Functional analysis of the T-cell-restricted protein tyrosine kinase Txk

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T lymphocytes express a range of tyrosine kinases that are involved in signalling processes driving cell activation, proliferation and differentiation. Two tyrosine kinases expressed only in T cells, the Itk/Emt and Txk gene products, are members of the Tec family of kinases. The role of Tec kinases in cellular function is poorly understood, although a Tec kinase specific to B cells, Btk, is essential for B-cell development. To explore the contribution of the T-cell-specific Tec kinases to lymphocyte function, we have expressed human Txk in the baculovirus system and conducted the first characterization of its activity. We

find that Txk exhibits a substrate preference *in vitro* quite distinct from that of the major T-cell kinases Lck and ZAP70, suggesting that Tec-family kinases might act on a distinct range of substrates. We also investigated the interactions of Txk with the cytoplasmic domains of the key signalling molecules CD3 ζ , CD28 and CTLA4 and find that none of these are phosphorylated by Txk, nor are they ligands for the SH2 or SH3 domains of Txk. We conclude that it is unlikely that Txk has a role in the early signal transduction events associated with these key pathways controlling T-cell activation.

INTRODUCTION

T cells contain a number of protein tyrosine kinases (PTKs), many of which have been demonstrated to have a vital role in the signalling cascades involved in cell activation. These have been analysed in most detail in CD4-positive T lymphocytes, in which activation by antigen-presenting cells requires two distinct classes of intracellular signal [1,2]. One signal is produced when antigen in association with class II MHC ligates the T-cell receptor complex. This results in the activation of Src-family PTKs, principally Lck and Fyn; phosphorylation of the paired tyrosine residues of the immunoreceptor tyrosine activation motifs (ITAMs) present in CD3 ζ and subsequently the recruitment and activation of another T-cell PTK, ZAP70 [3,4].

The outcome of this signal, i.e. whether the cell undergoes full activation, enters a non-responsive state or becomes destined to undergo apoptosis, is determined by a second, or co-stimulatory, signal arising from the ligation of various accessory molecules on the T-cell surface [5–9]. The best studied of the co-stimulatory signals is produced by the ligation of CD28 on the T-cell surface with one of two ligands expressed on APCs, CD80 (B7-1) [10] or CD86 (B7-2) [11–14]. In combination with the T-cell receptor signal, CD28 triggering drives T cells into full activation and protects them from apoptotic death [15,16]. Ligation of the CD28 molecule is associated with the phosphorylation of the cytoplasmic domain and the recruitment of a set of signalling intermediates (reviewed in [17]), including the p85 subunit of PI 3-kinase [18,19] and Grb2 [20].

The identity of the PTK(s) responsible for CD28 phosphorylation remains unclear. CD28 contains four tyrosine residues, occupying positions 173, 188, 191 and 200. Members of the Src family of PTKs, in particular Lck, have been shown to phosphorylate Tyr-173 [21,22], and the YMN motif containing this residue is the site of p85 binding [19]. The T-cell-specific Tec family PTK Itk/Emt [23–25] has been shown to be capable of phosphorylating CD28 at all four tyrosine residues [22]. Fur-

thermore Itk also interacts with CD28 through a PXXP-SH3-mediated mechanism, leading to an up-regulation of kinase activity [26].

In addition to Itk, T cells express another member of the Tec PTK family, Txk/Rlk [27–29]. Expression of Txk is restricted to T cells and mast cells, both of which express CD28 [27,28,30]. The two T-cell Tec kinases differ in their regulation: Itk is induced by the treatment of T cells with interleukin 2 [23,24], whereas Txk is expressed in resting lymphocytes [29] and down-modulated after cell activation [28]. These aspects of Txk regulation are consistent with a role in early activation events. However, nothing is known of the pathways that control its activation or the identity of its substrates.

To begin to analyse the contribution made by Txk to T-cell function, we have expressed catalytically active human p62TXK protein in the baculovirus system. We now report the first functional characterization of this enzyme, including a preliminary substrate preference analysis that suggests that Txk operates on a set of substrates distinct from those used by the other major T-cell kinases. Unlike other members of the Tec family, the activity of Txk is not modulated by an Src-family kinase.

We have also investigated the potential interaction of Txk with the major T-cell-signalling molecules CD3 ζ , CD28 and CTLA4, and show that none of these are substrates for Txk *in vitro*; neither can they serve to recruit Txk to a signalling complex via its SH3 or SH2 domains. We therefore conclude that Txk is unlikely to have an important role in the earliest signalling events through any of these three major pathways.

