Univerzita Karlova
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Autoreferát dizertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

Studium trojrozměrné organizace signálních molekul na T buňkách pomocí kvantitativních metod fluorescenční mikroskopie

Quantitative fluorescence microscopy techniques to study three-dimensional organisation of T-cell signalling molecules

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SUMMARY

Proteins represent one of the basic building blocks of all organisms. To understand their function at the molecular level is one the critical goals of current biological, biochemical and biophysical research. It is important to characterise all aspects that affect the localisation of proteins into different compartments with specific functions, the dynamic structure of proteins and their role in multiprotein assemblies, because altering these properties can lead to various diseases.

Most of the proteomic studies are nowadays performed using biochemical approaches that allow us to study multicellular organism or tissue at once. The disadvantage of these methods is complex preparation of sample and the need for a large number of cells, which leads to the loss of information at the molecular level and in individual cells.

On the contrary, microscopy can provide rather detailed information about proteins of interest and at the level of a single cell. A variety of fluorescence microscopy methods in combination with recombinant DNA techniques were applied to elucidate subcellular localisation of transmembrane adaptor proteins (TRAPs) in human lymphocytes and their nanoscopic organisation at the plasma membrane. Linker of activation of T lymphocytes (LAT), phosphoprotein associated with glycosphingolipid-enriched membrane microdomains (PAG) and non-T-cell activation linker (NTAL) were selected for our studies. These are single spanning proteins that lack significant extracellular domain, are palmitoylated and share comparable hydrophobicity and length of their transmembrane domain (TMD).

The necessity of palmitoylation for plasma membrane sorting of LAT, but not for other studied TRAPs, was demonstrated using transient expression of fluorescent fusion proteins. This different behaviour could be caused by the presence of helix-breaking residues introducing a dynamic kink in the centre of the structure of LAT TMD. The positive charge inside-rule was confirmed using model TRAP-like proteins carrying artificial TMD with different symmetry at the ends of the transmembrane segments. Also the presence of the DxE/ YxxØ amino acid sequence motifs responsible for release of proteins from endoplasmic reticulum was confirmed in the C-terminal part of LAT and PAG.

New sample preparation procedure for super-resolution microscopy was developed. This procedure radically improved the quality of acquired single molecule fluorescence data and, in addition, preserved the surface morphology of lymphocytes immobilised on the optical surface. The origin of previously described microclusters of CD4 was discovered by combination of our new protocol and improved and simplified version of the quantitative 3D analysis of single molecule localisation microscopy data. These accumulation patterns of CD4 at the tips of microvilli are palmitoylation dependent. In comparison, CD45 molecule was shown to be randomly distributed over the shaft and the base of microvilli. Indeed, its segregation from the tips of microvilli suggests the mechanism how surface morphology can regulate signalling in T cells.

In summary, the presented data indicate the importance of quantitative fluorescence microscopy for characterisation of proteins in their physiological environment, in individual cells and, in some cases, at the single molecule level.

SOUHRN

Proteiny patří mezi základní stavební jednotky všech organismů. Proto je pochopení jejich funkce na molekulární úrovni jedním z klíčových cílů současného biologického, biochemického a biofyzikálního výzkumu. Je důležité charakterizovat aspekty ovlivňující lokalizaci bílkovin do vnitrobuněčných částí se specifickými funkcemi a jejich samotnou dynamickou strukturu, včetně multiproteinových komplexů. Jakékoliv narušení těchto proteinových vlastností může vést ke vzniku různých onemocnění.

Většina proteomických studií je dnes prováděna pomocí biochemických přístupů, které nám umožňují studovat mnohobuněčný organismus nebo tkáň najednou. Nevýhodou těchto metod je složitá příprava vzorku a potřeba velkého počtu buněk. Tato kombinace vede ke ztrátě informací z jednotlivých buněk na molekulární úrovni.

Oproti tomu mikroskopické techniky mohou poskytnout poměrně podrobné informace o sledovaných bílkovinách, navíc z jednotlivých buněk. Pro studium lokalizace proteinů v různých částech lidských lymfocytů jsme vybrali trans-membránové adaptorové proteiny (TRAPy). Kombinací metod DNA manipulace a fluorescenční mikroskopie jsme sledovali i jejich nanoskopickou organizaci na plazmatické membráně. Jako zástupci byly vybrány tyto proteiny: „linker of activation of T lymphocytes" (LAT), "phosphoprotein associated with glycosphingolipid-enriched membrane microdomains" (PAG) a "non-T-cell activation linker" (NTAL). Jedná se o bílkoviny s jednou trans-membránovou doménou (TMD), která je palmitoylovaná a u všech má podobnou hydrofobicitu a délku. Navíc tito zástupci postrádají extracelulární doménu, která by měla vliv na jejich vlastnosti.

