INTRODUCTION

The question of how the signalling machinery downstream of the T-cell Receptor (TCR) rapidly integrates the binding information resulting from the encounter of a T-lymphocyte with an antigen presenting cell (APC) is central to our understanding of the adaptive immune response. Many hypotheses attempting to explain the speed and sensitivity with which a T-cell discriminates the antigens it encounters, include a notion of relative spatial and temporal control of particular biochemical steps involved in the process. These hypotheses include a notion of relative spatial and temporal control of particular biochemical steps involved in the process. This work was supported by institutional grants from Centre National du Cancer (BM), and Ministère de la Recherche (AT & BM).

Several steps in T-cell receptor (TCR) mediated signalling are the activation of the protein tyrosine kinase ZAP-70. ZAP-70 is recruited to the TCR upon receptor engagement and, once activated, is responsible for the phosphorylation of the protein adaptor, Linker for Activation of T-cells, or LAT. LAT phosphorylation results in the recruitment of a signaling complex including PLCγ1, Grb2/SOS, GADS and SLP-76. In order to examine the real time spatial and temporal evolution of ZAP-70 activity following TCR engagement in the immune synapse, we have developed ROZA, a novel FRET based biosensor whose function is dependent upon ZAP-70 activity. This new probe not only provides a measurement of the kinetics of ZAP-70 activity, but also reveals the subcellular localization of the activity as well. Unexpectedly, ZAP-70 dependent FRET was observed not only at the T-cell -APC interface, but also at the opposite pole of the cell or “antisynapse”.

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dynamic behavior of ZAP-70 activity in the immunological synapse more directly, we present here the creation of a novel biosensor, ROZA (for Reporter Of ZAP-70 Activity) which is comprised of elements of mouse Grb2, mouse LAT and the CFP-YFP pair of fluorescent donor and acceptor. Here we report the use of ROZA expressing cells to directly visualize ZAP-70 dependent phosphorylation in T-cell lines and primary human lymphocytes with subcellular resolution during the formation of an immunological synapse. Furthermore, we observe that ZAP-70 dependent activity revealed by ROZA displays a bipolar distribution, appearing at times at the pole opposite that of the lymphocyte-APC contact. To our knowledge, ROZA is the first biosensor engineered for a TCR dependent tyrosine kinase activity and as such represents a key step forward towards bringing the wealth of biochemical information available about TCR mediated signal transduction into the realm of live cell microscopy.

RESULTS AND DISCUSSION

Design of the FRET probe ROZA

As previously described [13,14], a ratiometric FRET based probe for a tyrosine kinase activity can be prepared by fusing a peptide sequence encoding a known substrate sequence for the targeted kinase with its cognate phospho-tyrosine binding domain (e.g. a Src homology (SH) 2 domain). When assembled with the appropriate linker sequences and flanked by compatible donor and acceptor autofluorescent proteins, such an engineered protein can undergo a phosphorylation dependent change in its FRET signal, presumably due to a phosphorylation dependent intramolecular binding event which changes the relative distance and/or orientation of the autofluorescent protein domains (See Figure 1B). In order to create a ZAP-70 specific probe, we targeted peptide sequences derived from mouse LAT encompassing tyrosine residues Y132, Y175 or Y195, known to be substrates for ZAP-70 [15]. The peptides were fused to their respective SH2 domain binding partners such as the N-terminal SH2 domain of mouse PLCγ1 for Y132 and the SH2 domain of mouse Grb2 for Y175and Y195 [16]. ROZA variants were produced in which the substrate peptide, the SH2 binding partner as well as the length and composition of the interdomain linkers were varied. Constructs were screened for their expression levels in Jurkat J-Tag cells and their ability to generate a phosphorylation dependent change in FRET signal upon pervanadate (PV) and anti-CD3 stimulation. The sequence resulting in the most significant phosphorylation dependent FRET change (see Fig. 1A) was used for all subsequent experiments. In order to facilitate the encounter of active ZAP-70 with the substrate probe, ROZA was targeted to the plasma membrane by incorporating the N-terminal residues of mouse Lck. As shown in Fig. 1C, the protein is indeed largely restricted to the membrane of Jurkat T-cells. A variant was also prepared incorporating the N-terminal 35 residues of LAT, encompassing the transmembrane domain and palmitoylation sites, however this anchor led to a largely vesicular probe expression (data not shown).

