INVESTIGATION OF FUNCTIONAL DIFFERENCE BETWEEN TecIII and TecIV IN MAMMALS COS-1 CELLS USING GFP FUSION PROTEINS

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ABSTRACT

Signal transduction cascades are critical components of intra- and inter-cellular communication. Key component of such cascades includes tyrosine kinases. One such family of tyrosine kinases is the Tec family of tyrosine kinases. This family of tyrosine kinases is expressed mainly in cells of the hematopoietic lineage, and mutations in at least one of its one member of this family, Btk, has so far been associated with the human immunodeficiency disorder X-Linked Agammaglobulinemia. Two major isoforms of the Tec transcript, referred to as *TecIII* and *TecIV*, have been detected in various mouse embryonic and adult tissues, as well as in a number of different hematopoietic cell lines: Tec IV is the full length Tec protein with functional PH, TH, SH3, SH2 and Kinase domains, while *TecIII* is generated by the splicing out of exon 8 sequences to yield a shorter peptide with a non-functional SH3 domain. Using GFP-TecIII fusion proteins, this shorter isoform of Tec was shown to have biological characteristics that differed from TecIV.

Keywords: tyrosine kinase, Tec, GFP fusion

INTRODUCTION

Tyrosine kinases are important constituents of the various signal transduction pathways that operate within cells. The Tec family of protein tyrosine kinases is a member of the much larger family of non-receptor/intracellular protein tyrosine kinases depicted in Figure 1. Tec family kinases are generally restricted to cells of the hematopoietic lineage with some relatively high degree of lineage specificity, though various members of the family have been detected in a subset of non-hematopoietic tissues(Mano et al., 1990, Siyanova et al., 1994; Heyeck and Berg, 1993; Smith et al., 1994; Tamagnone et al., 1994; Sommers et al., 1995).

The Tec family includes Tec (Mano et al., 1990), Btk (Thomas et al., 1993), Bmx (Tamagnone et al., 1994), Itk (Siliciano et al., 1992) and Txk (Sommers et al., 1995), making it the second largest family of intracellular Protein Tyrosine Kinases (PTKs). They are characterised by the presence of Src Homology (SH-)3, Src Homology (SH-) 2 and kinase domains. The N-terminal region of Tec kinases typically consists of a Pleckstrin Homology (PH) domain which is followed by a Tec Homology (TH) region (Vihinen et al., 1994) and one or two proline rich regions (PRR), except in Txk which lacks the characteristic PH region and has a shorter cysteine rich sequence instead (Sommers et al., 1995). Together with the PH domain, the TH region forms a structural unit that is highly characteristic of the Tec family and is referred to as the PHTH domain with 38-50% sequence identity shared between Tec family members (excluding Txk).

Mano first reported the isolation of *Tec* in 1990, since then at least five *Tec* mRNA isoforms have been reported in the literature that differ in the PHTH, SH3 and kinase regions (Mano et al., 1990; Mano et al., 1993). These transcripts give rise to proteins that vary in their PHTH, SH3 and kinase domains respectively. The study