MATERIALS AND METHODS

Materials

Restriction and modification enzymes were purchased from New England Biolabs (Hitchin, Herts., U.K.) and Boehringer Mann-

Abbreviations used: EUSA, europium-linked immunosorbent assay; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine activation motif; PTK, protein tyrosine kinase; sulfo-NHS-LC-biotin, sulpho-*N*-hexylsuccinimide-long-chain-biotin.

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heim (Germany). Chemicals were obtained from Sigma (Poole, Dorset, U.K.), as were ELISA reagents. Oligonucleotides were synthesized at Glaxo Wellcome (Stevenage, Herts., U.K.). Peptides were from Peptide and Protein Research (Exeter, Devon, U.K.) or synthesized in-house at Glaxo Wellcome and were supplied at > 95% purity. Europium-labelled anti-phosphotyrosine antibody was from Wallac (Turku, Finland).

Cloning of human Txk

A gene encoding human Txk was isolated by PCR from a purified CD4-positive T-cell cDNA library. In brief, CD4-positive T cells were purified from 50 ml of normal donor peripheral blood as previously described [31]. Total RNA was recovered from these cells with the Stratagene RNA Isolation Kit (Stratagene, La Jolla, CA, U.S.A.), and polyadenylated RNA purified by oligo(dT) chromatography (Dynal, Oslo, Norway). Reverse transcription was primed with oligo(dT) and performed with Superscript II (Life Technologies, Paisley, Renfrewshire, Scotland, UK). From this cDNA library three clones, between them encompassing the coding sequence of Txk, were amplified, sequenced and then reassembled to recreate a single Txk cDNA. Although the primers used in this strategy created a construct tagged with a C-terminal EEF epitope, which was originally intended to facilitate detection of the recombinant protein [32], the tag was not used in the present study as the anti-tag monoclonal antibody YL1/2 was found not to detect the epitope efficiently in this context (R. Suttmüller, unpublished work).

Expression of PTKs in baculovirus

For expression of recombinant human Txk, an *EcoRI*–*NotI* cassette containing the gene for Txk was cloned into pVL1393 (PharMingen, San Diego, CA, U.S.A.) and the resulting plasmid was co-transfected with *Bsu*36I-digested BacPAK6 baculovirus DNA (Clontech, Palo Alto, CA, U.S.A.) into Sf9 insect cells by using lipofectin (Life Technologies). Supernatant from these cultures was collected after 60 h and was used to infect fresh dishes of cells. Cultures were overlaid with low-melting point agarose (SeaPlaque; Flowgen, Lichfield, Staffs., U.K.) and plaques were identified by staining with 0.08% Neutral Red (Sigma). Virus was recovered from plugs of agarose containing single plaques, scaled up through a second round of infection and then stored for future use. To identify clones of recombinant baculovirus, infected cells were analysed by SDS/PAGE on 10% (w/v) polyacrylamide gels. These were blotted on PVDF membrane, then probed with anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY, U.S.A.).

For the production of recombinant p62TXK, Sf9 cells were infected with recombinant baculovirus and harvested after 48 h. The cell pellet was lysed by sonication on ice in lysis buffer A [300 mM NaCl/50 mM Tris (pH 7.4)/5 mM EDTA/1 mM Na₃VO₄/0.5% (v/v) Triton X-100/10 µg/ml leupeptin/10 µg/ml aprotinin/100 µg/ml trypsin–chymotrypsin inhibitor/2 mM PMSF]. The lysate was clarified by centrifugation then stored in aliquots at –70 °C.

Kinase assay *in vitro*

For the assessment of kinase activity, a highly sensitive europium-linked immunosorbent assay (EuISA) was used to detect the phosphorylation of biotinylated substrates. Typically, kinase reactions were conducted in 40 mM Hepes (pH 7.4)/20 mM MgCl₂/1 mM MnCl₂/1 mM Na₃VO₄/200 µM ATP, with sub-

strate peptide added at 25 µM. Na₃VO₄ was omitted from some reactions; dithiothreitol was added to some reactions at 2 mM. Lysate from cells infected with recombinant baculovirus expressing PTK was added at the indicated concentration. Non-peptide substrates were used at concentrations of 2.5% (v/v) (amino acid copolymers) and 3.2% (v/v) [glutathione S-transferase (GST) fusions]. In other experiments, the substrate concentration was titrated, with the enzyme held constant. Reactions (50 µl) were prepared in a microtitre plate, incubated at 37 °C for 2 h with gentle shaking and then stopped by dilution 10–40-fold in cold 20 mM EDTA. Samples (20 µl) were then diluted 1:5 in assay buffer (Wallac) and transferred to wells of a microtitre plate coated with 2 µg/ml streptavidin (STAR1B; Serotec, Oxford, Oxon., U.K.). After incubation at room temperature for 10–30 min, the plate was washed; bound phosphotyrosine was detected with Eu-conjugated anti-phosphotyrosine monoclonal antibody. The amount of bound Eu label was determined by time-resolved fluorescence with the DELFIA system (Wallac).

