TRAVEL INFORMATION ON THE TEC KINASES DURING LYMPHOCYTE ACTIVATION

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1. INTRODUCTION

Antigen receptor signal transduction is a critical step to the development and function of T and B lymphocytes. The non-receptor protein tyrosine kinases (PTKs) of Tec family are emerging as key players of antigen receptor signaling (see reviews [1–4]). The initial interest for working on this family came from the discovery of mutations affecting Btk linked to the human genetic disorder X-linked agammaglobulinemia (XLA) and murine X-linked immunodeficiency (see review [5]). Btk is a member of the Tec family that includes Tec (tyrosine kinase expressed in hepatocellular carcinoma), Btk (Bruton’s tyrosine kinase), Itk (inducible T-cell kinase, also known as Emt or Tsk), Bmx (Bone Marrow tyrosine kinase gene in chromosome X, also named Etk), and finally Rlk (resting lymphocyte kinase, also named Txk). Tec family members are expressed in cells of the hematopoietic lineage. Tec family kinases are involved in several aspects of the lymphocyte development, differentiation and activation (see reviews [1–4]).

Upon antigen receptor engagement, these PTKs become activated: in T cells these include Tec, Itk and Rlk, whereas in B cells, Btk and Tec. Initially, these PTKs have been shown to regulate Ca²⁺-dependent pathways by participating to the activation of PLC-γ. More recent reports demonstrated a contribution of Tec family kinases in actin cytoskeletal reorganization and cell adhesion.

These PTKs are characterized by an NH2-terminal pleckstrin homology (PH) domain (absent in Rlk), a proline-rich region, Src-homology 3 (SH3) and SH2 domains, and a COOH-terminal PTK domain. These domains can be

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involved in targeting the PTKs at a specific subcellular compartment and in contributing to the formation of signalosomes containing the Tec kinases. Whereas most of the Tec family members reside in the cytoplasm of resting T and B cells, an antigen receptor engagement induces different patterns of subcellular localization. In this chapter, we focus our attention in the regulation of the Tec kinases translocation from cytosol to or near to the plasma membrane, or to the nucleus. To evaluate this dynamic regulation of the Tec family kinases, different methods should be used; we will summarize the techniques available to visualize these intracellular proteins.

2. TEC KINASES LOCALIZATION AT THE PLASMA MEMBRANE

Upon antigen receptor engagement, activation of the Tec kinases involves two events: first, membrane localization via interaction of the PH domain with phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P3] generated by phosphoinositide 3-kinase (PI3-K); and, second, phosphorylation by the Src kinases on a tyrosine in the activation loop, resulting in enhanced Tec kinase activity. Thus, in this general scheme, membrane recruitment corresponds to the first step of Tec kinase activation and the presence of a PH domain on the amino-terminal sequence of Btk, Tec and Itk is essential for this recruitment in B and T cells upon antigen stimulation.

