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Expression of tyrosine kinase genes in mouse thymic stromal cells

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Abstract

Amongst the most important signal transduction molecules involved in regulating growth and differentiation are the protein tyrosine kinases (PTK). Since T cell development is a consequence of interactions between thymic stromal cells (TSC) and thymocytes, identification of the PTK in both compartments is required to dissect the mechanisms that control this process. Here we report a search for PTK in mouse TSC, using RT-PCR to survey the repertoire of PTK mRNAs expressed in a freshly isolated TSC preparation. We identified 10 different PTK cDNAs among the 216 cDNAs sequenced, and demonstrate that transcripts of three of those (*ufo*, *fyn* and *fer*) are widely expressed among a large panel of immortalized thymic epithelial cell lines (TEC) and in primary cultures of TSC. Of the other seven, none were expressed in established TEC lines but, instead, displayed distinct expression patterns in cell types likely to have contaminated the fresh TSC preparation, i.e. macrophages, B cells, T cells and fibroblasts. Among the three PTK expressed in TEC lines, only one, *ufo*, exhibited expression exclusively in cells of non-hemopoietic origin. Although expression of *ufo* (also known as *tyro 7*, *axl* or *ark*) is not thymic-specific, in that it is also expressed in cell types of mesodermal origin in other tissues, its presence in TEC suggests a role for *ufo* in differentiation of the TSC compartment. Consistent with this notion, high-level expression of this receptor PTK at the protein level could be documented in every TEC line investigated, as well as in fresh thymus tissue sections. These data provide the first example of a receptor PTK in TSC and open new approaches to study the regulation of TSC differentiation.

Introduction

Stromal cells in the thymus provide specialized microenvironments which support the differentiation of bone marrow-derived precursors into functional T cells (for reviews see 1–4). While there have been considerable advances in understanding the progressive steps in early T cell development, much less is clear about the role of the thymic microenvironment in this process. As thymocytes migrate through the thymus, niches formed by stromal cells allow for extensive communication with the developing T cells. Development of the most prominent of these, the αβ TCR-expressing population, can be subdivided into multiple stages based on the membrane expression of proteins such as the TCR itself and its co-receptors, CD4 and CD8 (3–5). Most thymocytes develop from $TCR^-CD4^-CD8^-$ precursors via $TCR^{lo}CD4^+CD8^+$ intermediates into either $TCR^{hi}CD4^+CD8^-$ or $TCR^{hi}CD4^-CD8^+$ mature T cells. Cytokines provide some of the early differentiation-inducing signals for $TCR^-CD4^-CD8^-$ T cells (6), with later (selection) steps more dependent on cell–cell interactions. These interactions are facilitated by the intricate microenvironment created by thymic stroma and lymphocytes, but information on the signal transduction molecules involved is lacking. Here we report on an analysis of the protein tyrosine kinases (PTK) expressed in freshly isolated thymic stromal cell (TSC) preparations.

Stromal cells encompass ~1% of the total cell number in the thymus and constitute a large yet poorly characterized variety of cell types. Thymic epithelial cells (TEC), unique amongst other epithelial cells because they create a three-

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dimensional network structure, can be further classified into a yet unknown number of subtypes based on localization (medulla, cortex or subcapsular area), morphology (1,2), and the expression of various intracellular and transmembrane proteins as defined by mAb (7). The same holds true for thymic macrophages, which are present in all mentioned thymic compartments and have an unknown variety of thymic subtypes. In addition, interdigitating or dendritic cells are present in the thymic medulla and also fibroblasts can be detected. The role of this exceptional cellular diversification in the thymic microenvironment is not clear, but the hypothesis that special functions can be attributed to the different compartments has been entertained. In negative and positive selection, arguments both for and against the notion that specific thymic cell types are responsible for these alternative forms of selection have been presented (reviewed in 8,9). For early T cell differentiation, cells of both mesenchymal and epithelial origin are required (10), while for the later steps of development, after the αβ TCR has been produced, the mesenchymal compartment can be missed. Macrophages do play a special role, to the extent that they have long been thought to be engaged in removal of apoptotic cells (11), firmly documented recently by *in situ* visualization of apoptotic cells in thymic macrophages (12). Overall, however, it is not clear what purpose is served by the large diversity of thymic stromal subtypes.

To complicate matters further, recent evidence supports the view that the communication between TSC and thymocytes is in fact bidirectional (13,14). Thus, not only are thymocytes dependent on the thymic stromal microenvironments, but TSC need properly developing thymocytes for their correct spatial organization. This is particularly illustrated by the finding that mice carrying targeted mutations in T cell differentiation-associated genes also exhibit, as a secondary effect, an altered thymic stromal environment (13). For example, mice with deleted RAG genes not only show a specific block in maturation of their thymocytes at the $CD44^-CD25^+$ stage of development (3–6,15,16), but these animals also lack normal thymic medullary epithelium (13). Likewise, mice with an even earlier block in T cell development, i.e. at the $CD44^+CD25^-$ stage, fail to develop a functional thymic cortex (17).