Preparation of GST fusion proteins

cDNA inserts encoding the cytoplasmic domains of human CD28 and CTLA4 and mutants thereof were prepared by PCR from an overlapping oligonucleotide template. These were fused in-frame to the C-terminal end of GST by cloning into a derivative of the vector pGEX-4T3 (Pharmacia, St Albans, Herts., U.K.). A cDNA encoding the cytoplasmic domain of human CD3ζ was obtained by PCR from a random hexamer-primed human peripheral blood lymphocyte cDNA library and cloned in-frame into pGEX4T3. Clones with the correct sequence were transformed into *Escherichia coli* strain BL21 (R&D Systems, Abingdon, Oxon., U.K.); fusion protein production was induced by the addition of isopropyl β-D-thiogalactoside to the culture medium at 1 mM. After several hours of growth at 37 °C the bacteria were pelleted, lysed by sonication in lysis buffer B [25 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.2% (v/v) NP40/100 µg/ml chymotrypsin/trypsin inhibitor/100 µg/ml chymostatin/10 µg/ml leupeptin/10 µg/ml pepstatin/10 µg/ml aprotinin/2 mM PMSF] and the GST fusion proteins were purified by affinity chromatography on glutathione–Sephacrose 4B (Pharmacia), in accordance with the manufacturer's instructions, followed by dialysis into PBS.

Substrate biotinylation

To create biotinylated substrates for the kinase assay *in vitro*, purified GST fusion proteins were treated with sulpho-*N*-hexylsuccinimide-long-chain-biotin (sulfo-NHS-LC-biotin; Pierce and Warriner, Chester, Cheshire, U.K.). Purified GST proteins were buffer-exchanged by dialysis against 50 mM bicarbonate buffer, pH 8.3. Sulfo-NHS-LC-biotin was prepared in water at 1 mg/ml and added to the protein solution to give a 10-fold molar excess. The reaction was allowed to proceed at room temperature for 30 min; the unconjugated biotinylation reagent was then removed by dialysis against PBS.

Amino acid copolymers poly(Glu,Tyr) (Sigma catalogue number P0275) and poly(Glu,Ala,Tyr) (Sigma catalogue number P3899) were prepared as approx. 1 mM solutions in 50 mM bicarbonate buffer, pH 9.6, and then reacted with a 20-fold molar excess of sulfo-NHS-LC-biotin at room temperature for 2 h. Unincorporated biotin was removed by gel-filtration chromatography on a PD10 column (Pharmacia) equilibrated in 40 mM Hepes, pH 7.4.

Yeast two-hybrid system

Genes encoding the cytoplasmic domains of human CD28 and CTLA4, and mutants thereof, were prepared as described above and cloned as an in-frame fusion with Gal4BD in pAS2-1 (Clontech). Inserts encoding the SH2 (residues 144–252) (Clontech). Inserts encoding the SH2 (residues 144–252) and both SH3 and SH2 domains (residues 83–252) of human Txk were obtained by PCR with the full-length clone as a template. These were cloned into pACT2 (Clontech) to produce a Gal4AD fusion protein. A Gal4AD fusion protein incorporating the C-terminal SH2 domain of human p85 α was prepared in a similar fashion. All inserts were verified by fluorescent dye-terminator sequencing. Plasmids encoding the cytoplasmic domain of human CD3 ζ fused to Gal4BD and the dual SH2 domains of human ZAP70 fused to Gal4AD were gifts from Dr. M. Morse (GlaxoWellcome, Stevenage, Herts., U.K.). Selected pairs of Gal4BD and Gal4AD plasmids were transfected into *Saccharomyces cerevisiae* strain Y4.11ck and two-hybrid interactions were detected by staining for β -galactosidase expression as described [33].

RESULTS

Expression of active p62TXK in insect cells

A full-length cDNA encoding human Txk was generated by PCR amplification from a cDNA library prepared from CD4-positive T cells. The sequence of the cDNA matched exactly that published previously [27], with the exception of a single silent mutation at position 1542 of the coding sequence [GCC (Ala) to GCG (Ala)].

Infection of Sf9 insect cells with recombinant baculovirus expressing human Txk under the polyhedrin promoter resulted in the appearance of a prominent pair of phosphoprotein bands at approx. 65 kDa, most probably autophosphorylated p62TXK, and a number of fainter bands, presumably reflecting cellular proteins phosphorylated by the kinase. These bands were not present in uninfected Sf9 cells, nor in Sf9 cells infected with wild-type baculovirus (Figure 1, upper panel).