The PH domain was first identified in 1993 as a 100–120 residue stretch of amino-acid-sequence similarity that occurs twice in pleckstrin and is found in several proteins involved in cellular signaling. The PH domains of the Tec kinases fall in the category of high-affinity phosphoinositide-binding PH domains. The importance of this domain has been highlighted by the mutations in the Btk PH domain known to cause impaired B cell development. The mutations that correlate with XLA in humans are known to decrease the affinity of the Btk PH domain for PI(3,4,5)P3, illustrating the importance of this event in B cell receptor (BcR) signalling. Membrane recruitment of the Btk PH domain is generally weak and occurs rapidly and transiently after BcR crosslinking, and correlates with the kinetics of PI(3,4,5)P3 production. PI3-K activation is critical in the signalling pathway leading to the plasma membrane translocation of the molecules containing a PH domain. A targeted disruption of the p85α regulatory subunit of PI3-K in mice (p85α KO mice) leads to the same phenotype as developed in Xid mice expressing Btk harbouring mutations in its PH domain. Nevertheless, the recruitment of activated Btk to the plasma membrane is not affected by PI3-K inhibitors or in p85α KO B cells. The Xid phenotype in these mice with a loss of the p85α-mediated PI3-K activation could be induced by the impairment of the membrane recruitment of another PH domain-containing protein: the Akt/PKB serine/threonine protein kinase. These data suggest that other domains of Btk can be involved for its translocation,
The PH domain-containing Tec family kinases are predominantly cytosolic in resting lymphocytes. The amino-acid sequence of these proteins reveals the presence of several domains, starting at the amino terminus with a PH domain, followed by a domain named TH (for Tec homology) containing one or two proline-rich (PxxP) regions. This TH domain is able to bind the SH3 domain; these interactions are involved in dimerization of the Tec kinases. As in Src family kinases, the SH3 domain is followed by an SH2 domain and the carboxy terminus at the sequence contains the tyrosine kinase domain. Upon receptor engagement, the Tec family kinases are phosphorylated on a tyrosine residue within the activation loop of the kinase domain by an Src kinase, then Tec kinases autophosphorylate a tyrosine residue within its SH3 domain to give a protein with full catalytic activity. Conditioning this activation step, the Tec kinases should be recruited from the cytosol to the plasma membrane. An established model (model A) for Tec localization at the plasma membrane, corresponds to a first step (A-1) where the PH domain interacts with the phospholipids: PI(3,4,5)P3 (PI3P) generated by the PI3-kinase activation, then the SH3 domain can interact with partners at the plasma membrane containing proline-rich regions (PxxP) and/or the SH2 domain will interact with tyrosine-phosphorylated (pY) proteins (A-2). We suggest an alternative model (model B) where the PH domain is not necessary for Tec recruitment and is initially replaced by other domains such as the SH3 and/or SH2 domain (B-1). However, all the data are converging to the point that the PH domain is important for Tec activation, suggesting that also in this second model, PI3P binding to the PH domain is required for Tec activation (B-2).
either to maintain Btk at the membrane or to target directly the Tec kinases at the plasma membrane.

For two of the Tec kinases present in T cells, Itk and Tec, a general activation model is established where membrane recruitment is mediated by their PH domains, thus the first step is controlled by PI3-K activation, and then an Src family member (Lck or Fyn) will phosphorylate and activate these Tec kinases (see Figure 1A). The Itk and Tec kinase activation is controlled by the PI(3,4,5)P$_3$ levels at the membranes: both SHIP, an SH2-containing inositol phosphatase, and PTEN, a phosphatase and tensin homologue that catalyzes respectively hydrolysis of PI(3,4,5)P$_3$ to PI(3,4)P$_2$ or to PI(4,5)P$_2$, downregulate the Itk and Tec kinase activities$^{13,14}$. Both Itk and Tec co-localize with the activated T cell receptor (TcR) and the deletion of their PH domain abrogates or impairs the translocation of these PTKs to the plasma membrane$^{15,16}$. Moreover, the Tec PH domain alone is able to co-localize partially with the activated TcR in Jurkat T cells (F. Garçon and J.A. Nunès, unpublished data). These studies were performed using antibodies against the TcR/CD3 complex. As a receptor engagement with antibodies or a natural ligand can mediate different intracellular signals$^{17}$, we and others have performed experiments using antigen-presenting cells (APCs) loaded with either agonist peptide or superantigen to stimulate the TcR expressed on T cells$^{18,19}$. Upon T cell–APC contacts, the presence of the PH domain is not necessary to induce membrane recruitment of Tec. All these data are showing that the PH domain of Tec is critical for the phosphorylation of PLC-γ1, a substrate of Tec, and activation of gene transcription. However, depending on the strength of T cell activation (antibodies crosslinking versus cellular contacts), the PH domain can be dispensable for the first step of Tec activation such as its targeting at the plasma membrane. In this new activation model (see Figure 1B), the first step of Tec activation could depend of another domain, for instance, the SH2 or SH3 domain. It has been reported that the SH2 domain contributes to localization of Tec at the membranes$^{16,18}$. We also described that the SH3 domain is essential for targeting Tec at the plasma membrane$^{19,20}$. Several partners of the SH2 and SH3 domains of Tec kinases are present at the T cell–APC contact zone, such as the adaptor molecules LAT (linker for activation of T cells) and SLP-76 (SH2-containing leukocyte protein of 76 kDa), Vav a Rac GDP/GTP exchange factor, WASP (Wiskott-Aldrich syndrome protein), or the CD28 co-stimulatory receptor (see review [3]). Several of these interacting proteins are involved in actin cytoskeleton reorganization, and the Tec kinases are good candidates to make the link between the receptors expressed at the cell surface and the assembly of the actin network (see review [21]). Vice-versa, some proteins participating in this network could bring Tec kinases from the cytosol and target them to the membranes.

