meanwhile, RTK2 seemed to belong to the insulin receptor family, and, consistent with this assignment, the closest PTKs were the receptors for neurotrophic factors (60% to mouse TrkB), Cak precursor (58% to human Cak precursor), and insulin-like growth factor 1 receptor (57% to human IGF1 receptor precursor). However, since the identities of the two RTKs to different types of receptor-type PTKs were as small as between 46 and 62%, it is difficult to exactly predict their ligands at present.

Meanwhile, each of the subfamilies of non-receptor PTKs seemed relatively highly conserved during the evolution, and it was rather easy to identify each of the five NRTKs (Table 1). Actually, the closest human PTKs turned out to have relatively high identities (mostly from 70 to 80%) to sea urchin NRTKs with other subfamilies of human PTKs showing as low as approximately 60% or less identities.

NRTK1 and NRTK2 were close to each other with an identity of 78%, and both showed high similarity to the Src family. Interestingly, NRTK1 was most similar to the Src-family kinase of *Hydra* (Stk, 79%), while the PTKs closest to NRTK2 reported to date were Srk1-4 of *Spongilla* (73%-82%) and Stk of *Hydra* (79%). What is interesting with regard to both NRTK1 and 2 is the sequence AAR in subdomain VI which is RAA for all the Src family PTKs reported besides Stk of *Hydra*, Srk1-4 of *Spongilla*, and Src64B of *Drosophila*. NRTK3 showed the highest identity to the members of Btk family which is another family of non-receptor PTKs including *Drosophila* Src28C (76% identitiy to NRTK3), while NRTK4 seems to belong to the Syk/ZAP70 family. Both Btk and Syk/ZAP70 families have been examined closely in mammals and considered to be involved in the regulation of immune cells (DeFranco, 1995). NRTK5 was significanly similar to Csk kinase of vertebrates (73% to chick Csk, 71% to mouse and human Csk) and considered a member of the Csk family. Taken together, four subfamilies were identified from sea urchin embryos out of the 8 subfamilies of non-receptor PTKs known to date (Src, Csk, Btk, Syk, Fak, Abl, Jak, Fps; Superti-Furga and Courtneidge, 1995). Molecular phylogenetic tree of non-receptor PTKs supported the above identification (Fig. 2).

Temporal regulation of the expression of PTK genes

In order to know the developmental expression of each PTK gene, we conducted a Northern blotting analysis under stringent conditions and quantitated the signal obtained by a Molecular Imager (Fig. 3, Table 2). The identities of the base sequences among the seven PTK cDNA fragments used as probes were less than 65% (not shown) except for *NRTK1-NRTK2*, *NRTK2-NRTK3*, and *NRTK1-NRTK5*, for which the values were 71%, 71%, and 65%, respectively. Moreover, *NRTK2*, which is thus relatively similar to *NRTK1* and *NRTK3*, gave only faint bands which were hard for visual presentation (not shown) in contrast to *NRTK1* and *NRTK3*.. These showed that the hybridization condition was basically specific and allowed discrimination at least among transcripts from the seven PTK genes. Despite this specificity, however, all the probes

Table 1. Identities of sea urchin partial PTK fragments with representative PTKs (% at the amino acid level). The highest identity for each sea urchin PTK is underlined.

PTK	RTK1	RTK2	NRTK1	NRTK2	NRTK3	NRTK4	NRTK5
EGFR	54	46	55	54	46	54	50
FGFR	62	54	52	53	46	45	47
IR	50	57	44	44	44	42	43
PDGFR	53	50	49	52	47	48	44
Abl	46	49	61	66	61	48	55
Btk	44	40	54	52	67	46	51
Csk	46	43	52	57	50	52	71
Fak	49	50	51	54	52	52	52
Fes	48	47	53	53	50	47	53
Jak	44	46	48	48	48	54	44
Src	49	50	79	75	57	44	54
ZAP70	44	48	51	49	52	78	52