Despite this evidence for 'cross-talk' between TSC and thymocytes, the surface receptors and intracellular signal transduction pathways that operate in TSC are still largely unknown. In contrast, several PTK have been implicated in T cell development (18–24) by virtue of their function in signal transduction through the TCR (25). Mice deficient in $p56^{lck}$ (21) or expressing a dominant negative form of $p56^{lck}$ (22) display an early block in thymocyte development at the $CD44^-CD25^+$ stage (21,22). ZAP-70 (23) and *itk* (24) deficient mice display a phenotype more consistent with a later block, i.e. at the transition of double-positive ($CD4^+CD8^+$) to single-positive stages. Interestingly, also proper development of the TSC compartment is affected by the *lck* mutation (13). The latter phenomenon might be an indirect consequence of the absence of specific thymocyte subpopulations which support the proper maturation of TSC. Alternatively, the tyrosine kinase genes expressed by TSC and thymocytes overlap; the TSC compartment would then be directly affected by the above mutations.

To begin to address which signal transduction pathways underlie TSC differentiation, we sought to define the repertoire of tyrosine kinase genes expressed by mouse TSC. In this report, we used homology-based cloning by PCR of PTK cDNAs out of fresh TSC cDNA. Transcripts of 10 different PTK were identified and their expression characteristics in TSC and other thymic cell types are presented. Three of these PTK are expressed by thymic stroma cells, i.e., *fer*, *fyn* and *ufo*. *ufo* represents a receptor-like PTK (also named *axl* or *tyro 7*) and is the only one of these PTK of which expression is restricted to non-hemopoietic cell types. Every TSC line we investigated manifests expression of Ufo/Axl/Tyro 7 at the protein level, as did primary cultures of TSC. Also, thymus tissue sections exhibit staining for Ufo in a reticular pattern predominantly in the medullary, but also in the cortical region. This receptor PTK may therefore be involved in the regulation of differentiation of the TSC compartment. Of the two other PTK expressed in stroma, one (*fer*) is also expressed in B cell and macrophage cell lines, while we found *fyn* to be universally expressed throughout the hemopoietic system. Identification of the physiological role of these kinases in TSC will be a challenge for the future.

Methods

Mice

Ten 8-week-old female C57BL/6 mice (B6) were used for isolation of TSC and thymus tissue section analysis. For the isolation of other organs we used 16-week-old B6 males.

Isolation of mouse TSC

Fresh C57BL/6 TSC were isolated as described (26, 27). In short, 10 thymi from female B6 mice were minced in cold PBS, subjected to enzymatic digestion with 400 IU/ml collagenase and 10 μg/ml DNase for 30 min at 37°C, and filtered through a 100 μm mesh to remove cell debris. Subsequently, the cells were incubated in 5 mM EDTA for 30 min at 37°C to disrupt rosettes and centrifuged on FCS (containing 5 mM EDTA) at 50 *g* for 3 min. The cells that had entered the FCS were harvested at 450 *g* for 3 min and washed twice in cold PBS (with 5 mM EDTA). Thus, $\sim 4 \times 10^5$ TSC were isolated.

Isolation of cDNA from mouse TSC

TSC were lysed in 600 μl solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) as described (28). DNA was depurinated by adding 60 μl 2 M sodium acetate, pH 4.0. Next, 600 μl phenol saturated with water was added, followed by 120 μl 50:1 solution of chloroform:isoamyl-alcohol. After 15 min on ice and 15 min centrifugation at 12,000 r.p.m. the water phase was transferred to a clean tube and RNA was precipitated overnight at –20°C by adding 1 vol. isopropanol. The pellet was collected by 15 min centrifugation at 12,000 r.p.m. and dissolved in 300 μl solution D to repeat the above procedure once more. Finally, the RNA pellet was washed with 70% ethanol and dissolved in 50 μl DEPC-treated water. Then, 5 μl RNA was mixed with 0.5 μg oligo-dT (Pharmacia, Uppsala, Sweden) and DEPC-treated water was added to

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tyro 8   DSDLSVKVSDFGMTRYV*LDDQYVSSVGTKF*PVKWSAPEVFHYFKYSSKS

lck      SDT--C-IA---LA-LI*E-NE-TARE-A--*-I--T---AIN-GTFTI--
fgr      GEY-IC-IA---LA-LI*E-NE-NPQQ----*-I--T---AALFGRFTV--
hck      SAS-VC-IA---LA-II*E-NE-TARE-A--*-I--T---AINFGSFTI--
lyn      SES-MC-IA---LA-VI*E-NE-TARE-A--*-I--T---AINFGCFTI--
fyn      GNG-IC-IA---LA-LI*E-NE-TARQ-A--*-I--T---AAL-GRFTI--

blk      SET-CC-IA---LA-II**-SE-TAQE-A--*-I--T---AI-FGVFTI-A

fes      TEKNVL-I-----S-EE*A-GI-AACS-LRQV----T---ALN-GR---E-
fer      GENNTL-I-----S-QE*DGGV-S--GLKQI*-I--T-A-ALN-GR---E-

ufo-R    NENMSVCVADFGLSKKIYNGDYYRQGRIAKMPVKWIAIESLADRVYTSKSDVWSFGVTMWEIATRG
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Fig. 1. Predicted amino acid sequences of the partial tyrosine kinase *tyro 8* cDNA (top) and the other tyrosine kinase cDNAs isolated in this study. Tyro 8 is shown as reference since it was previously isolated (29) using the same set of degenerate primers utilized in the present study. *Src* family: *lck*, *fgr*, *hck*, *lyn* and *fyn* and *blk*; *fes* family: *fes* and *fer*. The one receptor PTK isolated, *ufo*, is shown separately at the bottom. An asterisk indicates the absence of an amino acid residue. A dash indicates identity with the Tyro 8 reference sequence.

