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Isolation of tyrosine kinase related genes expressed in the early hematopoietic system

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Abstract

Transmembrane tyrosine kinase receptors are involved in cellular interactions which promote proliferation and differentiation of many cell types. To identify receptor tyrosine kinases important in embryonic hematopoietic cell development we have utilized the polymerase chain reaction (PCR) and degenerate oligonucleotides for isolation of such genes from mouse yolk sac and fetal liver. Sequence analysis of PCR amplified cDNAs from these hematopoietic sites of day 8 and 14 embryos, resulted in the isolation of nine tyrosine kinase and three serine/threonine kinase related clones. Two of these receptors, *tek* and *flk-1*, are expressed in both yolk sac and fetal liver and have been shown previously to be important for endothelial cell development. Two other clones, 9B4 and 9A2 appeared novel upon isolation but have been recently described as *ryk* and SK2 (rat homologue). Here we describe the twelve isolated kinases, the specific expression patterns of *flk-1*, *tek* and *ryk* kinases and their potential relationship to the development of the hematopoietic system.

Key words: Receptor tyrosine kinase; Mouse hematopoiesis; RT-PCR

1. Introduction

In the vertebrate embryo, cellular interactions and subsequent intracellular events promote the pluripotent hematopoietic stem cell to expand and differentiate into the diversity of cell types found in circulating blood and hematopoietic tissues. Cells with hematopoietic activity have been found at day 7 in the embryonic yolk sac, a few days later in the fetal liver and finally in the adult bone marrow [1–3]. In addition, the aorta, gonad, mesonephros region (AGM) in the developing embryo has recently been reported to contain hematopoietic precursors [4]. The timing and degree of multipotency of hematopoietic stem cells in these different regions has been studied extensively. However, the molecular mechanisms which control the early steps of development in the vertebrate blood lineages are not well understood.

A number of specific growth factors and their cognate receptors have been found to be involved in the production of functional hematopoietic cell types [5]. One family of genes, the receptor tyrosine kinase (RTK) gene family, have been shown to be important components of the signalling pathways by which cells interact [6,7]. These molecules are structurally and functionally related enzymes that show a high degree of conservation between species [8]. The structure of these proteins includes an extracellular ligand binding domain, a transmembrane domain and an intracellular portion containing the catalytic domain. When a specific ligand binds to its receptor, the effect is mediated by the catalytic activities of the receptor through phosphorylation of specific intracellular protein targets [6].

Several members of this large gene family have demonstrated function in the hematopoietic system. Amongst the best characterized are the colony-stimulating factor-1 receptor (CSF-1R) [9], the platelet-derived growth factor receptor (PDGFR) [10] and *c-kit* [11,12]. These RTKs and their ligands play multiple roles. The pivotal roles of these RTKs during development is supported by the mouse mutants *W*/Dominant white spotting (c-kit receptor mutants), *Sl* (SCF mutants) [2], *patch* (a PDGFα receptor mutant) [13], and *op* (a CSF-1 mutant) [14]. Other RTKs that may have some overlapping functions in several cell types include fetal liver kinase-1 and -2 (*flk-1* and *flk-2*) and *tek* [15–17]. The *flk-1* and *flk-2* genes were cloned from a population of fetal liver cells enriched for hematopoietic stem cell activity and may therefore be important in hematopoiesis. The receptors *tek* and *flk-1* have been implicated in endothelial cell development and yolk sac blood island formation, which may suggest that these receptors are utilised by both endothelial and hematopoietic precursors.

With the importance and abundance of RTKs in hematopoiesis as a precedent, we initiated studies to isolate novel tyrosine kinase genes that may play an important role in embryonic yolk sac and fetal liver hematopoietic development by reverse transcription and polymerase chain reaction (RT-PCR). We report here the abundance of various tyrosine kinases in these hematopoietically active tissues and the expression patterns of three RTK genes in tissues of the developing mouse embryo and adult and various hematopoietic cell lines.

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2. Materials and methods

2.1. Mice

Mice (A Thy 1 and CBA) were obtained from the breeding unit at NIMR, Mill Hill and all animal care was in accordance with the Animals Scientific Procedures Act, Home Office, UK.