Pomocí transientní transfekce buněk fluorescenčně označenými proteiny jsme prokázali efekt palmitoylace na cílení LATu na plazmatickou membránu. Podobné působení nebylo u ostatních zástupců pozorováno. Takto odlišné chování může být způsobené přítomností aminokyselinových zbytků, které narušují spirální strukturu TMD. Jejich přítomnost vede ke vzniku dynamického zlomu uprostřed α-šroubovice. Transfekcí umělých proteinů s vysoce hydrofobní TMD jsme potvrdili pravidlo „pozitivního náboje na vnitrobuněčné straně" tohoto segmentu. Účinnost jejich transportu na membránu byla snížena, pokud byl elektrostatický náboj symetrický na obou stranách. Dále jsme potvrdili funkci DxE / YxxØ aminokyselinových sekvenčních motivů, které jsou kódovány v sekvenci C-terminální části LATu a PAGu. Tyto motivy slouží proteinům k uvolnění z endoplazmatického retikula.

Byl vyvinut nový postup zpracování vzorku pro mikroskopii s vysokým rozlišením. Naše metoda radikálně zlepšila kvalitu získaných jedno-molekulárních dat a navíc zachovala morfologii povrchu lymfocytů přichycených k mikroskopickému sklu. Díky nové přípravě vzorku a vylepšené a zjednodušené trojrozměrné kvantitativní analýze dat jsme zjistili, že dříve popsané mikrostruktury CD4 jsou ve skutečnosti akumulací tohoto koreceptoru na vrcholcích mikrovilů. Tato organizace je závislá na palmitoylaci CD4. Oproti tomu CD45 molekula byla distribuována náhodně na těle a bázi mikrovilů. Segregace těchto dvou molekul naznačuje mechanismus regulace signalizace T buněk pomocí uspořádání jejich plazmatické membrány.

V souhrnu všechny prezentované výsledky ukazují důležitost metod fluorescenční mikroskopie při charakterizaci proteinů v jejich přirozeném prostředí. Jak v jednotlivých buňkách, tak na úrovni individuálních studovaných molekul.

1   INTRODUCTION

Integral membrane proteins of eukaryotic cells comprise almost 30% of all proteins encoded by the human genome (Almén *et al.*,2009). These undergo sorting into target compartments such as the endoplasmic reticulum (ER), Golgi complex, mitochondria or the plasma membrane to perform their function. Mis-localisation can lead to a loss-of-function of these proteins, resulting in cel lmalfunction and even development of diseases (Hung and Link, 2011; Lee *et al.*, 2013; Wang and Li, 2014). Therefore, it is important to understand their localisation and effect of all signals that influence the sorting of proteins. Most of the studies were nowadays done using the biochemical, biophysical or proteomic approaches. The disadvantage of these methods is the complex sample preparation and the need of huge amount of cells. This leads to total loss of information concerning the single molecules and single cells.

In comparison, microscopic techniques can provide very detailed spatial information about molecules in individual cells. In combination with molecular engineering techniques, especially fusion with fluorescent protein, it opened the new ways of studying of proteins on single molecular level.

Application of these techniques into molecular and cell biology allowed us to study the basic problems such as impact of palmitoylation on final localization of the protein. Because the existence putative sphingolipid and cholesterol-enriched membrane microdomains, also known as lipid rafts (Hořejší, 2005, 2004; Hořejší *et al.*, 2010; Hořejší and Hrdinka, 2014; Levental *et al.*, 2010; Štěpánek et al., 2014) is still deeply discussed. The introduction of state-of-the-art super-resolution techniques into biology (Hell and Wichmann, 1994, Betzig *et al.*, 2006; Gustafsson, 2000) opened the possibility to study these nanoscopic structures on cells without demanding sample preparation like for electron microscopy.

Nowadays super-resolution techniques are able to study molecules with the resolution up to 10 nm, which correspond to the size of single protein. But most of them lack the spatial information in all three dimensions. It is important to take into account also the complex of the cell surface as this aspect plays can play a role in the final protein localisation, respectively their segregation (Jung *et al.*, 2020).