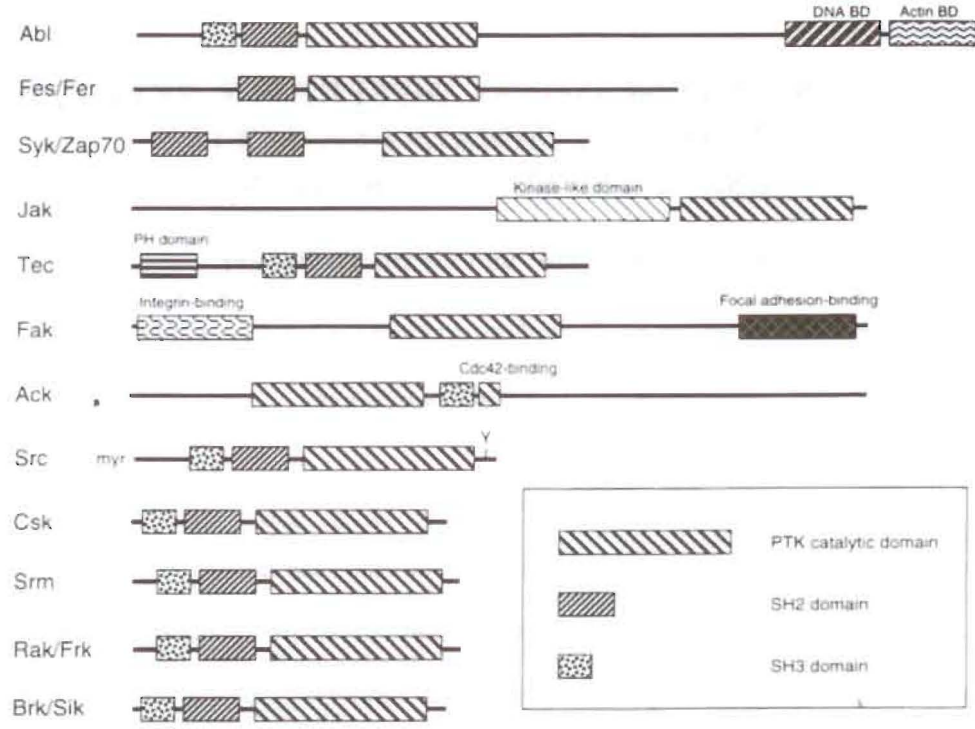

Figure 1. Nonreceptor protein tyrosine kinases (adapted from Hunter, 1998). Intracellular protein tyrosine kinase families are arranged according to their secondary structure. Common domains are depicted as shaded boxes. The various domains represented in this diagram include the SH3, SH2 and Kinase domains as well as the PH and TH domains characteristic of the Tec family of protein tyrosine kinases. (SH: Src Homology, PH: pleckstrin Homology, TH: Tec Homology)

described in this paper concentrates on two of these transcripts, *TecIII* and *TecIV*. Translation of *Tec III* and *IV* occurs in reading frame 1 and initiates at the first ATG codon located in exon 2. The full length *TecIV* transcript encodes functional PHTH, SH3, SH2 and kinase domains, but no Src-like negative regulatory Tyr residue is present in the C-terminal tail of the kinase domain. Compared to TecIV, TecIII has a truncation of 22 amino acids that is predicted to remove critical structural components of the SH3 fold. Studies by Andreotti et al. (1997) have revealed that the SH3 domain of Tec kinases is capable of interacting with poly-proline rich sequence(s) in the PRR region, of which Tec has two. Similar interactions have been reported for Tec (Pursglove et al., 2001). This interaction is predicted to prevent the docking of other SH3 binding proteins thus effectively sequestering the SH3 from other signalling molecules.

Because of the difference in their SH3 domains, TecIII and IV potentially differ in their biological characteristics, including in the range of protein-protein interactions in which they can participate. Some of these differences are addressed in this study.

MATERIALS AND METHODS

Plasmid pEGFP-C2 was a kind gift from Dr. S. McColl (Department of Molecular Biosciences, University of Adelaide). Plasmids pEGFP-C2-TecIII pEGFP-C2-TecIV were provided by Dr Anita Merkel (Department of Molecular Biosciences, University of Adelaide). Chinese Hamster Ovary (CHO) cells were maintained at 37°C in DMEM (GIBCO, Life Technologies) supplemented with 10%(v/v) fetal bovine serum, under 10% CO_2. For fluorescence microscopy, cells were plated on glass cover slips in 6-well plates at 10^5 cells/well.

Large scale preparation of plasmid DNA. Large scale DNA preparations were obtained from 500

ml cultures of LB supplemented with ampicillin (100μg/ml) inoculated with a single bacterial colony and grown at 37°C with shaking overnight. Bacterial cultures were centrifuged at 5,000 r.p.m. for 10 min. Pellets were resuspended in 16 ml Solution 1 (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0) and incubated at room temperature for 5 min. Cells were lysed in 40 ml Solution 2 (0.2 M NaOH, 1% (w/v) SDS) for 10 min on ice, and neutralised on ice with 30 ml Solution 3 (5M Potassium acetate, 11.5% (v/v) glacial acetic acid) for 10 min. Cellular proteins and chromosomal DNA were removed by centrifugation at 13,000 r.p.m. for 15 min. To precipitate plasmid DNA, equal volumes of isopropanol were added and pellets were subsequently collected by 15 min centrifugation at 12,000 r.p.m. (4°C), resuspended in 400μl 1x TE and transferred to fresh 1.5 ml eppendorf tubes. Bacterial RNA was removed with 50 μl RNase A (10mg/ml) at 37°C for 30 min, and DNA preparations were further cleaned by phenol/chloroform and chloroform extractions. Plasmid DNA was finally precipitated with 2.5 volumes 95% ethanol at maximum speed. Pellets were then washed with 70% ethanol and resuspended in 1x TE.