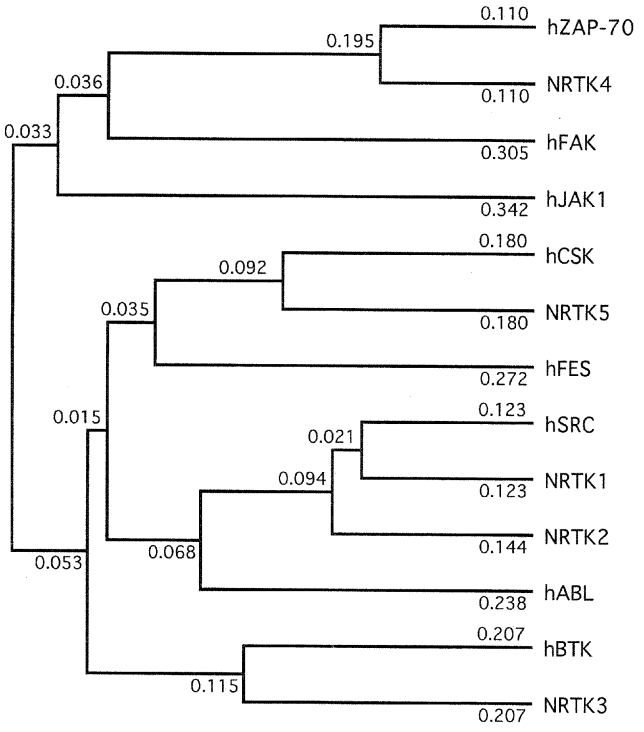

Fig. 2. Phylogenetic relationship among the four sea urchin non-receptor PTKs and eight human non-receptor PTKs. Each of the human PTKs represents one of the eight subfamilies of non-receptor PTKs known till now (Superti-Furga and Courtneidge, 1995). The tree was drawn by the UPGMA method, based on the amino acid sequences for the PCR fragments of NRTKs and those of the subregions of human PTKs which correspond to the amplified region.

detected more than one bands. The lengths and numbers of the transcripts, and the temporal changes in amount for different sea urchin PTKs were distinct (Fig. 3, Table 2). In most cases except for *NRTK5*, multiple bands for each probe assumed similar temporal patterns, suggesting they are derived from the same gene. Actually, a number of PTK genes are known to yield more than one transcript, some of which are due to alternative splicing (FGF receptor, EGF receptor, Src, Fyn, Lyn etc.; Hunter and Lindberg, 1994; Lowell and Soriano, 1996). The possibility of detecting closely related gene transcripts cannot be excluded at present, however, which may demand employing larger probes to confirm the present result in the future.

The transcript of *RTK1* was not detected in unfertilized eggs and was activated only after blastula stage. Meanwhile, all the other PTK genes were expressed both maternally in unfertilized eggs and zygotically after blastula stage, though each gene showed a distinct temporal pattern. *RTK2*, *NRTK2,* and *NRTK3* showed much higher expression in unfertilized eggs, while *NRTK1* and *NRTK4* were expressed strongly after the blastula stage. *NRTK5* transcripts were detected at similar levels in the embryos all through the early development. In contrast to the other sea urchin PTK genes examined, for which muitiple transcripts assumed parallel temporal patterns, smaller *NRTK5* transcripts (3.1 kb, 2.2 kb) were detected mainly zygotically together with the constantly expressed transcript of 6.8 kb (Fig. 3, Table 2).

Table 2. Summary of the results of Northern analysis

Genes	Class	Transcript size (kb)
RTK1	FGF receptor/Ret	5.7, 3.1
RTK2	Insulin receptor	8.6, 3.0
NRTK1	Src	8.0 (5.4, 3.1, 2.6, 1.5)[1]
NRTK2	Src	3.6, 2.5, 1.5
NRTK3	Btk	6.1, 5.4, 2.7
NRTK4	Syk/ZAP70	8.6, 6.3, 3.1
NRTK5	Csk	6.8 (3.1, 2.2)[2]

[1]Transcripts of the sizes shown in parentheses were detected in some batches.
[2]Shorter transcripts were specific to zygotic expression.

DISCUSSION

We surveyed the PTK families expressed in early embryos of the sea urchin using PCR strategy and identified two receptor PTKs and five non-receptor PTKs. More than two PCR clones were obtained for all the PTKs except NRTK4 and 5. The mRNA expression of these clones, detected by the Northern analysis, showed characteristic temporal accumulation patterns. Thus, we conclude that all these PTKs are really derived from sea urchin embryos.