Stimulation of ROZA-expressing Jurkat T-cells with pervanadate (PV), a phosphatase inhibitor that unveils constitutive tyrosine kinase activity, triggered a simultaneous increase of the 436 nm signal and a decrease of the 436→535 nm signal.

Figure 1. Structure and subcellular localization of ROZA. (A) Scheme of the ROZA sequence including the N-terminal residues from p56 Lck, the linker sequence and residues 171-178 of LAT (underlined) surrounding Y175 (in bold). (B) Model showing the schematic structure of ROZA (without its membrane anchor), and illustrating that, in the absence of ZAP-70 activity, ROZA may adopt several conformations, some of them allowing FRET. Following phosphorylation of the LAT based sequence by ZAP-70, ROZA adopts a constrained conformation incompatible with FRET. (C) In Jurkat T-cells transiently transfected with ROZA, the probe is mainly located at the plasma membrane as visualized by CFP fluorescence.

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(Fig. 2A upper panel). Stimulation thus triggers a decrease of the 535 nm/470 nm ratio, i.e., of the FRET signal (Fig. 2A lower panel). Changes in FRET signals could also be detected in Jurkat T-cells (Fig. 2B upper panel) or human PB T-cells (Fig. 2B lower panel) after stimulation with anti-CD3. The ROZA expressing Jurkat T-cells were similarly stimulated by either anti-CD3 or PV and subsequently lysed and subjected to western blot analysis. Both phosphorylated forms of ZAP-70 and endogenous LAT where readily detectable 2 minutes (120 seconds) after stimulation, confirming that ZAP-70 is indeed activated under these conditions in the ROZA expressing cells (Fig. 2C). The stimulation dependent decrease in FRET is compatible with a model in which the autofluorescent proteins of the non-phosphorylated form of the probe have a relative distance or orientation which allows FRET, whereas the phosphorylated form of the probe assumes a conformation less compatible with FRET. Other FRET based kinase activity probes described in the literature function with a phosphorylation dependent decrease in FRET capacity [17,18]. The most relevant example is the second generation Src kinase reporter [18], which like ROZA incorporates an SH2 phospho-tyrosine binding domain, and also undergoes a phosphorylation dependent decrease in FRET. The authors postulate that the close positioning of the N and C termini of the SH2 domain would allow a relative proximity of the linked autofluorescent protein domains and thus FRET in the probe’s basal state. This relative proximity would be disrupted during the phosphorylation dependent intramolecular binding event and the FRET ratio would decrease. The N and C termini of the SH2 domain of Grb2 are also found on the same face of the domain in its crystal structure which is consistent with the FRET decrease observed in ROZA [19]. Fig 1B illustrates such a model, taking into account the possibility that in the basal state, the probe may adopt several conformations. In the following figures, ZAP-70 activity is arbitrarily expressed as the inverse of FRET ratio (Fig. 2B inset). Using this representation a FRET decrease corresponds to an increase in net ZAP-70 dependent activity.

Both ROZA FRET and phosphorylation are ZAP-70 dependent

In order to establish that the observed FRET changes were due to a ZAP-70-dependent phosphorylation of the probe, Jurkat T-cells transiently expressing ROZA were stimulated, lysed, and subsequently subjected to anti-phosphotyrosine immunoprecipitation and anti-GFP immunoblotting (Fig. 3A). The resulting immunoblots of the transfected Jurkat T-cell samples displayed an

Figure 2. (A) In ROZA-expressing Jurkat T-cells, PV induces changes in the 436→535 and in the 436→470 signals (top) and in their ratio (bottom). Traces correspond to the mean of 3 different T-cells. (B) In Jurkat T-cells (top), anti-CD3 induces a partial activation of ZAP-70 that can be completed by PV. In peripheral blood T-cells (bottom), anti-CD3 triggers a large and rapid FRET decrease. Inset: ZAP-70 activity is shown as the inverse of the FRET signals. Traces correspond to the average of 8–17 individual cells. (C) In Jurkat T-cells stably expressing ROZA and stimulated with PV or anti-CD3, ZAP-70 (site 319) as well as endogenous LAT (site 175) are phosphorylated after a 2 minute stimulation.