CsCl purification of plasmid DNA. Plasmid DNA required for transfections into mammalian cells are required to be of high quality and purity. Typically, approximately 300 μg of plasmid DNA prepared following the protocol described in section 2.3.2 was resuspended in 720 μl 1x TE with 1.26 g CsCl. Following the addition of 120 μl of the DNA intercalating agent EtBr (10mg/ml), the DNA-containing solution was underlaid into a TL100 tube (Beckman) containing 1.4 ml CsCl (65% (w/v)). The tubes were sealed and centrifuged at 100,000 r.p.m. for 3 hrs at 20°C. Plasmid DNA was drawn with a 1 ml syringe, transferred to 1.5 ml eppendorf tubes and extracted 3x with equal volumes of H_2O saturated butan-1ol, and precipitated with two volumes of 95% Ethanol. DNA pellets were collected by centrifugation at maximum speed at room temperature for 10 min. Pellets were finally rinsed with 70% ethanol and the DNA was resuspended in 1x TE.

COS-1 cell Culture. COS-1 cells were routinely maintained at 37°C in DMEM (GIBCO, Life Technologies) supplemented with 10%(v/v) fetal bovine serum, under 10% CO_2. For immunofluorescence, cells were plated on glass cover slips in 6-well plates at 10^5 cells/well.

Fugene™ transfection of GFP construct in COS-1 Cells. Transient transfection required for immunohistochemical analysis of Tec isoforms were carried out in 6 well plates. Briefly, 10^5 cells were plated on glass cover slips 24 hours prior to transfection. Cells were transfected according to the manufacturer's instruction. Typically, 3μl transfection reagent were diluted in 97μl serum-free DMEM and incubated at room temperature for 5 min. The diluted transfection reagent was subsequently added to 1μg of purified plasmid and complex formation was carried out at room temperature for 15 min. This mixture was finally added to freshly changed medium on adherent cell layer.

Fluorescence microscopy. GFP and GFP fusion proteins were visualised after 24 hours in PBS using a Nikon inverted (ECLIPSE TE 300) microscope. To visualise nuclei, cells were fixed in pre-chilled methanol for 2 min, rehydrated in PBS for 15 minutes and permeabilised in PBS/ 0.1% TritonX-100 for 10 min. Nuclei were stained with Hoechst (1μg/ml) for 30 sec and thoroughly washed in PBS/0.1% Tween-20.

RESULTS AND DISCUSSION

Characterisation of the subcellular localisation of GFP-TecIII and GFP TecIV

Biological differences between *TecIII* and *TecIV* were investigated using fusion genes consisting of *TecIII* or *IV* cDNA sequences fused in frame to the 3' end of the green fluorescent protein (GFP) coding region. The fluorescent GFP moiety enables direct visualisation of the fusion protein in living cells by fluorescence microscopy without the need for fixation and immunostaining. This permits comparative studies of the subcellular distribution of the two isoforms of Tec to be carried out in living cells, in real time, and facilitates the characterisation of anticipated translocation(s) in the presence of specific stimuli. All transfections were transient to avoid the selection of non-representative clones that might arise from the procedure of establishing stable transfectants. Plasmids carrying GFP-Tec fusion sequences

were transfected into COS-1 cells. COS-1 cells are routinely used as a model system for transient transfection experiments. They grow adherently as flattened cells with large cytoplasmic volumes which facilitates the visualisation of the subcellular distribution of fusion proteins in transfected cells. The subcellular localisation of Tec fusion proteins is expected to reflect that of its endogenous counterpart, although it is possible that the exogenous overexpression system used in these transfections might affect the fluorescence pattern observed.