Lysate from cells infected with the Txk baculovirus was then used in a kinase assay *in vitro* with the biotinylated substrate poly(Glu,Tyr). Dose-dependent phosphorylation of the substrate was seen (Figure 1, lower panel), indicating that catalytically active Txk was present in the lysate. A similar result was obtained with the biotinylated substrate poly(Glu,Ala,Tyr) (results not shown).

The buffer requirements for activity of PTKs *in vitro* are modest: physiological pH, a low concentration of bivalent cations and substrate (ATP and phosphate acceptor). The bivalent cations normally employed are Mg²⁺ and Mn²⁺. To determine the favoured cation for p62TXK activity, we compared the effects of altering both the absolute bivalent cation concentration and the relative balance between Mg²⁺ and Mn²⁺ ions on phosphorylation by p62TXK of poly(Glu,Tyr). We found that a minimum bivalent concentration of 5 mM was required to maintain full activity, and that p62TXK displayed no strong preference between Mg²⁺ and Mn²⁺ (Figure 2).

Substrate preference of Txk

To obtain an indication of the type of tyrosine-containing sequences likely to serve as substrates for p62TXK, we examined its activity against a bank of peptide substrates (Table 1). pOPT and pEAY4 are good substrates for the Src-family PTKs, including p56LCK and p59FYN [34]. pEGF and pB3 are substrates for ZAP70 [35]. pSYK is the preferred substrate for p72SYK [36]. None of the substrates was phosphorylated by p62TXK at peptide concentrations up to 100 μ M. Because the

Figure 1 Active Txk can be expressed in insect cells

Upper panel: samples of cell lysate obtained from Sf9 cells infected with Txk baculovirus (lane 1) or control baculovirus (lane 2), or uninfected (lane 3), containing roughly equivalent amounts of cellular protein were separated by SDS/PAGE [12% (w/v) gel], blotted to nitrocellulose and probed for the presence of phosphotyrosine. The positions of molecular mass standards are shown at the left. Lower panel: samples of uninfected Sf9 lysate and Txk-infected Sf9 lysate were incubated in a kinase assay *in vitro* with the poly(Glu,Tyr) substrate; phosphorylation was determined by EuISA. Points are means $\pm$ S.E.M. for duplicate determinations.

p62TXK was active against poly(Glu,Tyr), and in control reactions the peptides were efficiently phosphorylated by PTKs of the Src and Syk families as predicted (Table 1), these results suggest that p62TXK has a substrate sequence spectrum quite distinct from these other classes of T-cell PTK.

Is Txk activity controlled by phosphorylation?

The activity of the related Tec family kinase Btk has been shown to be regulated by interaction with Src family kinases [37–40]. Transphosphorylation of Btk by Src family PTKs at a regulatory site within the kinase domain (Tyr-551) is followed by an activating autophosphorylation at a second site (Tyr-223) [41]. Itk also seems to require an interaction with Src-family PTKs before developing full activity [42,43]. Because a tyrosine residue homologous to Btk residue 551 is conserved in Txk (Tyr-420) and all other family members (Figure 3, upper panel), a requirement for Src-family phosphorylation at this site might represent a potential general control mechanism for Tec-family kinases.

Figure 2 Bivalent ion preferences of p62TXK

Constant amounts of Txk lysate and poly(Glu,Tyr) substrate were incubated in kinase buffers containing the indicated total bivalent ion concentration in the indicated ratios of Mg²⁺ and Mn²⁺; the degree of phosphorylation was determined by EuISA. Points are the means $\pm$ S.E.M. for triplicate determinations.

As we had expressed p62TXK in the absence of Src PTKs, we therefore predicted that the basal level of Txk kinase activity seen previously might be up-regulated on Src-family transphosphorylation of regulatory sites. To test this hypothesis, we co-incubated p62TXK lysate with a small amount of p56LCK lysate and looked for augmentation of kinase activity against poly(Gly,Tyr), a substrate of both kinases. The degree of substrate phosphorylation observed was consistent with independent activity of the two PTKs (Figure 4), with no evidence for any regulatory interactions. A similar set of experiments designed to examine whether p62TXK could influence p56LCK activity, with the peptide pOPT, which is a substrate for p56LCK but not p62TXK, produced the same result (results not shown).