The Tec kinases cannot only translocate to the plasma membrane; it has been reported that Tec, but not Itk or Btk, is able to accumulate in intracellular vesicles under the plasma membrane of T cells$^{18}$. The nature of these vesicles has been recently characterized as a compartment expressing the early en-
dosomal antigen 1 marker (EEA1), and the PH domain is necessary to locate Tec in these cellular structures\textsuperscript{22}. These intracellular compartments can accumulate several proteins involved in antigen receptor signaling\textsuperscript{23,24}. Interestingly, the translocation of Tec from the cytosol to these vesicles could involve Tec in the regulation of endocytosis and/or regulation of signal transduction at the plasma membrane.

The presence of the Tec kinase family member Btk has been described in a subcellular fraction from primary B cells corresponding to the caveolae\textsuperscript{25}. The caveolae are invaginations of the plasma membrane that serve as cell-surface microdomains\textsuperscript{26}. Smith and colleagues reported some evidence that this localization would downregulate Btk activity and that Btk associates with caveolin-1\textsuperscript{25}. The larger isoform of Rlk that lacks the PH domain contains an amino-terminal cysteine-string motif where palmitoylation occurs\textsuperscript{27}. This isoform is accumulated also in a detergent-insoluble fraction that is corresponding, in this case, to the lipid rafts\textsuperscript{28}.

3. TEC KINASE LOCALIZATION IN THE NUCLEUS

In addition to their membrane recruitment, Tec kinases have been shown to travel from the cytoplasm to the nucleus.

The first evidence of nuclear localization of Tec kinases appeared in 1999 with the identification and characterization of Txk/Rlk\textsuperscript{27}. Looking at the intracellular localization of Rlk, the authors showed by immunofluorescence that the shorter form, which lacks the cysteine string motif, is located in the nucleus when expressed without the longer form. In fact, inspection of the amino-acid sequence of Txk/Rlk revealed a nuclear localization site (NLS) — (K(N)\textsubscript{10}KRKPLPP) — present in both forms of Rlk. Mutation of this NLS induces cytoplasmic localization of Rlk, while mutation of the cysteine string motif induces nuclear localization of the larger form, suggesting that this motif is responsible for maintaining the larger form in the cytoplasm. Thus, the NLS and the cysteine-rich motif play the role of antagonist in the localization of Rlk. Upon TCR activation of Jurkat T cells, a subpopulation of Rlk migrates from the cytoplasm to the membrane, while a second subpopulation translocates in the nucleus. However, after long-term stimulation the membrane fraction disappears and only the nuclear fraction remains. These data suggest that Rlk may have distinct cytosolic and nuclear function.

Indeed, the nuclear localization of Rlk has been shown to be important for induction of IFN-\(\gamma\), as a mutation of the NLS of Rlk abrogates induction of IFN-\(\gamma\) promoter activity\textsuperscript{29}. In fact, Rlk directly binds to the IFN-\(\gamma\) enhancer region and acts as a transcription factor\textsuperscript{30}. Remarkably, the region of the IFN-\(\gamma\) enhancer that responds to Rlk is conserved between the human and other mammalian IFN-\(\gamma\) genes, and similar sequences are present in several Th1 cell-associated genes. This suggests an important role of the nuclear translocation of Rlk for
Th1 development. Interestingly, the longer form seems to be the main isoform that binds to the IFN-γ promoter. However, there is still no evidence that the nuclear function of Rlk is solely dependent of one isoform or the other.

Similarly, following a first description of the nuclear translocation of Btk in HeLa cells after SDF-1α stimulation, the nuclear shuttling of Btk in pre-B cell line, B cells, and mast cells has been shown. Like Rlk, Btk presents an NLS-like sequence in the PH domain. However, this sequence is not necessary or required for translocation of Btk. Instead, Btk uses an exportin-1-dependent nuclear export signal to shuttle between nucleus and cytoplasm. Indeed, an NES-like sequence (PLNFKKRLFLL) can be found in the PH domain. It is of interest that this sequence is also found in sequences of the other Tec kinase family members. The PH domain and the Src kinases are critical in the signaling pathway leading to nuclear translocation of Btk. Thus, nucleocytoplasmic shuttling of a PH-deleted form of Btk is dependent on Src activity. Interestingly, deletion of the SH3 domain induces nuclear localization of Btk, suggesting that this domain could also present an NES. However, the precise mechanisms of the shuttling are still not known at the moment. How the PH and the SH3 domain act to regulate the shuttling of Btk is still to be elucidated. Data obtained so far would suggest either that Src-dependent protein, which sequesters Btk in the cytoplasm, may release it when tyrosine is phosphorylated, or that interaction of Btk with other Src substrates may expose masked NLS.