The sizes of the fusion protein products were confirmed by Western blotting analysis of whole cell lysates (Figure 2G). GFP fusion partner alone yielded the predicted 29 kD GFP product, while GFP-TecIV and GFP-TecIII migrated at the 100kD mark. Surprisingly GFP-TecIII appeared to migrate slower than GFP-TecIV despite its 2kD deletion. This is proposed to reflect differences in post-translational modification of the two peptides, most likely phosphorylation

Visualisation of GFP transfected cells was carried out on a Nikon inverted (ECLIPSE TE300) microscope. GFP was present diffusely in the cytoplasm and nucleus of transfected cells and showed no specific subcellular localisation (Figure 2A). The GFP-PHTH fusion protein was also present diffusely in the cytoplasm and nucleus of transfected cells (Figure 2B). Rarely, in less than 5% of transfected cells, GFP-PHTH expressing cells were identified that were phenotypically distinct from the rest of the population. These cells were mostly larger than other transfectants and displayed extensive

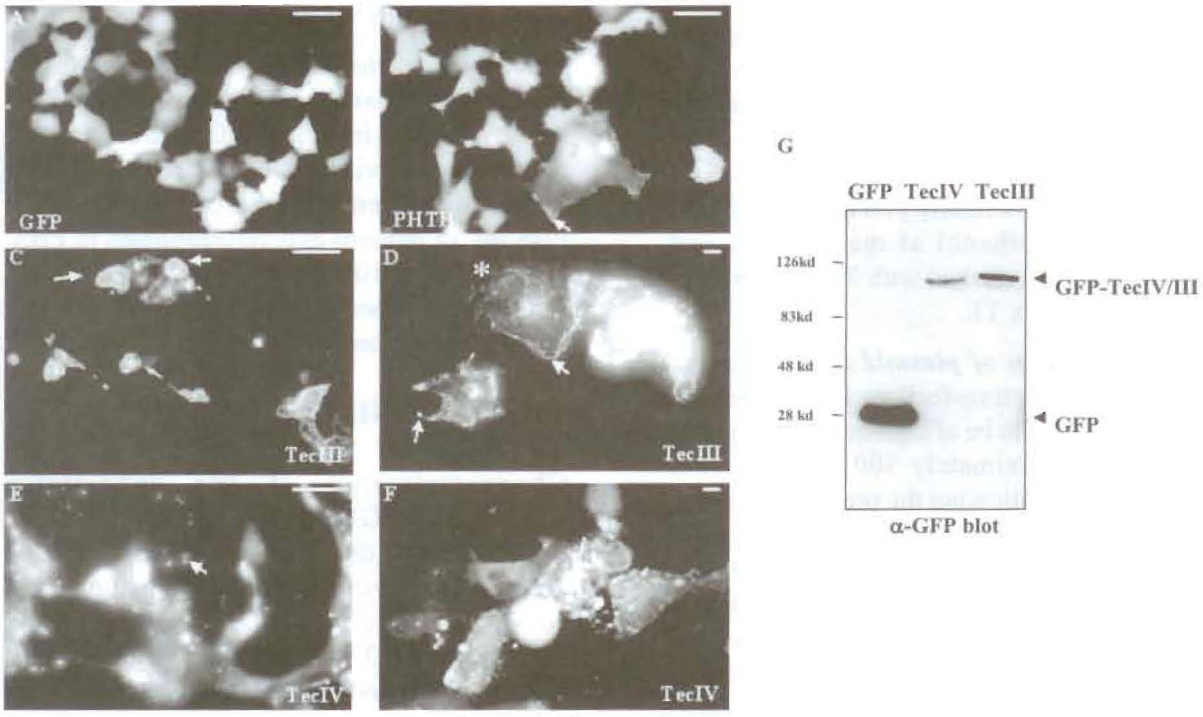

Figure 2. Characterization of the subcellular localisation of GFP fusion proteins in COS-1 cells. Fluorescence analysis of COS-1 cells expressing (A) GFP, (B) GFP-PHTH, (C-D) GFP-TecIII and (E-F) GFP-TecIV. $5x10^6$ COS-1 cells were transfected with 2µg plasmid DNA (A) pEGFP-C2, (B) pEGFP-C2-PHTH, (C-D) pEGFP-C2-Tec3 and (E-F) pEGFP-C2-Tec4 for 18 hours at 37°C using the Fugene™ transfection system. Cells were grown at 37°C in complete medium for at least 24 hours prior to visualisation. The arrows in (B) mark membrane ruffle-like region characterised by plasma membrane fluorescence. The arrow in C marks cells with rounded morphology. The * in (D) highlights cells with asymmetrical distribution of plasma membrane GFP-TecIII and the arrows highlight fluorescent filopodia extensions. The arrow in (E) points to vesicular-like, punctate fluorescence of GFP-TecIV and the arrow in (F) shows juxtanuclear GFP-TecIV. Bar = 100?M (A-C; E) and 10?M (D & F). (G) Western immunoblot analysis of total cell lysates prepared from COS-1 cells expressing GFP, GFP-TecIV and GFP-TecIII. Protein samples were separated by SDS-PAGE electrophoresis on an 8% Tris-tricine polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond-C), probed with GFP-specific antibody and HRP-conjugated anti rabbit antibody, and detected by enhanced chemiluminescence.

cytoplasmic extensions and membrane ruffle-like structures that were enriched with GFP-PHTH (Figure 2B arrow).