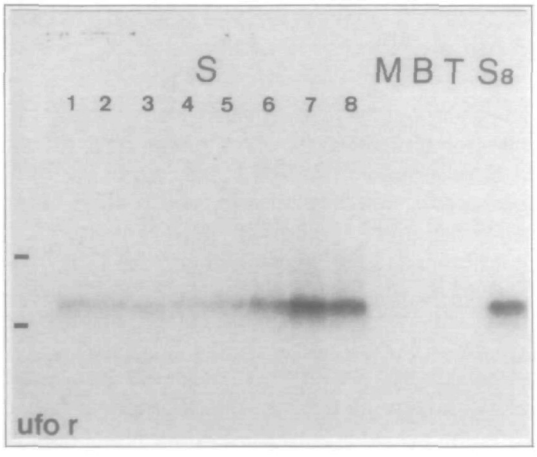

Fig. 2. Northern blot analysis of mouse TSC lines MTE-1 (S1), B6TE/A (S2), TNCR3.1 (S3), 2.4B6 (S4), A2T (S5), TEC (S6), 1D4 (S7) and Tepi (S8), macrophage cell line P388D1 (M), B cell line SP2/0 (B), T cell line RMA-S (T) and 8 days cultured TSC (S8) hybridized to a partial *ufo* cDNA probe. Migration positions of 18S and 28S rRNA are indicated by horizontal lines.

12 µl. After 10 min incubation at 70°C, followed by chilling on ice, a cDNA reaction was performed in a 20 µl volume using 200 µ M-MLV reverse transcriptase (BRL, Grand Island, NY) for 1 h at 37°C. The reaction was stopped by 3 min at 85°C, 180 µl DECP water was added and 20 µl of the cDNA was used for PCR amplification.

Degenerate PCR amplification and cloning of TSC tyrosine kinase cDNA

Amplification of TSC cDNAs was performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) using degenerate oligonucleotide primers based on conserved tyrosine kinase domains as described (29). The primers contained *Eco*RI and *Bam*HI cloning sequences and increasing annealing temperatures were used during the amplification. Two pools of products were generated of 148 and 213 bp (A series and B series respectively). A series primers: forward 5′-CCGGAATTCCAYCGGGACCTGCGGGCTGCCAACWKYYTN-GT-3′, reverse: 5′-CCCGGATCCCTCRGGGGCYRTCCACTT-DATNGG-3′. B series primers: forward same as A-series, reverse: 5′-CCCGGATCCCTCYSWCAGCAGGATGCCRAAG-GACCANACRTC-3′. The pools were separated from the primers by 1.2% agarose gel electrophoresis and re-amplified using corresponding fresh primers by 30 rounds of 45 s 94°C, 45 s 60°C and 1 min 72°C. The PCR products were extracted with Tris–HCl, pH 8.0, saturated phenol, precipitated, digested with 5 µ *Eco*RI and *Bam*HI at 37°C for 1 h, phenol extracted, and precipitated again. The digested products were purified by 1% low melting agarose gel electrophoresis (30) and ligated into the *Eco*RI and *Bam*HI sites of pUC18. Ligates were transformed into electrocompetent *Escherichia coli* DH5a bacteria by electroporation at 25 µF capacitance and 200 ohm resistance. Thus two libraries (A series and B series) of partial tyrosine kinase domain cDNAs were generated of ~5000 clones each.

DNA sequencing of TSC tyrosine kinase cDNA clones

Plasmid DNA was isolated from single colonies as described (30) and subjected to PCR-aided non-radioactive automated DNA sequencing in a DNA sequencer 373A-36 (Applied Biosystems, Foster City, CA). Databases were searched with nucleic acid sequences (GenBank) and predicted protein sequences (GenBank, Protein Identification Resource and Swis-Prot).

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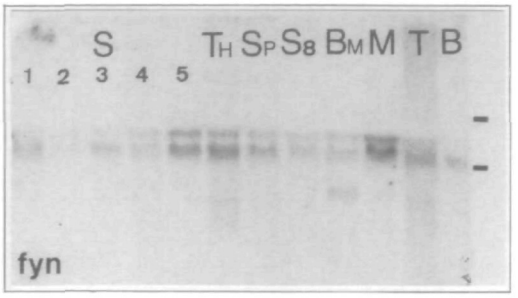

Fig. 3. Northern blot analysis of mouse TSC lines 1D4 (S1), Tec (S2), Tepi (S3), 1C2 (S4) and TNCR3.1 (S5), thymus (Th), spleen (Sp), 8 days cultured TSC (S8), bone marrow (Bm), macrophage cell line P388D1 (M), T cell line RMA-S (T), and B cell line SP2/0 (B) hybridized to a complete *fyn* cDNA probe. Migration positions of 18S and 28S RNA are indicated by horizontal lines.