To investigate further the properties of the predicted Src-family phosphorylation site, we used a peptide encompassing Tyr-420 (Figure 3, upper panel) as a substrate in a kinase assay *in vitro* with p56LCK. No phosphorylation was seen, despite full activity of the p56LCK preparation towards a control substrate (Figure 3, lower panel). In a preliminary experiment, this peptide was also not phosphorylated by p62TXK (results not shown). These results offer no support for Tyr-420 being a facile site of

Figure 3 Phosphorylation of potential control sequences

The protein sequences of human Txk (GenBank accession number L27071), Itk (S65186), Tec (D29767) and Bmx (X83187), and murine Btk (L29788) were aligned with the use of the CLUSTAL algorithm (upper panel). Dots indicate sequence identity with Txk. A region from the alignment is shown, centred on the Btk Src family phosphorylation site (arrowed): this is conserved in all family members. Lower panel: a peptide based on this region in Txk (boxed in the upper panel: pTxk-SP [biotin]-KVLDDVEYVSSFGA) was incubated with p56LCK lysate; phosphorylation was analysed by EuISA. The peptide pOPT was used as a positive control for p56LCK activity. Points are means $\pm$ S.E.M. for triplicate determinations.

either Txk autophosphorylation or transphosphorylation by Src-family PTKs.

Is Txk activity involved in the proximal T-cell activation pathways?

Because the Tec-family kinase Itk has been implicated in the proximal signalling pathways associated with the co-stimulatory receptor CD28, we investigated whether p62TXK could phosphorylate peptides derived from the cytoplasmic domains of human CD28. The peptide pCD28.1 encompasses Tyr-173, whose phosphorylation is required for p85 binding [19]. pCD28.2 includes two tyrosine residues, Tyr-188 and Tyr-191, which have

Table 1 Peptide substrate phosphorylation by Txk

Lysates from Si9 cells infected with baculovirus expressing either Txk, other T cell PTKs or no PTK (Wt lysate) were used in *in vitro* kinase reactions with substrate at the indicated concentrations. In control kinase reactions using poly(Glu,Tyr) as the substrate and 2-fold more Si9 lysate, Txk lysate produced a signal of 15387 ± 1442 c.p.m., and Wt lysate produced a signal of 2303 ± 119 c.p.m. pOPT and pEGF are variants of the optimal substrate sequences for p56LCK and the EGF receptor respectively [34]. pB3 comprises residues 2–16 of the cytoplasmic fragment of erythrocyte band 3 [35]. pSyk is a variant of the optimal p72SYK phosphorylation motif [36]. Phosphorylation of substrate was assessed by the EuISA method as described in the Materials and methods section. Results are given as means $\pm$ S.E.M. for triplicate determinations.

Substrate	Sequence	Peptide concentration (μ M) ...	Txk phosphorylation (c.p.m.)			Positive control PTK phosphorylation (c.p.m.) at 2.5 μ M substrate
			100	10	1	
pOPT	[biotin]-KSAESIYGVLFSG		1244 $\pm$ 184	1159 $\pm$ 64	1009 $\pm$ 68	498010 $\pm$ 11114 (Lck)
pEAY4	[biotin]-KEAYEAYEAYEAY		1261 $\pm$ 238	822 $\pm$ 75	1160 $\pm$ 219	461015 $\pm$ 11807 (Lck)
pEGF	[biotin]-EEEEYFELV		882 $\pm$ 48	1203 $\pm$ 123	1038 $\pm$ 75	45577 $\pm$ 2151 (Zap70)
pB3	[biotin]-EELQDDYEDMMEENL		1145 $\pm$ 242	863 $\pm$ 63	808 $\pm$ 82	127438 $\pm$ 11605 (Zap70)
pSyk	[biotin]-KEDPDYEWPS		845 $\pm$ 111	843 $\pm$ 61	894 $\pm$ 50	569323 $\pm$ 10594 (Syk)
						234642 $\pm$ 23534 (Fyn)
						268374 $\pm$ 16524 (Fyn)
						227198 $\pm$ 15280 (Syk)

Figure 4 Effect of Src-family kinase activity on p62TXK activity

Lysates containing active p62TXK or p56LCK were used either singly or in combination to phosphorylate poly(Glu,Tyr); the degree of phosphorylation was determined by EuISA. Points are means $\pm$ S.E.M. for triplicate determinations.

been implicated in the CD28-stimulated production of interleukin 2 [44] and are substrates for Itk [22]. Neither peptide was phosphorylated by p62TXK (Figure 5, upper panel).

To eliminate the possibility that other parts of the CD28 cytoplasmic domain absent from the peptides might be required to enable Txk phosphorylation, we prepared biotinylated GST fusion proteins containing the full-length cytoplasmic domains. These were incubated with p62TXK and analysed for phosphorylation by EuISA (Figure 5, lower panel). No phosphorylation was seen, confirming the result from the biotinylated peptides. A similar experiment performed with a GST fusion protein containing the cytoplasmic tail of CD3 ζ , the major signalling chain of the T-cell receptor, was also negative (Figure 5, lower panel). In another series of experiments the same CD28 and CD3 ζ GST fusion proteins were successfully phosphorylated by p56LCK and detected with the same EuISA (J. H. Ellis, unpublished work).

Can Txk be recruited to the CD3 ζ or CD28 signalling complexes?