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activation-dependent phosphorylated species with the expected molecular weight of the ROZA probe (69 kD). Pre-treatment of transfected cells with the Syk family inhibitor piceatannol, or with the Src kinase inhibitor PP2, resulted in inhibition of ROZA phosphorylation. Similarly, mutation of the probe’s target tyrosine residue LAT 175 to phenylalanine resulted in significant inhibition of the probe phosphorylation (Fig. 3A upper gel). Stripping and reblotting with an anti-GFP antibody confirmed that the most prevalent GFP containing species had a molecular weight consistent with the full length ROZA (Fig. 3A lower gel).

Additional specificity tests were performed by imaging. As expected, the FRET changes triggered by anti-CD3 stimulation were strongly inhibited in cells pretreated with PP2 for 30 minutes before stimulation (data not shown). The strong fluorescence of piceatannol precluded its use in FRET experiments. Cells expressing the YF175 ROZA mutant also displayed no significant change in the FRET signal upon PV stimulation confirming that phosphorylation of the LAT sequence found within ROZA was necessary for the FRET ratio change. Finally, in ZAP-70-deficient Jurkat T-cells, (P116) cells expressing ROZA or in normal Jurkat T-cells transfected with the mutated probe (YF).

Speculation: Is double signalosome formation a simple biological pattern?

The appearance of a double signalosome could be considered as a simple case of biological pattern formation. In the theory of biological pattern formation initially proposed by Turing and later revisited by others, a pattern-forming reaction uses a combination of self-enhancing local activator and a long-range inhibitor triggered by the activator, and may create a dead zone for activator accumulation around the initial accumulation [24,25]. However little information is currently available about either the dynamics or the mechanisms underlying this phenomenon.
Construction of ROZA

The sequence encoding ROZA was constructed from three fragments. A CFP fragment was created by PCR amplification of mCFP residues 1-228 using a coding primer incorporating a HindIII site, a kozak sequence, and the 13 N-terminal residues of mouse Lck and a non-coding primer incorporating a SphI restriction site. Mouse Grb2 (residues 56-152) cDNA was amplified using a coding primer incorporating a SphI site and a non-coding primer which incorporated the linker sequence, mouse LAT residues 171-178 and a SacI site. The specific amino acids created were as shown in Fig. 1A. A YFP fragment was created by amplifying full length mYFP with a coding primer including a SacI site and a non-coding primer incorporating a stop codon and an EcoRI site. The three fragments were ligated simultaneously into the HindIII/EcoRI sites of pCDNA3.1. The Tyr to Phe mutation of the LAT tyrosine 175 present in central fragment of the ROZA sequence was created using nested amplifications of the Grb2-linker LAT sequence. The complete nucleotide sequence encoding this structure has been deposited in Genebank (accession number EU035753).

Cell transfections

Jurkat T-cells and PBT were transfected by Amaxa nucleofection with solution V / Program G-10 or S-18 and human T solution / Program U-14, respectively. Typically 5×10⁶ cells were transfected with 5 μg plasmid DNA. Cells were used 24–48 hours after nucleofection. Stable clones of the Jurkat J77 cl20 expressing ROZA were established first sorting the YFP positive cells with a Becton Dickenson FACSAria cell sorter followed by cloning by limiting dilution in the presence of 1.5 mg/ml G418 (Invitrogen).