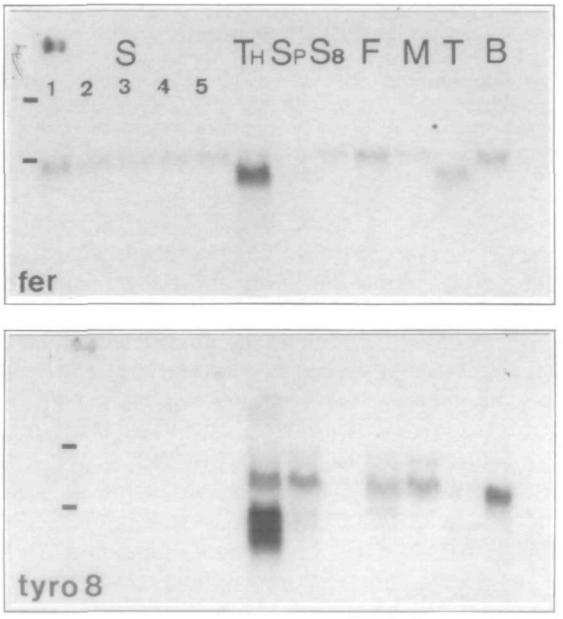

Fig. 4. (A) Northern blot of Fig. 5(A) rehybridized to *fer* cDNA probe (*lck* signal still visible). (B) Northern blot of Fig. 5(B) rehybridized to *tyro 8* probe (TCRβ signal still visible).

Northern blot analysis of RNA isolated from mouse tissues and cell lines

RNA was extracted by using the guanidinium–HCl method essentially as previously described (31). Cell lines were harvested (if necessary, by treatment with 0.05% trypsin/0.5 mM EDTA), washed twice in PBS and lysed in 6 M guanidinium hydrochloride. The following cell lines were used: TEC lines 1D4 (27), Tec (32), Tepi (33), TEC1-2C1 (gift of Dr Kasai), TNCR3.1 (34), MTE-1 (35), B6TE/A (36), 2.4B6 (36), A2T (37), fibroblast cell line NIH 3T3 (ATCC CRL 1658), macrophage cell line P388D1 (38), T cell lines RMA-S (39) and 18.2 (day 18 fetal thymus cell line), and B cell line SP2/0 (ATCC CRL 1581).

Sixteen-week-old BALB/c males were sacrificed to obtain complete organs (thymus, spleen, bone marrow, liver, testis,

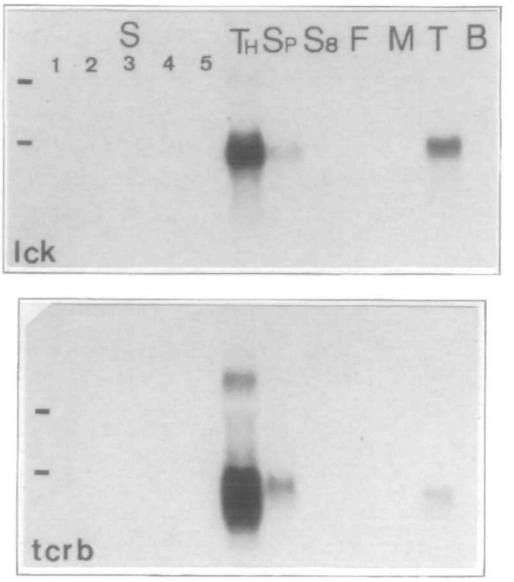

Fig. 5. (A) Northern blot analysis of mouse TSC lines 1D4 (S1), Tec (S2), Tepi (S3), 1C2 (S4) and TNCR3.1 (S5), thymus (Th), spleen (Sp), 8 days cultured TSC (S8), fibroblast cell line NIH/3T3 (F), macrophage cell line P388D1 (M), T cell line RMA-S (T), and B cell line SP2/0 (B) hybridized to *lck* cDNA probe. (B) Northern blot analysis as above; hybridization to TCRβ probe. Fibroblast RNA replaced by mouse bone marrow (Bm). Migration positions of 18S and 28S RNA are indicated by horizontal lines.

heart, lung, kidney and brain) which were directly frozen in liquid nitrogen and crushed in a mortar just prior to the addition of 6 M guanidinium–HCl. Aliquots of 30 µg of RNA were separated by electrophoresis for 4 h at 120 V in 1.2% agarose/formaldehyde gels (30). RNA was transferred to nylon membranes (Amersham Hybond N^+) by overnight capillary blotting in 0.05 N NaOH. Hybridization was performed according to the manufacturer's instructions and washing was done with 1×SSPE/0.1% SDS at 65°C. Blots were exposed to Kodak XAR-5 films at –80°C. The following probes were used: a 0.6 kb *Eco*RI fragment containing TCRβ C-region cDNA, a 2.1 kb *Eco*RI fragment containing a complete *lck* cDNA (gift of Dr Perlmutter), a 3.8 kb *Eco*RI fragment containing an almost complete *ufo* cDNA (gift of Dr Bartram), a 2.2 kb *Xho*I fragment containing a complete *lyn*A cDNA (gift of Dr Miller), a 2.0 kb *Eco*RI fragment containing a complete *hck* cDNA (gift of Dr Perlmutter) and a 2.6 kb *Eco*RI fragment containing a complete c-*fes* cDNA (gift of Dr Pawson). In addition, when full length probes were not available, DNA minipreps (including the pUC18 vector) of the various tyrosine kinase cDNAs that we cloned during this study were used as probes. Labeling was performed by random priming and Klenow DNA polymerase (Pharmacia).