Although Txk does not seem to have the capacity to phosphorylate the major stimulatory molecules CD3 ζ or CD28, it might be recruited to the complexes associated with activated forms of these proteins and might contribute to their signalling by the phosphorylation of downstream proteins. Txk possesses both an SH3 domain and an SH2 domain, giving the potential to bind to both proline-rich sequences and phosphotyrosine-containing motifs respectively. CD3 ζ possesses six potential phosphorylation sites; CD28 has four potential phosphorylation sites and two PXXP motifs.

To explore this possibility, we examined possible interactions between CD28, CD3 ζ and Txk in a form of the yeast two-hybrid system in which the potentially interacting proteins were expressed in the presence or absence of an active PTK [33]. Gal4AD fusion proteins containing either the SH2 domain or both SH3 and SH2 domains of Txk were coexpressed with Gal4BD fusion proteins containing the cytoplasmic domains of CD28 and CD3 ζ in a yeast strain containing p56LCK driven by a conditional promoter. The interaction between the fusion proteins was assessed with the p56LCK gene either active or repressed (Figure 6). No interactions with Txk were observed, even though control experiments with known SH2-binding

Figure 5 p62TXK does not phosphorylate CD3 ζ , CD28 or CTLA4 cytoplasmic domains

Upper panel: biotinylated peptides pCD28.1 ([biotin]-KLLHSDYMNMTTPR: residues 168–179 of human CD28), pCD28.2 ([biotin]-KRKHYQPYAPPRD: residues 185–196 of human CD28) and pCT4 ([biotin]-KLTTGVYVKMPPT: residues 159–170 of human CTLA4) were incubated at the indicated concentrations with p62TXK; substrate phosphorylation was determined by EuISA. In a parallel positive control reaction (results not shown), poly(Glu,Tyr) was phosphorylated by p62TXK. Lower panel: as for the upper panel, but with biotinylated GST fusion proteins containing the complete cytoplasmic domains of CD3 ζ , CD28 and CTLA4 instead of peptide substrates. Points are means $\pm$ S.E.M. for triplicate values.

partners demonstrated successful phosphorylation and accessibility of the CD28 and CD3 ζ fusion proteins.

Txk and CTLA4 signalling

In addition to the major stimulatory signals transduced by CD3 ζ and CD28, T cells also have a key inhibitory pathway controlled by the interaction of CTLA4 with its ligands CD80 and CD86 [45–49]. Because CTLA4 is strongly homologous to CD28, and Itk has been implicated in CD28 signalling, we investigated whether Txk might have an analogous role for CTLA4.

p62TXK did not phosphorylate either a peptide encompassing the putative p85-binding site from human CTLA4 (Figure 5, upper panel), or a GST fusion protein containing the entire cytoplasmic domain (Figure 5, lower panel). Additionally, the SH2 and SH3 domains of Txk were assayed for binding to CTLA4 in the yeast system: no interaction was seen whether or not p56LCK was present (results not shown). These results suggest that Txk does not contribute to the early stages of the CTLA4 signal, either by phosphorylation of CTLA4 or by direct recruitment to activated CTLA4.

Figure 6 Interactions between CD28, CD3 ζ and Txk

Gal4AD fusion proteins containing the SH2 or combined SH3 and SH2 domains of Txk were assayed for interaction in the yeast two-hybrid system with Gal4BD-CD28 or Gal4BD-CD3 ζ fusion proteins in the presence or absence of p56LCK. Interactions were detected by staining for β -galactosidase activity. Known binding partners for phosphorylated CD28 and CD3 ζ (C-terminal SH2 domain of p85 and dual SH2 domains of ZAP70 respectively) were included as positive controls.

DISCUSSION

Txk activity *in vitro*

The sequence that we obtained for human Txk is in almost complete agreement with that reported previously [27] (GenBank accession number L27071). The single silent mutation that we observed might reflect a natural allelic variant or a misincorporation error introduced during the cloning process. As the amino acid sequence was unaltered, we did not pursue this finding further. Expression of Txk cDNA by recombinant baculovirus produced a protein that was constitutively active, both within Sf9 cells and against two broad-specificity PTK substrates. The presence of at least 5 mM Mg²⁺ or Mn²⁺ was required for maximum activity, although there was no preference between these two cations.

The physiological substrate specificity of a PTK arises from a combination of factors. Foremost among these are the inherent properties of the enzyme's active site and the availability of substrate proteins, as affected by subcellular compartmentalization and complex formation. Simple kinase assays *in vitro* allow investigation of only the intrinsic specificity of the enzyme: they indicate whether a particular substrate is capable of being phosphorylated but do not permit the identification of physiologically relevant interactions. Despite this limitation, such assays have yielded significant insight into the properties of PTKs. In particular, through the use of libraries of short peptide substrates, Cantley and colleagues have demonstrated that phosphorylation by PTKs requires that the acceptor tyrosine residue lie within a particular sequence context [34]. Related kinases were found to display similar substrate sequence preferences, although unique optimal peptides could be distinguished for each enzyme.