2.2. Cell lines

WEHI-3 (monocyte/macrophage) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (Gibco) and antibiotics. The IL-3 dependent mouse cell line A4 (FDCP mix-A4) [18] established from a long-term bone marrow culture was grown in Fischer's medium supplemented with 20% horse serum (Gibco) and 10% WEHI-3 conditioned medium (containing IL-3) [18]. The pre-B cell line BaF3 [19] was cultured in standard DMEM medium supplemented with WEHI-3 conditioned medium. The AB1 embryonic stem cells (AB1-ES cells) were maintained on confluent feeder layers of mitotically inactivated STO-neo embryonic fibroblasts [20]. The growth medium used for ES-culture consisted of DMEM, 20% FCS, 0.1 mM 2-mercaptoethanol, 1 × non-essential amino acids, 10 U/ml penicillin and 10 μg/ml streptomycin. Differentiation of ES cells was carried out essentially as described by Doetschmann [29].

2.3. RT-PCR, cDNA cloning and sequence analysis

First strand cDNA was synthesized using 5 μg of RNA, oligo(dT)$_{15-18}$ primer (Amersham) and avian myeloblastosis reverse transcriptase (Promega). cDNAs were amplified using the degenerate oligonucleotides, PTK1 and PTK2 (1 μg each) described by Wilks [21] using a Techne PHC2 with the following parameters: denaturate 1.5 min at 95°C, anneal 2 min at 37°C and extend 3 min at 65°C for 30 cycles. Products were analysed on a 1.3% agarose gel (a ~210 bp fragment indicated amplified kinase related sequences) and cloned into Bluescript plasmids after ethanol precipitation, digestion with *Eco*RI and *Bam*HI (sites incorporated into the oligonucleotides) and gel purification. After screening transformants for the presence of the insert, positive clones were sequenced using the USB Sequenase kit (Stratagene, UK) and database (FASTA program) comparisons were performed. Yolk sac transformants were screened using a probemix of PCR products isolated from fetal liver prior to isolation, characterization and sequencing to avoid duplication of clones already isolated from fetal liver.

2.4. RNA extraction and Northern blot analysis

Total RNA was extracted using two different methods. For abundant tissues we utilized the LiCl/urea method [22]. Briefly, frozen or fresh tissues or cells were homogenised in 3 M LiCl, 6 M urea (5 ml/g), sonicated and kept overnight at 0–4°C. The RNA was pelleted (15,000 × *g*) and reprecipitated in a ½ volume of cold LiCl/urea. After centrifugation the pellet was dissolved in 10 mM Tris pH 7.6, 1 mM EDTA and 0.5% SDS, extracted once with phenol/chloroform/isoamylalcohol (25:24:1), once with chloroform and ethanol-precipitated.

Total RNA from small amounts of tissue (15–60 pooled yolk sacs and embryos) day 8.5 was extracted with RNAzol (Biotecx Lab. Inc., USA) according to the suppliers specifications. Briefly, the tissue was homogenised with RNAzol (0.8–2 ml depending on the amount of tissue), mixed with a 1/10 volume of chloroform and left on ice for 5 min. The suspension was centrifuged for 15 min, the aqueous phase collected and RNA precipitated with a ½ volume of isopropanol.

For Northern blot analysis, 20 μg of total RNA was fractionated on a 1.3% agarose gel containing 6% formaldehyde and transferred to a Genescreen Plus nylon membrane using standard conditions. The membranes were hybridised with ^{32}P-labelled antisense riboprobes synthesized from RT-PCR cDNA products of clones 9B4, *flk-1* and *tek*. The RT-PCR cDNA products and longer cDNA clones isolated from cDNA libraries representing 9B4 and *tek* were labelled by the random priming method (a *tek* cDNA was kindly provided by D. Dumont and M. Breitman). Hybridisation was carried out in 60% formamide, 1 × Denhardt's solution (0.1% of Ficoll, polyvinylpyrolidone and bovine serum albumin), 5 × SSC, 20 mM sodium phosphate buffer pH 6.8, 1% SDS, 7% dextran sulfate and 100 μg/ml of each of denatured salmon sperm DNA and baker's yeast tRNA and 10 μg/ml poly(A)RNA at 65°C for 16–24 h. Filters were washed in 2 × SSC, 1% SDS at 50°C and in 0.2 × SSC, 1% SDS at 80°C and autoradiographed.