Western blot analysis

Cells were lysed for 30 min on ice with lysis buffer containing: 1% Nonidet P-40, 0.1% SDS, 10 mM Tris–HCl, pH 8.0, 140 mM NaCl, 2 mM EDTA, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml of: chymostatin, leupeptin, aprotinin, antipain and pepstatin) and phosphatase inhibitors (2 mM sodium orthovanadate and 10 mM NaF). Lysates were

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Table 1. Expression characteristics of mouse tyrosine kinase genes in thymus (Th), spleen (Sp), 8 days cultured TSC (S8), TEC lines (S), bone marrow (Bm), macrophages (M), T cell lines (T), B cell (B) and fibroblast (F) lines, as assessed by Northern blotting analyses (sizes of the various mRNAs are indicated in kb).

PTK	Th	Sp	S8	S	Bm	M	T	B	F	Size (kb)	Reference
lck	++	+	–	–	ND	–	++	–	–	2.2	43
fgr	–	–	–	–	++	++	–	–	++	2.6	44
hck	+	+	–	–	++	+	–	++	+	2.4	45
lyn	–	+	–	–	ND	+	–	–	+	3.2	46
fes	–	+	–	–	++	++	–	–	–	2.8	47
ufo	+	+	++	++	+	–	–	–	+	4.2	48
fyn	++	+	+	+	+	+	+	+	+	2.8	49
tyro 8	+	+	–	–	++	+	–	++	–	2.3	29
fer	–	–	++	++	ND	++	–	++	++	2.4	50
blk	–	+	–	–	ND	–	–	+	ND	2.5	51

spun at 14,000 *g*, 15 min at 4°C, and the supernatants added to 2×SDS sample buffer. The proteins were separated on 7.5% SDS–PAGE gels, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked overnight at 4°C with 2% BSA, 0.2% Tween 20, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl. Ufo/Axl/Tyro7 protein was detected with affinity-purified rabbit polyclonal anti-Axl antibody (gift of Dr B. Varnum), followed by anti-rabbit peroxidase-conjugated secondary antibody staining. Blots were developed using enhanced chemiluminescence (Amersham, Amersham, UK).

Immunohistology

Frozen sections of thymuses from 8-week-old female B6 mice were incubated as described (7) with rat mAb ER-TR4 or ER-TR5 (staining cortical and medullary epithelial cells respectively) (7), or with affinity-purified rabbit-anti-Axl or -anti-Fyn polyclonal antibody after blocking with 10% normal rabbit serum. Negative controls were incubated with rat Ig (for ER-TR4 and ER-TR5) or rabbit Ig (for the polyclonal antisera). Second-step reagents were FITC–anti-rat Ig or FITC–anti-rabbit Ig. Sections were analyzed under a fluorescence microscope.

Results

Isolation of tyrosine kinase cDNAs from fresh TSC cells

Fresh mouse TSC were isolated from 10 female C57BL/6 mice. RT-PCR was performed with two different primer combinations (A and B) resulting in two pools of tyrosine kinase cDNAs of 148 and 213 bp in size. DNA sequencing was performed on 239 independent clones (59 from pool A and 180 from pool B). Database searches revealed that the following partial tyrosine kinase cDNAs were cloned: *lck* (23 A clones, 46 B clones), *fgr* (19 A, 43 B), *hck* (11 A, 16 B), *lyn* (1 A, 1 B), *tyro 8* (2A, 2B), *fes* (1 A, 25 B), *ufo* (0 A, 1 B), *fyn* (1 A, 12 B), *fer* (0 A, 13 B) and *blk* (1 A, 0 B). No novel kinases were identified and 21 clones appeared to be empty (10%). Figure 1 displays the amino acid sequences of the 10 tyrosine kinases that were sequenced. Comparison of the primary cDNA sequences reveals that nine cytoplasmic tyrosine kinases were isolated (*lck*, *fgr*, *hck*, *lyn*, *fyn*, *blk*, *tyro 8*, *fes* and *fer*) and only one transmembrane receptor type kinase: *ufo*. *Blk* and *tyro 8* belong to the cytoplasmic tyrosine kinase family encompassing *itk* (19), *tec* (40), *dsrc*28C (41) and *bpk* (42); *fes* and *fer* to the *fes* family; and *lck*, *fgr*, *hck*, *lyn*, and *fyn* to the *src* family kinases.