Though the assignment is not decisive at present due to the lack of information on the flanking region of the amplified region, it seems safe to say that at least two receptor-type PTKs are expressed in sea urchin embryos, suggesting the presence of regulatory mechanisms involving growth factors or similar ligands. In this regard, several lines of evidence have been presented which makes it highly probable that receptor-type PTKs play important roles in sea urchin develpment. First, Tomlinson and his colleagues showed that molecules related to TGF-α and PDGF, receptors of which are also protein tyrosine kinases, play important roles in both gastrulation and skeletogenesis in sea urchin embryos (Govindarajan *et al.*, 1995). Second, it was reported that insulin receptor is involved in the pseudopodial cable growth of skeletogenic cells of sea urchin embryos (Kuno *et al.*, 1994). Finally, we have shown that EGF-like peptides (exogastrula-inducing peptides, EGIP) are expressed in sea urchin embryos which have been implicated in gastrulation (Suyemitsu *et al.*, 1989; Yamasu *et al.*, 1995). EGIPs induce exogastrulation of embryos, and may require a receptor(s) with a PTK activity just like EGF.

Maternal expression of NRTK1/2 (Fig. 3) is also interesting, given the involvement of PTKs, especially Src-family kinases, in the activation of eggs at fertilization (Kamel *et al.*, 1986; Moore and Kinsey, 1994; Kinsey, 1996). Experiments examining the effects of a PTK inhibitor suggested that the activation of PTK following fertilization is required both for egg activation and gastrulation of the embryos (Moore and Kinsey, 1995; Kinsey, 1995). It is tempting to speculate that NRTK1 and/or NRTK2 are the kinases which have been implicated in fertilization of eggs.