Immunoprecipitation and immunoblotting

ROZA expressing cells were cultured at densities less than 0.5×10⁶ cells per ml, recovered, rinsed once in serum-free medium and suspended at 5×10⁶ cells per point in RPMI supplemented with 10 mM HEPES with or without kinase inhibitors. Cells were stimulated with the pervanadate or antibody for the indicated time, briefly spun, and resuspended in ice cold NP-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1mM PMSF, 0.4 mM sodium orthovanadate, and Complete protease inhibitor Mix (Roche) as per manufacturers instructions) at 4°C for 20 minutes. In the indicated experiment, post nuclear supernatants were subjected to immuno precipitation with an anti-GFP antibody to pull down the probe. Immunoprecipitates were subjected to SDS-PAGE transferred to PVDF membrane. Membranes were probed with indicated antibodies as per manufacturers recommendations for each specific antibody followed by HRP conjugated secondary antibody (Immunotech SA, Marseille, France). Images were revealed using ECL plus (Amersham).
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REFERENCES
1. Burroughs NJ, van der Merwe PA (2007) Stochasticity and spatial heterogeneity in T-cell activation. Immunol Rev 216: 69–80.
2. Moskva CR, Freiberg BA, Kupfer H, Scialy K, Kupfer A (1998) Three-dimensional segregation of supramolecular activation clusters in T-cells. Nature 395: 82–86.
3. Trautmann A, Valitutti S (2003) The diversity of immunological synapses. Curr Opin Immunol 15: 249–254.
4. Fried P, den Boer AT, Gunzer M (2005) Tuning immune responses: diversity and adaptation of the immunological synapse. Nat Rev Immunol 5: 532–545.
5. Dendl S, Kadlecek TA, Brdicka T, Cao X, Weis A, et al. (2007) Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell 129: 735–746.
6. Horjoi V, Zhang W, Schraven B (2004) Transmembrane adaptor proteins: organizers of immunoreceptor signalling. Nat Rev Immunol 4: 603–616.
7. Stefanova I, Hemmer B, Vergelli M, Marini R, Biddison WE, et al. (2003) TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. Nat Immunol 4: 288–294.
8. Ahtan-Bonnet G, Germain RN (2005) Modeling T-cell antigen discrimination based on feedback control of digital ERK responses. PLoS Biol 3: e356.
9. Sloan-Lancaster J, Presley J, Ellenberg J, Yamazaki T, Lippincott-Schwartz J, et al. (1998) ZAP-70 association with T-cell receptor zeta (TCRzeta): fluorescence imaging of dynamic changes upon cellular stimulation. J Cell Biol 143: 613–624.
10. Klosinski SC, Hong DH, Kardon JR, Yamazaki T, McGlade CJ, et al. (2002) T-cell receptor ligation induces the formation of dynamically regulated signaling assemblies. J Cell Biol 158: 1263–1275.
11. Kohler K, Leflouch AC, Vollmer S, Stoeverandt O, Hoff A, et al. (2005) Chemical inhibitors when timing is critical: a pharmacological concept for the maturation of T-cell contacts. ChemBiochem 6: 152–161.
12. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, et al. (2002) T-cell receptor signaling precedes immunological synapse formation. Science 295: 1539–1542.
13. Ting YW, Kain KH, Klenke RL, Tsien RY (2001) Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. Proc Natl Acad Sci U S A 98: 15003–15008.
14. Sato M, Ozawa T, Inukai K, Asano T, Umezawa Y (2002) Fluorescent indicators for imaging protein phosphorylation in single living cells. Nat Biotechnol 20: 287–294.
15. Paz PE, Wang S, Clarke H, Lu X, Stokoe D, et al. (2001) Mapping the ZAP-70 phosphorylation sites on LAT (linker for activation of T-cells) required for recruitment and activation of signalling proteins in T-cells. Biochem J 356: 461–471.
16. Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, et al. (2000) Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T-cell antigen receptor-mediated signaling. J Biol Chem 275: 23353–23361.
17. Violin JD, Zhang Z, Tsien R, Newton AC (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C gamma. J Cell Biol 161: 899–909.
18. Wang Y, Rotnicki EL, Zhao Y, Berna MW, Usami S, et al. (2005) Visualizing the mechanical activation of Src. Nature 434: 1040–1045.
19. Maiguen S, Guillaume JP, Fromage N, Arnoix B, Beequart J, et al. (1995) Crystal structure of the mammalian Grb2 adaptor. Science 268: 291–293.
20. Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science 296: 913–916.
21. Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE (1998) LAT: the ZAP-70 tyrosine kinase substrate that links T-cell receptor to cellular activation. Cell 92: 83–92.
22. Ray P, Sospedra M, Barbour B, Trautmann A (2001) Functional antigen-independent synapses formed between T-cells and dendritic cells. Nat Immunol 2: 925–931.
23. Costello PS, Gallagher M, Cantrell DA (2002) Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. Nat Immunol 3: 1082–1089.
24. Turing A (1952) The chemical basis of morphogenesis. Philos Trans R Soc London B 237: 37–72.
25. Meinhardt H, Gierer A (2000) Pattern formation by local self-activation and lateral inhibition. Bioessays 22: 753–760.

Author Contributions
Conceived and designed the experiments: BM CR AL. Performed the experiments: CR AL PM. Analyzed the data: CR AL. Contributed reagents/materials/analysis tools: DM. Wrote the paper: AT CR AL.