Expression of tyrosine kinase transcripts in TSC

In order to assess the expression of the above 10 tyrosine kinase cDNAs in TSC, Northern blotting experiments were performed on an extended panel of TEC lines that have been isolated by various groups of researchers from various mouse TSC preparations (see Methods). Since immortalized TEC lines often lose characteristics that are expressed by intact thymic epithelium, we also isolated RNA from complete thymus stromal cell preparations that had been cultured for 8 days, while regularly removing the unattached thymocytes (preparation S8) (26).

Surprisingly, only three of the isolated tyrosine kinases showed detectable expression in the short-term stromal cell culture S8 and TEC lines: *ufo*, *fyn* and *fer*. As is shown in Fig. 2, the 4.2 kb *ufo* mRNA is present both in S8 and in all TEC lines tested, while SP2/0 (B cell), RMA-S (T cell) and P388 (macrophage) are negative. Additional experiments revealed a rather broad expression pattern, *ufo* transcripts being present in spleen, heart, bone marrow and testis, but absent from kidney, liver and brain (not shown). The characteristic *fyn* mRNAs of 2.8 and 3.7 kb are clearly present in all TEC preparations tested, but the same mRNAs can also be detected in B cell, T cell, macrophage cell lines, thymus, spleen and bone marrow (Fig. 3). Liver and kidney are consistently negative for *fyn* (results not shown). Finally, 2.4 kb *fer* mRNA transcripts are present in S8, TEC lines, fibroblasts, macrophages and B cells (Fig. 4A), but could not be detected in whole thymus, T cells and spleen. Of the three PTK expressed in TSC, *ufo* is thus the only one of which expression is restricted to non-hemopoietic cell types.

None of the seven other isolated tyrosine kinases showed detectable mRNA levels in TEC lines as assessed by Northern blotting. For example, *lck* is clearly only expressed in mouse thymus, T cells and spleen (Fig. 5A) and absent from S8, TEC lines, B cells, macrophages and fibroblasts. Figure 5(B) displays an independent Northern blot hybridized to a mouse TCR β probe, showing the same expression characteristics as *lck*. This analysis confirms the lymphoid specificity of *lck* gene expression (43), and indicates that the original isolation of *lck* out of TSC cDNA was a result of contamination by thymocytes. Transcripts for *fgr*, *hck*, *lyn* and *fes* were easily detectable in the macrophage cell line P388 and other macrophage cell lines (data not shown), but not in any of the TEC lines or the TSC preparation S8 (data not shown). Presence of these PTK in fresh TSC is thus likely to have been a result of contaminating macrophages. The kinases *tyro 8* (Fig. 4B) and *blk* (data not shown) were differentially expressed in the different cell types tested, but not in TSC. Presence of *blk* and *tyro 8* in B cells suggests that B cell

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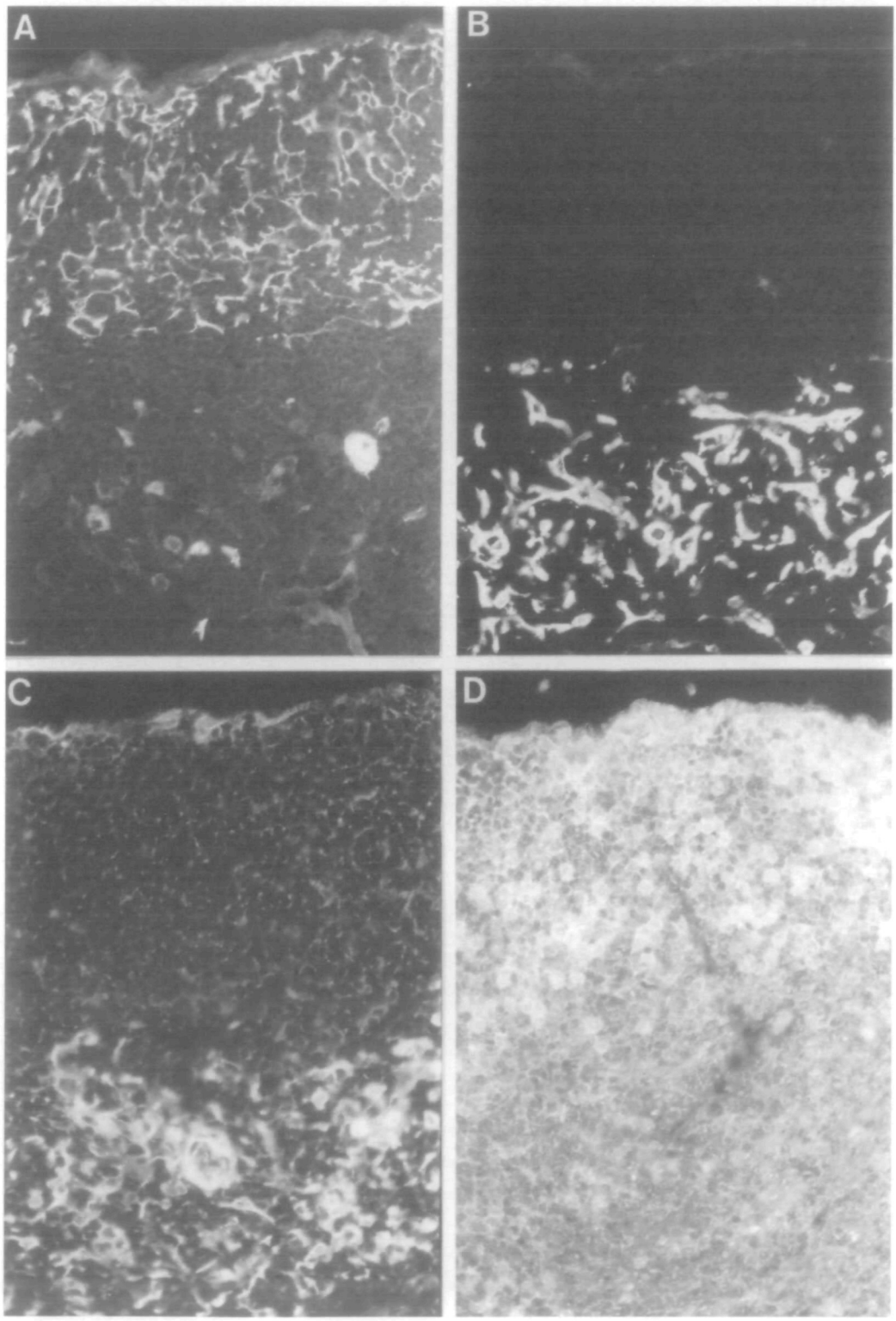