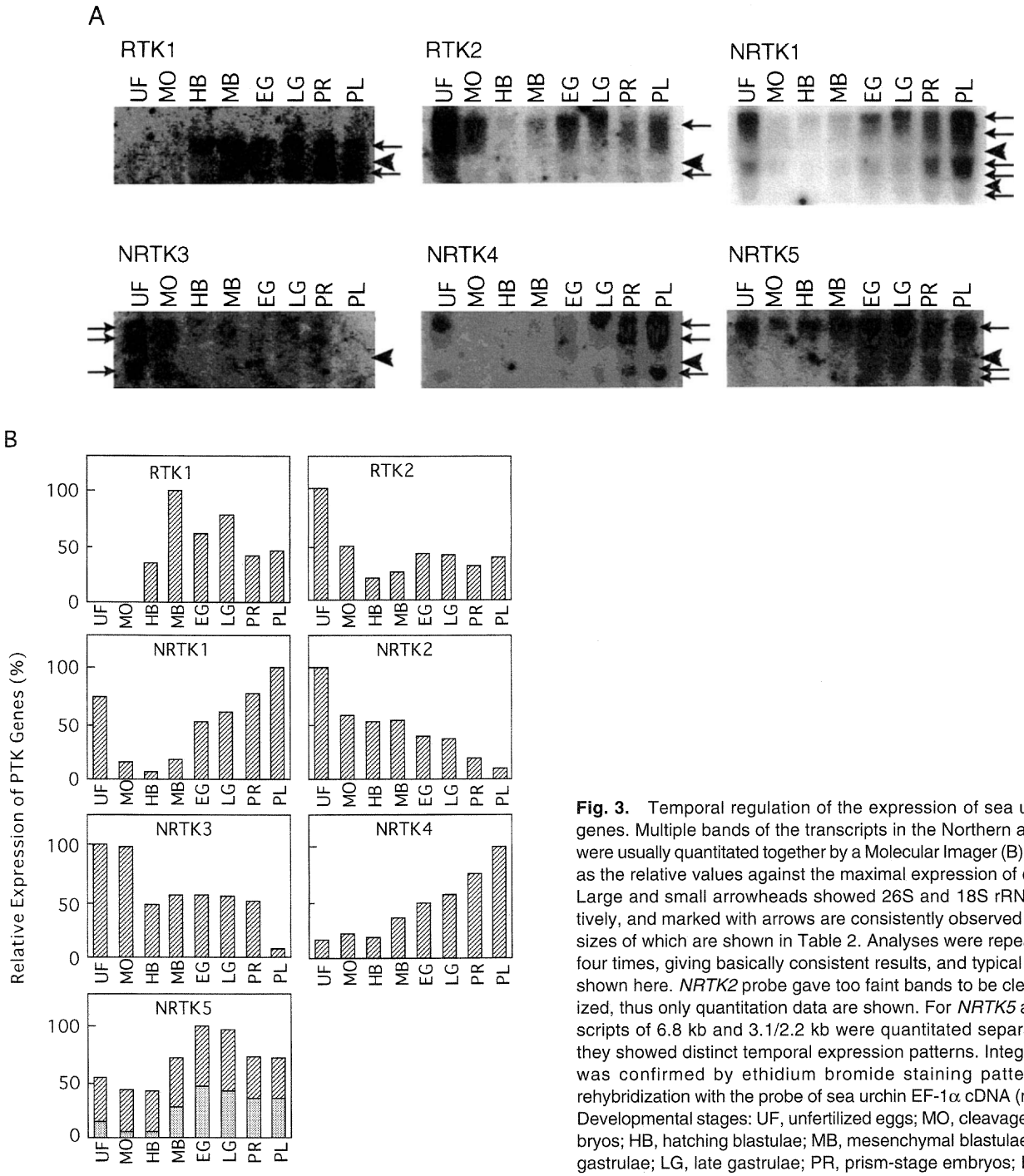

Fig. 3. Temporal regulation of the expression of sea urchin PTK genes. Multiple bands of the transcripts in the Northern analysis (A) were usually quantitated together by a Molecular Imager (B) and shown as the relative values against the maximal expression of each gene. Large and small arrowheads showed 26S and 18S rRNA, respectively, and marked with arrows are consistently observed bands, the sizes of which are shown in Table 2. Analyses were repeated two to four times, giving basically consistent results, and typical results are shown here. *NRTK2* probe gave too faint bands to be clearly visualized, thus only quantitation data are shown. For *NRTK5* alone, transcripts of 6.8 kb and 3.1/2.2 kb were quantitated separately since they showed distinct temporal expression patterns. Integrity of RNA was confirmed by ethidium bromide staining pattern and by rehybridization with the probe of sea urchin EF-1α cDNA (not shown). Developmental stages: UF, unfertilized eggs; MO, cleavage-stage embryos; HB, hatching blastulae; MB, mesenchymal blastulae; EG, early gastrulae; LG, late gastrulae; PR, prism-stage embryos; PL, plutei.

Recently, cloning of a PTK cDNA was conducted which is most close to the FGF receptor (SpFGFR; McCoon *et al.*, 1996). Meanwhile, we have obtained a partial cDNA clone for RTK1 which codes for the putative cytoplasmic region. However, since the identity of the cytoplasmic domain of SpFGFR to that coded by the partial cDNA clone for *RTK1* is only 46% (unpublished data), the identity of RTK1 remains to be defined. Wessel and his colleagues also recently performed a PCR analysis of sea urchin PTKs, and they obtained five PTK fragments (Wessel *et al.*, 1995). By comparison of the sequences, it is probable that their PTKSp15/PTKSp50 (Wessel's nomenclature) is the homologue of NRTK5 (Csk, 96%/98%), and SpPTK26 is the homologue of NRTK4 (Syk, 98%), though they considered them to be similar to Abl and Jak kinases, respectively, in contrast to our assignment.

They also obtained PCR fragments for protein tyrosine phosphatases and detected their expression all through the development of the embryos. This is consistent with the idea that tyrosine phosphorylation is dynamically regulated in the sea urchin embryos as it is in other animals (Sun and Tonks,

1994). Furthermore, it is highly probable that Csk kinase (NRTK5) is involved in the regulation of the Src kinases (NRTK1 and 2). In this regard, relatively constant expression of NRTK5/Csk may be important for the regulation of Src kinases which are always expressed from eggs through pluteus stage. Meanwhile, Syk/ZAP70 kinases and Btk kinase have been reported to be regulated by the Src family kinases (DeFranco, 1995), and Src kinases are activated by PDGF receptor and CSF-1 receptors, to which RTK1 has some similarity (Cooper and Howell, 1993). Taken together, the regulatory system of tyrosine phosphorylation of proteins exists in sea urchin embryos, suggesting that the modification of proteins by phosphorylation on tyrosine residues plays important roles in sea urchin embryogenesis like in other animals.

The availability of probes for several different PTKs now offers an opportunity to study the role of PTKs in sea urchin development by direct means. Furthermore, such studies on the sea urchin may provide important clues on the evolution of the deuterostome development.

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