2.5. Isolation and characterization of cDNA clones

The RT-PCR products representing clones 9A2, 9B4 and *tek* were used as probes to screen as oligo(dT)primed WEHI-3 cDNA library in λgt11 (kindly provided by G. Goodwin, London) in order to obtain longer cDNAs. Using standard conditions, three overlapping clones of 9A2 and one clone representing 9B4 were isolated.

2.6. Western blot analysis

Cell lysates were prepared from the BaF3 cell line and mouse embryonic heart (day 10–11). Cells and tissues were incubated in lysis buffer (10 mM Tris pH 7.5, 20 mM EDTA and 2% NP40) on ice for 30 min, followed by homogenisation and centrifugation for 45 min at 4°C. The extracts were separated on a 7% SDS-polyacrylamide gel, transferred to Hybond-C extra nylon filters (Amersham, UK) for 4 h at 80 V. The filters were blocked for 2 h with 10% bovine serum albumin-phosphate buffered saline pH 7.4, 0.1% Tween (BSA-PBT) followed by 2 h blocking in 5% milk-PBT. Thereafter, the filters were incubated with affinity-purified tek-antibodies (kindly provided by D. Dumont and M. Breitman) in 5% milk-PBT overnight at 4°C. After antibody incubation the filters were washed in PBS and incubated with horseradish peroxidase conjugated goat anti-rabbit Ig (Southern Biotechnology), diluted in 5% milk-PBT for 1 h at room temperature. The filters were washed extensively and developed using the chemiluminescence reaction (Amersham, UK).

3. Results

3.1. Isolation and sequence analysis of PCR amplified cDNA clones

To identify and characterize tyrosine kinases potentially important in early hematopoietic cell development, cDNAs were synthesized from RNA isolated from the known sites of embryonic hematopoiesis; day 8 yolk sac and day 14 fetal liver. Degenerate oligonucleotides corresponding to the IHRDL and DVWSFG conserved amino acid sequences in the catalytic domain, previously shown to amplify tyrosine kinases, were used as primers for PCR [21]. A total of 22 PCR-amplified clones (approximately 210 bp) from day 14 fetal liver were sequenced. This analysis resulted in the identification of 10 distinct cDNAs representing 8 tyrosine kinases and 2 serine/threonine kinases (Table 1). In addition, 4 clones did not contain any sequence similarity to protein kinases. Three clones represented in day 14 fetal liver were the intracellular tyrosine kinases *lyn, fyn* and *hck* (Table 1). Previously, these kinases have been shown to be expressed in cells of the adult hematopoietic system. The tyrosine kinase FD17, originally cloned from FDCP-1 cells was recovered from the fetal liver at a high frequency (5/22) which may indicate that this gene product may play an important role in multipotential hematopoietic cells during development. Three other clones isolated were of the RTKs type: *flk-1, tek* and IGFR (insulin growth factor receptor) (Table 1).

To reduce the possibility of isolating intracellular tyrosine kinase cDNAs from day 8 yolk sac, a probe mix containing PCR products representing *lyn, fyn, hck*, FD17 (intracellular kinases) and IGFR (isolated from day 14 fetal liver) was used in colony hybridization. Non-hybridizing transformants were isolated and sequence analysis of 16 cDNA clones from day 8 yolk sac revealed

4 distinct tyrosine kinases, all of which appeared to be of the receptor type (Table 1). Eight clones were homologous to *flk-1*, 5 to *tek* and 1 to the basic fibroblast growth factor receptor (bFGF) genes. Two clones, 9B4 isolated from both fetal liver and yolk sac and 9A2 isolated from fetal liver, appeared novel upon FASTA program sequence analysis.

Clone 9B4 contains the invariant DFG triplet, the conserved tyrosyl residue homologous to the autophosphorylation site Y-416 in src-kinases [23] and a methionine in the second position of the conserved motif W(M/T/L)A(A/P)E which is indicative of a transmembrane tyrosine kinase [8]. Furthermore, the upstream sequence IHRDLAARN strongly suggests that 9B4 is a tyrosine kinase, rather than a serine/threonine kinase [8]. This has been confirmed by recent cloning of the full length mouse peritoneal macrophages cDNA encoding a putative RTK,*ryk* [24,25] which shows 100% homology to clone 9B4.