Adopting a more restricted approach, we have used a set of prototype peptides that are good substrates for a range of well-characterized PTKs, including key members of the Src and Syk families. None of these peptides were phosphorylated by

Txk, even at high substrate concentrations. Since these experiments were conducted in conditions under which Txk is clearly active (Figure 3, upper panel), these results suggest that the preferred sequences for Txk phosphorylation are not similar to those for Src- and Syk-family kinases. We therefore predict that, *in vivo*, Txk will act on a set of substrate sites quite distinct from those of the other major T-cell PTKs Lck, Fyn and ZAP70.

Control of Tec-family kinases

Two other Tec-family PTKs have been studied *in vitro*: Btk, which is expressed in B cells, and Itk, which is a T-cell-specific kinase. These investigations have revealed a number of control mechanisms that modulate the kinase activity. First, all Tec kinases share a conserved Tyr residue in the activation loop of the kinase domain (see Figure 4, upper panel). For Btk this residue has been proposed to participate in a sequential activation mechanism [41]. Transphosphorylation by a Src family kinase at this position, Tyr-551, (termed site 1) is required for full activation, which is associated with subsequent autophosphorylation at a second tyrosine residue (Tyr-223, termed site 2) [37,39–41]. Consistent with this model, Itk activation has been reported to be greatly decreased in cells lacking Lck [42,43].

These results for Btk and Itk contrast significantly with the results reported here for Txk. Not only was p62TXK constitutively active in Sf9 cells, phosphorylating both itself and other proteins, but this activity could not be potentiated by co-incubation with highly active p56LCK under conditions permissive for both enzymic activities. These findings are consistent with our additional observation that a peptide encompassing the Txk homologue of site 1 (Txk residue 420) failed to be phosphorylated by p56LCK. Furthermore we note that, whereas the tyrosine residue itself is absolutely conserved within the Tec family, the residues either side of Tyr-420 in Txk are different from their homologues in other Tec PTKs (see Figure 4, upper panel); these are changes that might significantly alter the properties of Tyr-420 as a PTK substrate [34]. These structural differences, together with our functional results, lead us to suggest that Txk might differ from Btk and Itk in the scope for regulation by Src-family PTK phosphorylation, particularly at site 1.

Reconciliation of these observations and the Btk two-site model might be achieved if, in Txk, site 1 is actually a major target for autophosphorylation rather than Src-family transphosphorylation. In this case, our p62TXK preparations would be fully activated before incubation with Src-family kinase, and no further up-regulation would be possible. However, although we do not have definitive mapping data on the autophosphorylation sites of Txk, a preliminary peptide experiment (see the Results section) failed to show any autophosphorylation of the Tyr-420 site 1 sequence, as would be predicted by this model.

A second control mechanism has been described for Itk, in which an intramolecular interaction between a PXXP motif and the SH3 domain holds the molecule in a 'closed' form in which the kinase activity is suppressed [26,50]. Binding of the PTK SH3 domain by a proline-rich ligand disrupts this structure and allows the kinase domain to become active. The intramolecular regulatory motif for ITK comprises the sequence KPLPPTP, and this sequence is well conserved in all members of the Tec family, including Txk (Figure 7, box 1), suggesting that Txk activity too might be regulated in this fashion.

Although our results do not indicate directly whether Txk activity is regulated by the KPLPPTP motif, the degree of activity obtained from the expression of Txk in a non-lymphoid cell, in which no specific activating SH3 ligands might be

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hITK 154 KKPLPPTPEDN-----RRPLWEPEE-TVVIALYDY
mITK 160 KKPLPPTPEDN-----RRSFQEPEE-TLVIALYDY
hBTK 185 KKPLPPTPEDQIL-KKPLPPEEAAAPVSTSELKKVVALYDY
mBTK 185 KKPLPPTPEDQIL-KKPLPPEETAAPISTELKKVVALYDY
hTEC 154 KKALPPAHEE-----KKRRPPPIPLEEDNSEE--IVVAMYDF
hTXK  69 KKPLPPLH-----PSEVAEEKIQVKALYDF

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Box 1 Box 2

Figure 7 Proline-rich regions in Tec-family kinases

The proline-rich regions of the Tec-family PTK members Itk (human and mouse), Btk (human and mouse), Tec (human) and Txk (human) were aligned by the CLUSTAL algorithm followed by manual refinement. Regions with similarity to the ITK control sequence KPLPPT [50] are boxed.

expected, suggests that certainly for Txk any closed structure might not preclude significant enzymic activity. This might reflect a weaker inhibitory intramolecular interaction; consistent with this and uniquely among Tec-family kinases, Txk lacks the PH domain, which has been suggested to stabilize the 'closed' state by interacting with the kinase domain [50].