When TecIV was fused to the C-terminus of GFP, a distinct change in the subcellular distribution of the fusion protein compared to that of GFP or PHTH alone was observed (Figure 2E-F). In general, within a single population of transfected cells, three distinct fluorescence patterns were observed that included: (i) diffuse staining in the cytoplasm and nucleus of transfected cells, undistinguishable from that of GFP alone. (ii) Intense punctate staining generally in combination with diffuse fluorescence in highly fluorescent cells (Figure 2E arrow). This was suggestive of vesicular distribution and appeared to be associated with fine structures extending from the cytoplasm. An intense juxta-nuclear pool of fluorescence, suggestive of the endoplasmic reticulum/golgi network, was also observed in the majority of these cells (Figure 2F arrow). (iii) Plasma membrane staining that was in general accompanied by punctate fluorescence.

The pattern of fluorescence observed for GFP-TecIII fusion proteins was distinct from that described above. Although, overall the same three patterns were identified, a larger proportion of transfected cells showed intense plasma membrane localisation of the fusion protein in ruffle-like membrane projections, as well as in fine filopodia-like extensions (Figure 2C-D). Interestingly, two distinct populations of GFP-TecIII cells appeared to exhibit plasma membrane localisation of the fusion protein. The first population of GFP-TecIII cells had large cytoplasmic regions, generally accompanied by an intense juxta nuclear pool of GFP-TecIII fusion proteins and plasma membrane localised GFP-TecIII in plasma membrane ruffle-like regions (Figure 2D asterisk and arrow) suggesting a potential role for TecIII in the formation of such structures. The second group of GFP-TecIII cells were smaller and had typically round cytoplasms, with distinctive intense plasma membrane fluorescence (Figure 2C arrow). Because they often possessed smaller, condensed nuclei it was expected that these represent a subpopulation of poorly growing cells.

As previously described, the population of transfected GFP-TecIII and TecIV cells were heterogenous in their fluorescence pattern. The proportion of each characteristic pattern was thus quantified to provide an insight into the major characteristics that differentiate the two major isoforms of Tec. As described in Figure 3, 100% of GFP and approximately 98% of GFP-PHTH transfected cells showed diffuse cytoplasmic/nuclear localisation, while only 50% GFP-TecIV and less than 10% GFP-TecIII transfected cells showed the same diffuse staining pattern. Plasma membrane targeting was only observed in approximately 2% of GFP-PHTH cells, 15% of GFP-TecIV cells and 45% of GFP-TecIII cells. Plasma membrane targeting was thus most prominent in GFP-TecIII cells compared to GFP-TecIV cells. Punctate fluorescence was scored based on the presence of distinctive vesicular-like fluorescence, with or without the presence of an intense juxta-nuclear pool of fusion protein. When punctate fluorescence was observed in cells with an otherwise diffuse fluorescent pattern, those cells were counted as punctate. As seen in Figure 3, similar proportions of TecIII and GFP-TecIV expressing cells displayed vesicular punctate staining although the appearance of the punctate distribution was generally different (Figure 3C-F).