Clone 9A2 shows 100% homology to a serine/threonine kinase (SK2) isolated from a rat large granular lymphocytic cell line [26]. Some homology to the *Drosophila* serine/threonine kinase *ninaC* gene [27] was found but sequence does not belong to any of the classical subfamilies of serine/threonine kinases [28]. Further expression analysis of the 9A2 clone was not pursued.

3.2. *Expression of the tyrosine kinase receptors* flk-1, tek *and 9B4* (ryk) *in mouse tissues and hematopoietic cell lines*

To determine whether the isolated kinases were expressed in a hematopoietic cell-specific manner, Northern blot analysis was performed on embryonic, fetal and adult mouse tissues and on mouse cell lines for the RTKs *flk-1*, 9B4 and *tek* (Figs 1a, b and c, respectively). These data are summarized in Table 2.

In early stage embryos (day 8–10 of mouse gestation), transcripts for all three RTKs; *flk-1, tek* and *ryk* were detected at one or several stages in both the yolk sac and the body of the embryo. At later stages (day 12–14 of gestation), *flk-1* and *ryk* mRNA were observed in fetal liver. However, no *tek* expression was seen. Since we were able to clone a cDNA for *tek* from day 14 fetal liver using RT-PCR (Table 1), the level of *tek* expression must be below the limits of detection using total RNA in Northern blot analysis.

When the relative levels of expression were examined in these tissues, amounts of *tek* and *flk-1* mRNA appeared to decrease as the age of the embryo increased. *Tek* specific transcripts were detectable up to day 11 in the embryo but were not detected in day 11 yolk sac or day 14 fetal liver. *Flk-1* was found in day 10 yolk sac and embryo body but decreased greatly from day 12 to day 16 (undetectable) in the fetal liver. This correlates well with the abundant isolation of *flk-1* cDNA clones (eight) from day 8 yolk sac while only one *flk-1* cDNA was cloned from day 14 fetal liver by RT-PCR (Table 1). In contrast to *flk-1* and *tek*, *ryk* expression was found to increase in the yolk sac and embryo from day 8 to day 9 and remain relatively constant throughout development up to day 14 in fetal liver. In addition, we examined *flk-1* and *ryk* expression in ES cells which have been shown to differentiate into various hematopoietic lineages [29,30] and express hematopoietic specific genes in a developmentally controlled manner [31,32]. *Flk-1* transcripts were found in undifferentiated and day 9 differentiated cells but no or undetectable *flk-1* expression was observed after 11 days of differentiation. This decrease in expression during development corresponds to that seen in yolk sac (Fig. 1a) and in 6.5 day differentiated ES cells [33]. *Ryk* expression was observed in both day 9 and day 11 differentiated embryoid body cells (Fig. 1b).

In the adult mouse, high levels of *flk-1* and *tek* expression were observed in lung and heart and lower levels were observed in the brain. *Flk-1* was also expressed at abundant levels in kidney and muscle. In the hematopoietic tissues (BM, LN and thymus), *flk-1* could be detected at very low levels (see Table 2) while no *tek* expression was detected. The *ryk* RTK (Fig. 1b) appears to be expressed in almost all hematopoietic and non-hematopoietic tissues (no expression in spleen).

Finally, hematopoietic cell lines FDCP-A4 and WEHI-3 and a non-hematopoietic STO cell line were examined for RTK expression by Northern blot analysis. FDCP-A4 and WEHI-3 but not STO cells were found to express *flk-1*. Whereas all embryonic and adult tissues expressed only a 5.5 kb *flk-1* transcript [36,37], cell lines produced two additional *flk-1* transcripts. The *ryk* RTK

Table 1
Protein tyrosine kinase cDNAs isolated from day 14 fetal liver and day 8 embryonic yolk sac by reverse transcription and polymerase chain reaction

Protein tyrosine kinase	Number of clones	Reference
d14 fetal liver		
FD17	5	21
lyn	2	46
hck (bmk)	2	47
ryk (9B4)	2	24,25,40
IGFR	1	48
fyn	1	49
tek	1	17
flk-1	1	15
9A2*	1	26
9B1	2	Ser/Thr homologue
–	4	unrelated
d8 embryonic yolk sac		
flk-1	8	15
tek	5	17
ryk (9B4)	2	24,25,40
bFGFR	1	50

*A serine/threonine kinase.