Btk can phosphorylate and regulate the transcription factors TFII-I and STAT5 and can associate with the Bright transcription factor complex in a PH-dependent manner. Interestingly, while Bright is synthesized in activated spleen cells from Xid mice, it did not bind DNA or associate with Btk. Thus, these data suggest a potential mechanism by which Xid mutation could lead to defects in Ig synthesis. Nevertheless, it appears that Btk can still be found in the nucleus of Xid cells.

For Itk, an interaction with the nuclear import chaperone karyopherin/Rch1α has been demonstrated in Jurkat cells. This interaction implicates the SH3 domain of Itk and requires proline 242 of Rch1α. Interestingly, expression of a proline mutant of Rch1α decreased both Itk nuclear localization and CD3-mediated IL-2 production in Jurkat T cells. The nuclear localization of Itk has been shown to be enhanced in TcR-activated T cells. If the exact function and the nature of Itk targets in the nucleus still remain to be elucidated, post-transcriptional modifications could be one important step in the nuclear function of Itk. For instance, methylation of proteins on arginine by S-adenosylmethionine-dependent protein arginine methyltransferase (PRMT) may be important during nuclear import/export process. Thus, it has been shown that CD28 engagement induced arginine methylation of several proteins, including Vav1 and Itk. Interestingly, the R-methylated form of Vav1 is localized preferentially in the nucleus. These data suggest that this modification may require appropriate subcellular localization for Vav1. According to the authors, Itk was also found methylated upon CD28 stimulation with kinetics similar to Vav1. The role of R
methylation is just emerging, and the impact of this modification on protein function remains to be clarified.

Interestingly, even if the NLS sequence can be found in the sequence of all the Tec kinases, only translocation of Rlk has been shown to be NLS-dependent so far. Both Btk and Itk use different ways to shuttle between nucleus and cytoplasm.

Finally, Tec is also able to interact with Rch1α but the mutation of its SH3 domain does not abolish this interaction (F. Garçon and J.A. Nunès, unpublished data). However, Tec can be found in the nucleus, and this localization seems to be dependent on its SH3 domain19. The exact function of this localization is still not known.

4. VISUALIZATION METHODS

Over the past decade, genetically encoded fluorescent proteins have become widely used as markers in living cells. The development of these fluorescent proteins, coupled with advances in digital imaging has allowed addressing questions of the recruitment, colocalization and interactions of specific proteins within particular subcellular compartments. However, analyses of the localization of the Tec kinases poorly use these new technologies, whereas these techniques are now widely used in the field of immunology and more specifically in signal transduction.

In the following section, we will briefly review some recent developments in imaging that could be used to monitor subcellular distribution and colocalization of Tec kinases.

4.1. TIRF

In 2004 Tec was shown to have a unique pattern of subcellular localization as it was found in small vesicles at the plasma membrane18. Recently, the authors used TIRF (total internal reflection fluorescence) microscopy in order to examine these Tec-containing structures at the plasma membrane in live cells22. TIRF imaging relies on an evanescent wave generated when light reflects off a surface at an incident angle equivalent to or greater than the critical angle. This allows better resolution due to a smaller optical section depth. The major advantage of this method is a higher signal-to-noise ratio than the other fluorescence imaging method, e.g., less background and more sensitivity to small changes in fluorescence. Furthermore, as just a small number of molecules are excited, photobleaching and phototoxicity are reduced. Using TIRF, it has been possible to demonstrate that Tec segregates in specific vesicles containing several important signaling molecules like Lck and PLCγ2. Recently, such clusters of proteins have been described in Jurkat cells38. Once again, TIRF allowed the authors to follow and characterize the behavior of single signaling molecules like Lck,
LAT, or CD2 at the surface of a live cell. These clusters, or “microdomains,” are supposed to be formed by diffusional trapping through protein–protein interaction, and concentrate or exclude cell surface proteins to facilitate T cell signaling. One can wonder if these “microdomains” overlap with the Tec-containing vesicles, and where the others Tec kinases are. TIRF microscopy is certainly the best approach to answer this kind of question.