Fig. 6. Immunohistology analysis of thymus tissue sections. Frozen sections of thymuses from 8-week-old B6 mice were stained with ER-TR4 (cortical epithelial marker, A), ER-TR5 (medullary epithelial marker, B), anti-Axl (C) and anti-Fyn (D). Second-step reagents were FITC–anti-rat Ig (A and B) or FITC–anti-rabbit Ig (C and D); controls consisting of second step reagents alone or irrelevant rat or rabbit Ig followed by appropriate second step reagents did not reveal staining. Magnification: ×200.

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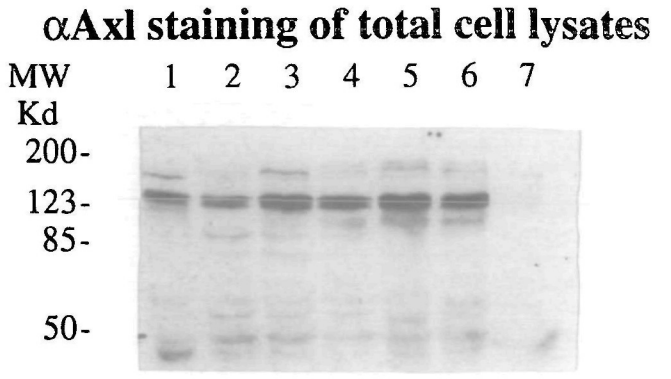

Fig. 7. Western blot analysis of Ufo/Axl expression on lysates from stromal cell lines BMS2 (lane 1), 2.4B6 (lane 2), 1D4 (lane 3), TEC (lane 4), MTE (lane 5) and TNCR3.1 (lane 6). Whole thymus lysate is shown in lane 7. Blotting with a negative control anti-*lck* antiserum gave no effect in lanes 1–6, consistent with the failure to detect *lck* mRNA in TSC lines on Northern blots (Table 1). Ufo/Axl appears in several species, consistent with earlier studies (53).

contamination of the TSC preparation may have contributed to the isolation of these kinases. Table 1 summarizes the expression characteristics of the isolated tyrosine kinases, as assessed by the above Northern blotting experiments. References to pertinent earlier descriptions of these kinases are listed as well (43–51). In summary, only *fyn*, *fer* and *ufo* are expressed in thymic stroma, and of these three PTK, the expression of *fyn* is probably the least relevant, since no defect in T cell development was noted in mutant mice that do not express *fyn* (52). The identification of *fer* and *ufo* as PTK expressed in thymic stroma should permit dissection of their role in signal transduction events in thymic stroma.

Protein expression of the TSC PTK Ufo

We next investigated the possible functional significance of *ufo* expression in thymic stroma by testing cell lysates of several TSC lines and thymic tissue sections for presence of Ufo/Axl protein. Such analysis was not possible for Fer because of our lack of success in generating a Fer-specific antiserum or monoclonal. Fyn protein was detectable in both lymphoid and non-lymphoid compartments of the thymus by immunohistology (Fig. 6D), consistent with the mRNA expression pattern (Table 1). Western blotting of lysates from 6 different stromal cell lines with an Axl-specific antiserum (kindly provided by Dr B. Varnum) clearly showed presence of the 140 kDa Ufo/Axl protein (Fig. 7, lanes 1–6), although it was barely detectable in lysates from whole thymus (lane 7). In addition, bands representing previously described (53) smaller immunoreactive species of Ufo/Axl at 120, 104 and 80 kDa were also found to a different extent in the various cell lines. It should be noted that Axl expression is not thymus-specific as also the bone marrow stromal cell line BMS (54) contains Axl (Fig. 7, lane 1). However, staining with anti-Axl on thymic tissue sections showed a reticular staining pattern (Fig. 6C), confirming that the expression in TEC lines is also reflected in the *in situ* thymus. The staining pattern obtained with anti-Axl (Fig. 6C) was different from that resulting from staining for the cortical epithelium marker detected by the ER-TR4 mAb (Fig. 6A). Staining resembled that obtained with the medullary epithelium-specific ER-TR5 mAb (Fig. 6B), although some staining with anti-Axl in the cortical compartment was also noted. In summary, Ufo/Axl is a suitable candidate for a receptor on thymic stroma receiving signals from the environment (13,14).