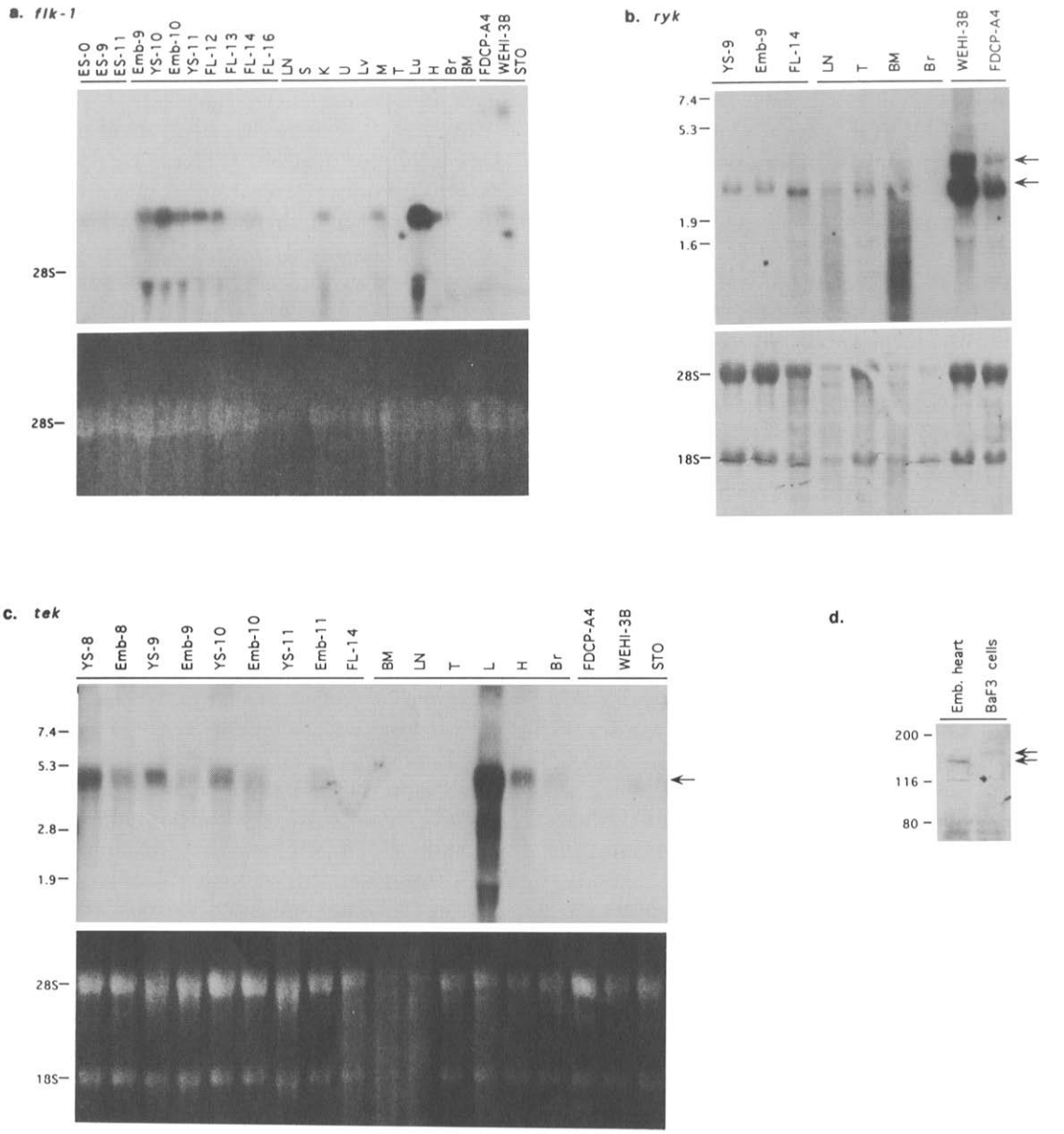

Fig. 1. Expression of: (a) *flk*-1, (b) *ryk* and (c) *tek* mRNA using Northern blot analysis. Embryonic, fetal and adult mouse tissues and three cell lines (FDCP-A4, WEHI-3B and STO) were tested. Twenty μg of total RNA was used in each lane. Abbreviations used for embryonic and fetal tissues: ES-0 = undifferentiated embryonic stem cells; ES-9/11 = embryonic stem cells differentiated for 9 respectively 11 days in vitro; YS = yolk sac; Emb = embryo; FL = fetal liver. Adult tissues: LN = lymph node; S = spleen; K = kidney; U = uterus; Lv = liver; M = muscle; T = thymus; Lu = lung; H = heart; Br = brain; and BM = bone marrow. Arrows indicate predominant transcript. (d) Western blot analysis of tek protein expression. Lysates of embryonic heart (day 10–11) and the BaF3 cell line were analysed for the presence of tek protein using purified tek antibodies. The tek antibody detects a 140 kDa protein in the embryonic heart preparation and a slightly bigger protein, 150 kDa, in the BaF3 cell line. The positions of the molecular weight markers are depicted on the left.

was found to be highly expressed in both FDCP-A4 and WEHI-3 cell lines. Two transcripts were observed, a 3.5 kb band and more abundant 2.8 kb band and correspond to those previously reported [24,25]. STO cells produced low levels of *tek* transcripts while FDCP-A4 and WEHI-3 cells were negative. The *tek* transcripts in all the tissue and STO cell RNAs tested were comparable to the previously published 4.5 kb size, except for the lung where multiple additional smaller transcripts were found. Some of these correspond to the other three transcripts reported by Dumont et al. [17] in embryonic heart.

To investigate whether tek protein was expressed in any hematopoietic cell lines, Western blot analysis was performed on WEHI-3 and P338D1 monocyte/macrophage lines, the EL4 T cell line, MEL cells (erythroleukemic) and the pre-B cell line BaF3. Embryonic heart extracts (day 10–11) were used as a positive control for tek protein expression. No expression was observed in either