4.2. FRET

The molecular dynamics of immunoreceptors and signaling proteins are thought to be important for cell activation but have not been extensively investigated in the environment of the immunological synapse. Classical biochemical approaches to the study of localized interactions cannot give a complete and dynamic picture of the events. One of the few techniques capable of giving such views of protein–protein interactions relies on Fluorescence Resonance Energy Transfer (FRET). FRET is a technique used to measure the interaction between two molecules labeled with different fluorophores (the donor and the acceptor) by the transfer of energy from the excited donor to the acceptor. This transfer is only possible when the distance between the donor and the acceptor is very small (about the nanometer range). In biological applications, this technique has become popular to map protein–protein interactions (for some example of applications in T cells see review [39]). So this technique can give dynamic information about the real interaction between molecules, as opposed to simply the subcellular colocalization provided by fluorescence microscopy. Based on the idea that mutation in the PH domain of Btk leads to changes in its affinity for PI(3,4,5)P3, a miniaturized FRET-based assay for drug screening has been developed by monitoring interaction between the PH domain of Btk and PI(3,4,5)P3. This system could be easily adapted in a cellular system and allow researchers to monitor the effects of the compounds of interest on Tec localization. Recently, a new FRET method using three chromophores instead of two has been developed. This technique allows multiple-interaction screening in living cells. Thus, the authors were able to detect and analyze interactions between EGF receptor and two intracellular partners: the adaptor molecule Grb2 and the E3 ubiquitin ligase Cbl. This 3-FRET technique could facilitate an understanding of the formation of protein networks containing Tec kinases during T cell activation.

By its sensibility, FRET will be a great help in visualizing the dynamic of the interactions of Tec kinases with their partners during T cell activation.

4.3. FRAP

The current knowledge of the changes in intracellular localization and dynamic movements of signaling molecules during lymphocyte activation is limited. Fluorescence recovery after photobleaching (FRAP) uses a short pulse of intense
laser light to irreversibly destroy fluorescence in a very small area. The recovery of fluorescence into that area occurs as a result of signaling molecule mobility. This technique is then very useful to study the dynamics of signaling molecules during lymphocyte activation. For example, LAT has been shown to have lower mobility when present at the site of aggregated rafts after TCR stimulation than that of LAT found in other areas of plasma membrane. Then, FRAP can provide useful information on the mobility of proteins in the membrane or between several subcellular compartments. It could then help us determine if Tec kinases are trapped into a specific location at the synapse or if they are able to diffuse in the plasma membrane like PI(3,4,5)P_3.

5. CONCLUDING REMARKS

A well-established mechanism of activation of Tec family kinases suggests that the first step of activation is driven by plasma membrane recruitment of these Tec kinases via their PH domains. In this chapter we provide some arguments that this first step can be driven by other domains. Thus, the notion that Tec kinases are effectors of PI3-K should be discussed. The PH domain of Tec kinases interacts with some 3-phosphoinositides generated by the PI3-K activation such as PI(3,4,5)P_3. The Akt/PKB serine/threonine kinase is a downstream target of PI3-K, and contains a PH domain that is necessary and sufficient to recruit Akt/PKB at the plasma membrane. Several animal models demonstrated that Akt/PKB, but not Tec kinases, is directly involved in PI3-K signaling. A major difference between the PH domain of Akt/PKB and those found on the Tec kinases corresponds to the specificity of these domains: the Akt/PKB PH domain recognizes both PI(3,4,5)P_3 and PI(3,4)P_2 in contrast to the Tec PH domain, which binds only PI(3,4,5)P_3 with high affinity (see review [46]). Upon antigen receptor engagement in lymphocytes, PI(3,4,5)P_3 production appears to be very transient compared to PI(3,4)P_2 production. Thus, the Tec kinases might develop other strategies to be located at the plasma membrane using their SH2 or SH3 domains.

Localization of the different Tec family kinases in the nucleus has been reported, but the issue of the nuclear functions of these kinases remains to be addressed. Many of the studies on the organization of cellular networks containing Tec kinases will profit from the now available methodologies (TIRF, FRAP, FRET, etc.) that allow visualization of two or more partners. As the Tec kinases are differently expressed during lymphocyte maturation (see review [4]), these studies should be addressed in the different stages of lymphocyte differentiation and activation.
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7. REFERENCES


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