Discussion

This study was performed to get insight into the repertoire of PTK that are expressed in normal mouse TSC. These molecules are likely to play a role in the signal transduction pathways that are induced in TSC through interactions with thymocytes (13,14), but PTK that are functional in mouse TSC have yet to be identified. Some *in vitro* experiments point towards specific phosphorylation of TSC molecules upon contact with thymocytes (55). Two partial PTK cDNA sequences have been cloned from a medullary TEC line (56). However, since both immortalization of cells and the use of certain culture medium components, such as fibronectin (57), can influence the expression of PTK genes, we chose to characterize PTK expression in fresh, unstimulated mouse TSC.

For three of the PTK cDNAs amplified from fresh TSC, i.e. *ufo* (48), *fyn* (49) and *fer* (50), expression could be confirmed by Northern blotting of RNA from multiple TEC cell lines and short-term TSC cultures. Of these three PTK, only *ufo* exhibited an expression pattern restricted to non-hemopoietic cells. Expression at the protein level was confirmed for *ufo* by staining on tissue sections and Western blotting. Ufo/AxL/Tyro 7 represents a receptor PTK (48) with previously reported transforming ability (58,59), that has been implicated in development of the nervous system (29). It is broadly expressed in other developing tissues (60) and the product of the growth-arrest-specific gene 6 (*gas*6) (61) was recently shown to function as its ligand (62,63). Nothing is known about the function of *gas*6, although a role as a positive regulator of fibroblast growth has been suggested (63). Likewise, the related protein S has been reported to function as a growth factor for smooth muscle cells (64). The response of stromal cells to *gas*6 will be next investigated.

The choice of using degenerate PTK domain PCR primers to amplify cDNAs from freshly isolated TSC implies several drawbacks. First, the TSC preparation contains thymocytes, macrophages, B cells and fibroblasts. Second, the design of the PCR primers was based on PTK cDNA sequences that are known to date (29) and thus a bias will be introduced towards amplification of TSC kinases that have similar DNA sequences. Also, the primers used were biased towards cytoplasmic kinases (29). Therefore, it cannot be excluded that TSC express various other, novel PTK that are structurally more divergent from the ones detected here. Thirdly, a bias due to the use of degenerate PTK domain PCR primers was illustrated by variations in frequencies of PTK cDNAs amplified by the A series and B series of primers. For example, the A series proved unable to amplify *fer* cDNA because of several 3′ mismatches, while the B series performed well (13 clones). Finally, TSC are very heterogeneous in cellular composition, which implies that the exact cell type that expresses a certain PTK needs to be further defined after cloning. Most likely all of the above factors played a role in the present study,

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necessitating the conclusion that it probably underestimates the number of PTK operative in TSC. Nevertheless, this study identified three PTK present in TSC, *ufo*, *fyn* and *fer*, and expression of one of those, *ufo*, is restricted to cells of non-hemopoietic origin.

The other PTK cDNAs isolated from fresh TSC are not expressed by TSC. The notion that thymocyte cDNA was present in our original TSC cDNA preparation was confirmed by a positive PCR amplification of TCR β chain cDNA under high stringency conditions. Combining the Northern blot data with the various PTK cDNAs we isolated from fresh TSC cDNA, the following conclusions can be drawn: *lck* most probably derived from thymocytes, *fgr* and *lyn* derived from macrophages or fibroblasts, *hck* from macrophages, fibroblasts or B cells, *fes* from macrophages, *ufo* from fibroblasts and TEC, *tyro 8* from macrophages and B cells, *fer* from TEC, macrophages, fibroblasts and B cells, and *blk* from B cells. Finally *fyn* was found to be ubiquitously expressed in all cell types of either hemopoietic or stromal cell origin. Thus, for many PTK the exact TSC types of expression still need to be established. Transcripts for *ufo*, *fer* and *fyn* could easily be detected in both short-term TSC cultures devoid of hemopoietic cells (as detected by absence of CD45 staining), as well as in long-term lines. Given the observation that *fyn* knockout mice do not express any thymic phenotype (52), Fer and Ufo-R are therefore the best candidates for PTK operative in TSC, be it in signal transduction pathways that create the thymic microenvironment or in interactions that induce thymocyte differentiation directly. Our present efforts focus on the possible differential expression of these PTK in mutant mice in which the thymic microenvironment is affected. Also, the effects of interference in T cell development on these PTK with mAb against known interaction molecules will be studied. Together these experiments should clarify the functional significance of these PTK.

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Abbreviations

PTK	protein tyrosine kinase
RAG	recombinase activating gene
TEC	thymic epithelial cells
TSC	thymic stromal cells

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