of the macrophage cell lines, the T-cell line or the MEL cells (data not shown). However, in the BaF3 pre-B cell line, the affinity-purified tek antibodies recognized a protein of approximately 150 kDa (Fig. 1d). A 140 kDa protein can be detected in the control embryonic heart extract and corresponds to the previously reported size of the tek protein in day 13.5 embryonic heart and endothelial cell lines [38]. The unglycosylated, predicted size of tek estimated from the deduced amino acid sequence is 126 kDa [36]. The slightly larger size protein detected in the BaF3 cell line may indicate that tek occurs in an alternative glycosylated form as compared to that found in heart or endothelial cells.

4. Discussion

We have described the isolation of 12 distinct protein tyrosine kinase cDNAs from embryonic yolk sac and fetal liver using degenerate oligonucleotides and RT-PCR and have also described the expression patterns of three of these: *flk-1*, *tek* and *ryk* in the mouse and in tissue culture cells. The *flk-1* RTK, isolated from both the yolk sac and the fetal liver, was detected in RNA from embryonic stem cells, embryoid bodies, yolk sac and the developing embryo. It appears that *flk-1* is one of the most abundant RTKs in the yolk sac (representing 8 of 16 cloned sequences). While its expression level decreases during development, it is highly expressed in the adult heart and lung but can also be detected in some hematopoietic tissues and cell lines. Similarly, we have shown that *tek* is a frequently represented RNA in the early developing mouse yolk sac (5 of 16 cloned and sequences). Its expression decreases as the embryo matures. In the adult, it is expressed predominantly in the heart and lung. Although *tek* RNA is not detected in any adult hematopoietic tissues we have found tek protein in a pre-B hematopoietic cell line.