In summary, in the absence of external stimuli, full length GFP-TecIV is diffuse in the cytoplasm of transfected cells and shows lower levels of tyrosine phosphorylation than GFP-TecIII which shows higher incidence of plasma membrane targeting. This suggests a role for a functional SH3 domain in preventing plasma membrane targeting and activation of Tec.

Under normal conditions, it is expected that a functional SH3 domain, such as that of TecIV, interacts with negative regulators and/or internal SH3 binding sites to prevent the redistribution of TecIV to the plasma membrane. Phosphorylation of the activation loop tyrosine and subsequent phosphorylation of the SH3 domain tyrosine residue is predicted to displace such interaction(s) thus permitting plasma membrane targeting and activation of Tec. The truncated SH3 domain of TecIII is unable to mediate these negative regulatory interactions. It is possible that plasma membrane targeting motifs of the PH domain of TecIII are largely exposed, and/or that SH3 binding sites in the PRR are also available to potential SH3 binding

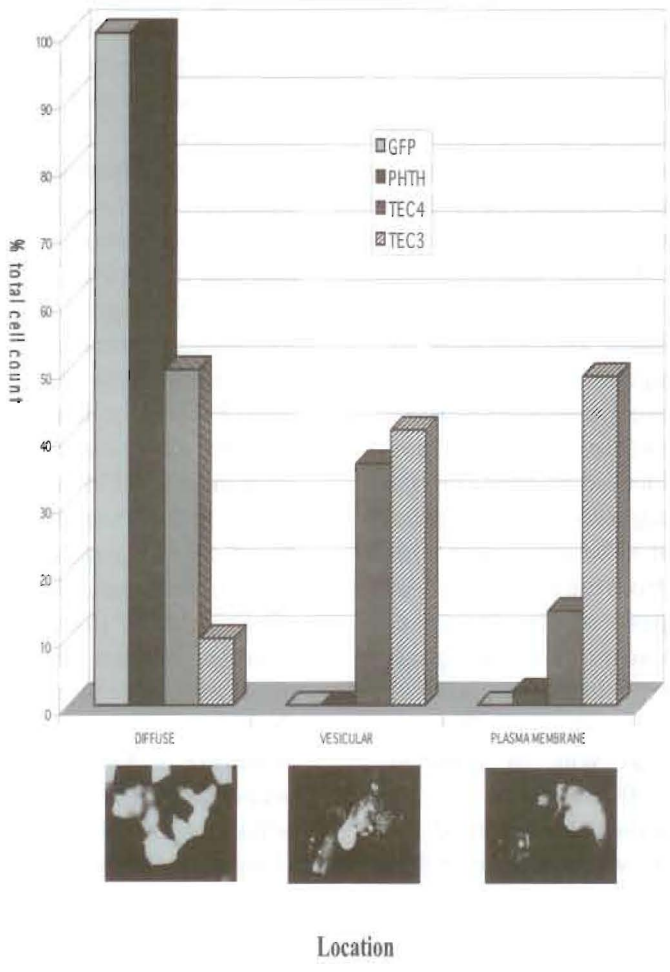

Figure 3. Quantitation of subcellular pattern of GFP fluorescence in the population of COS-1 transfected cells. $5x10^6$ COS-1 cells were transfected with 2µg of the following plasmid DNA pEGFP-C2, pEGFP-PHTH, pEGFP-TecIII and pEGFP-TecIV as described in Figure 3.7. Staining patterns were quantitated from images captured using a Nikon (ECLIPSE TE300) inverted microscope. Briefly, cells were visualised using Adobe Photoshop 5.5 and approximately 200 cells were counted in each case. Cells expressing diffuse as well as punctate fluorescence were scored as punctate. An example of each subcellular localisation pattern is shown beneath the graph.

proteins. Either one, or the combination of these interactions, might then facilitate the targeting of the truncated protein to the plasma membrane. Redistribution of TecIII to the plasma membrane in turn spatially positions TecIII in proximity to plasma membrane localised Src family kinases that are then able to phosphorylate and activate TecIII.

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