Interestingly, we note that Btk, and to a smaller extent Tec, possesses a second sequence that is highly similar to the regulatory motif (Figure 7, Box 2), suggesting that these kinases might have extra scope for interactions not available to Itk and Txk. The distance between the repeats is similar to that between the phosphotyrosine motifs of an ITAM (reviewed in [51]), raising the intriguing possibility: that of a co-ordinate interaction with a bidentate paired-SH3 partner.

Tec-family kinases have also been reported as having regulatory interactions with the G-protein system. The catalytic activity of Btk is stimulated by G_{α} in the GTP-bound state [52], and Itk has been reported to be stimulated by $G\beta\gamma$ subunits [53]. Because G-protein subunits are known to bind to PH domains [54], it might be that these interactions are not relevant to Txk control, although the present results do not bear directly on this question.

In summary, our results showing that p62TXK is constitutively active *in vitro* and is not influenced by a Src-family PTK activity suggest that Txk might differ from other members of the Tec family in the way that the kinase activity is controlled. This hypothesis is further supported by several structural differences between Txk and the other Tec PTKs.

Tec-family kinases in T-cell signalling

The only well-characterized contribution of a T-cell-specific Tec PTK to signalling lies in interactions between Itk and the CD28 co-stimulatory system. The results obtained so far indicate that Itk contributes to CD28 signalling in two ways. First, it is recruited to the activated CD28 complex, binding through its SH3 domain to a PXXP motif in the CD28 cytoplasmic domain [26]. Once co-localized with the signalling complex, Itk might phosphorylate downstream effector molecules. Secondly, CD28 is itself a substrate for Itk, with all four tyrosine residues able to be phosphorylated by the Itk kinase activity [22]. These extra phosphotyrosine residues might then form SH2 docking sites for the recruitment of adaptor proteins and other molecules to the signalling complex, although this has yet to be demonstrated other than for Tyr-173.

Despite these findings, the physiological importance of the interaction between CD28 and Itk is brought into question by the observation that Itk-deficient mouse T cells are not defective in proximal CD28 signalling [55]. These results can be reconciled by postulating that there is some redundancy in the Itk-CD28 interaction that allows another protein to compensate for the Itk

deficiency. As a related T-cell-specific PTK, Txk would be a prime candidate for this role.

However, the present results are not consistent with this hypothesis. In kinase assays *in vitro*, CD28 cytoplasmic domain is not phosphorylated by active p62TXK, suggesting that it is unlikely to form a substrate *in vivo*. Furthermore, in the yeast two-hybrid system, we were unable to obtain any evidence for any direct association between CD28 and the Txk SH2 or SH3 domains. These results do not support the suggestion that Itk and Txk might have interchangeable functions in CD28 signalling.

In a similar vein we have investigated whether Txk has the capability to contribute to the TCR or CTLA4 signalling complexes. With respect to the kinase activity, neither CD3 ζ nor CTLA4 cytoplasmic domains were phosphorylated by p62TXK *in vitro*. Assays for SH2- or SH3-mediated associations between these molecules and Txk were also negative, suggesting that Txk does not contribute to the formation of, or participate in, the primary signalling complexes.

It is possible that the yeast two-hybrid system employed to investigate these potential interactions might not be permissive for some essential structural aspect of the interaction between the Txk SH2 and SH3 domains and their ligands. To partly control for this, the yeast experiments were conducted with the full-length cytoplasmic domains of CD3 ζ , CD28 and CTLA4. Furthermore the CD28 and CD3 ζ domains were shown in control reactions to be both phosphorylated and subsequently bound by physiologically relevant SH2-domain fusion proteins (Figure 6), indicating that the yeast system is compatible with these SH2-phosphoprotein interactions. Moreover, to corroborate our negative results in the yeast system, we have also examined anti-phosphotyrosine Western blots of immunoprecipitates of CD3 and CD28. These do not show a phosphorylated band at 62 kDa (results not shown), as would be expected if active phosphorylated p62TXK were associated with these receptors.

In summary, the present results indicate that Txk might be quite different from other members of the Tec family with respect to its control mechanisms, reflecting structural differences between the proteins. The substrates for the Txk kinase activity are likely to be distinct from those phosphorylated by Src- and Syk-family kinases. Furthermore, even within the Tec family, it seems unlikely that Txk and Itk have overlapping functions, at least with respect to CD28 signalling. Our results also suggest that Txk is not involved in the earliest signalling events associated with the CD3 ζ or CTLA4.

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