Both *flk-1* and *tek* have been implicated in endothelial cell differentiation [17,33,34] by in situ hybridisation of endothelial cells and their presumptive precursors. In the yolk sac blood islands of day 7.25–8.5 embryos, studies have shown that *flk-1* is expressed earlier and in a more primitive endothelial precursor cell than *tek* [33]. This primitive precursor, the hemangioblast, is believed to be the common precursor for both the endothelial and hematopoietic cell lineages. Angioblasts (the committed endothelial progenitor) of the yolk sac, express both *flk-1* and *tek*. While day 8.5 embryonic yolk sac contains hemangioblasts, angioblasts and endothelial cells, hematopoietic progenitors for the B lymphoid, myeloid and erythroid lineages are also present in the blood islands [1,2,36]. Due to this colocalization, *flk-1* and *tek* may play a role in hematopoiesis or lineage commitment events. It is interesting to note that *flk-1* is expressed in fetal liver cells enriched for hematopoietic stem cell activity [15] and in the bone marrow derived hematopoietic progenitor cell line FDCP-A4 (Fig. 1c). Also, ES cells differentiated for 9 days which contain identifiable hematopoietic blood islands [29] as well as endothelial cells [38,39] were found to express *flk-1*. These data together with the published data [33,34] may suggest a role for *flk-1* in early hematopoietic cell development. We have found tek protein in a pre-B cell line suggesting that this putative RTK may have a very limited or secondary role in the adult hematopoietic system. Perhaps the *flk-1* and *tek* RTKs, like many other growth factor receptors (*c-kit*, for example), have multiple roles in different cell types.

We have shown that the 9B4 clone, *ryk*, is ubiquitously expressed in most hematopoietic and non-hematopoietic tissues in the embryonic and adult mouse and at very

Table 2
RNA expression analysis of the tyrosine kinase receptors *flk-1*, *tek* and *ryk* (9B4) in mouse tissues and cell lines

Cell line/Tissue	*flk-1*	*tek*	*ryk* (9B4)
Embryonic/Fetal			
d0 ES	+	nt	nt
d9 ES	+	nt	+
d11 ES	–	nt	+
d8 YS	nt	+	–[1]
d8 embryo	nt	+	–
d9 YS	nt	+	+
d9 embryo	+	+	+
d10 YS	+	+	+
d10 embryo	+	+	+
d11 YS	nt	–	+
d11 embryo	nt	+	+
d12 FL	+	nt	+
d13 FL	+	nt	+
d14 FL	+	–[2]	+
Adult			
thymus	–[3]	–	+
LN	–[3]	–	+
spleen	–	nt	–
BM	–[3]	–	+
liver	–	nt	+
kidney	+	nt	+
uterus	–	nt	+
muscle	+	nt	+
lung	+	+	+
heart	+	+	+
brain	+	+	+
Cell lines			
FDCP-A4	+	–	+
WEHI-3	+	–	+
STO	–	–	+

[1] *ryk* was cloned from d8 YS.
[2] *tek* was cloned from d14 FL.
[3] These tissues have shown expression of *flk-1* but at very low levels (data not shown).
nt = not tested; ES = embryonic stem cells; YS = yolk sac; FL = fetal liver; LN = lymph node; BM = bone marrow.

high levels in hematopoietic cell lines. Both the mouse and human full-length cDNAs have recently been isolated [24,25,40] and analysis of the extracellular and catalytic kinase domains revealed that *ryk* does not fall into any of the previously defined classes of protein tyrosine kinases [8] although retaining associated features. The human homologue was cloned from the human leukemia cell line K562 and was shown to be widely expressed in both adult human tissues and tumor cells of different origins [40,41]. What specific role *ryk* may play in the hematopoietic system remains to be elucidated.

It appears from our studies and those of others [17,41,42,43] that the degenerate oligonucleotides PTK1 and PTK2 have reached the extent of their usefulness in the isolation of novel embryonic and fetal RTK genes. We were unable to isolate even a single novel clone by sequencing the 210 base region. Perhaps only the most abundant receptor and intracellular tyrosine kinases are easily amplified or alternatively, some cDNAs may be cloned more easily with respect to the degenerate oligonucleotides used. It is interesting to note that the same spectrum of clones has been isolated from many different tissue types [17,24,26,41,43,44]. Using this particular cloning strategy, a level of saturation has been reached. Therefore in order to isolate new RTKs it could be more useful to design oligonucleotides for other regions of conserved domains or perform more stringent subtraction procedures with existing kinase genes. A procedure with nested PCR primers has been used for the isolation of the *ryk* RTK [24]. In parallel, it will probably be useful to isolate the cell population or single cells [45] of interest prior to applying a particular cloning strategy.

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