Several approaches were introduced. Stimulated emission depletion (STED) or adjusted structured illumination microscopy (SIM) allow to study the biological sample into several micrometres depth. But their disadvantage is limited achievable resolution (up 30 nm for STED, 100 nm for SIM). The 3D abilities of these methods are even more limited (Lukeš *et al.*, 2014, Schermelleh *et al.*, 2010).

Therefore there is a need to find method with resolution comparable to the size of protein molecule, but in all three dimensions. This could be possible with the application of Temporal, radial-aperture-based intensity estimation (TRABI Franke *et al.*, 2017) that offers nearly isotropic resolution in 3D.

In hand with the development of microscopy techniques, it is necessary to focus also to sample preparation. Because existing protocols stress the cells and force them to spread over the surface (Maverakis *et al.*, 2015; Mazia *et al.*, 1975; Santos *et al.*, 2018). Therefore it is important to find proper conditions to immobilize the cell to imaging surface, suppress background and preserve the plasma membrane surface structure.

2   AIMS OF THE WORK

The objective of this work was to demonstrate the impact of TMD and proximal sequences of single-spanning membrane proteins on their higher order organisation on the T-cell plasma membrane. For the ability to study single cells and to avoid biochemical methods, we used advanced fluorescence microscopy techniques. In addition to methodological development, two biological questions were studied in this work.

Biological Aim 1: How does protein palmitoylation and transmembrane domain properties influence sorting of TRAPs to the plasma membrane?

Experimental approach: Expressing of a large panel of fluorescent fusion proteins and their mutants in T cells together with markers of subcellular compartments. Subsequent quantitative analysis of protein localisation together with the markers. Application of super-resolution microscopy to further determine nanoscopic organisation of proteins at the T-cell surface.

Biological Aim 2: How are signalling receptors CD4 and CD45 organised at the T-cell plasma membrane with complex morphology determining nanotopography of CD4 and CD45 on the surface of resting T cells?

Experimental approach: New surface coating development to preserve the 3D nanostructures at the T-cell surface. Adaptation of the original TRABI method for quantitative, three-dimensional single molecule localisation analysis of receptor nanotopography.

Methodological Problem 1: How to adapt sample preparation protocols to improve the signal-to-noise by reducing background and achieve reproducibility in super-resolution microscopy?

Experimental approach: Extensive optimalisation of coverslip cleaning, storage and liquid handling protocols.

Methodological Problem 2: How to adapt sample preparation for super-resolution microscopy and preserve complex morphology of studied cells?

Experimental approach: Development, optimisation and in-depth characterisation of a new coverslip coating method together with the protocols for immunohistochemistry of the cells.

3   MATERIALS AND METHODS

3.1   Cell culture

HeLa cells and Jurkat cell line and its derivatives lacking (LAT) and CD4 (Jurkat CD4 KO) were used for the whole study

3.2   Transient transfection of above listed cell lines

Above mentioned cell lines were transiently transfected with DNA encoding proteins of interested (LAT, PAG, NTAL, LW19, LW25, CD4ex-LAT, NTAL etc.) fused with GFP or other fluorescent proteins. Cells were also co-transfected with DNA for intrecellular protein markers along with proteins of interest.

3.3   Genetic engineering

DNA coding proteins of interest was transferred into vector pXJ41 (Xiao *et al.*, 1991) carrying coding sequences for fluorescent proteins (GFP, mEOS2, PSCFP2).

3.4   Optimization of super-resolution sample preparation

Sample preparation was optimised for our cells.

3.5   Confocal microscopy

Single or multicolour confocal microscopy was performed on living or fixed cells, respectively. Data were then analysed for localisation and quantitative analysis. New system of high throughput epifluorescence microscopy for viability assay based on the principle from flow cytometry was developed.

3.6   Super-resolution microscopy

Single molecule localisation microscopy was used to elucidate the nanoscopic organisation of proteins of interest. LAT mutant versions were studied using photo-activation localisation microscopy (PALM). CD4 and CD45 molecules were studied using direct stochastic reconstruction microscopy approach (dSTORM). Data were analysed either by localisation software (Thunderstorm; Ovesný *et al.*, 2014) or adapted super-resolution optical fluctuations (SOFI; Geissbuehler *et al.*, 2012; Lukeš *et al.*, 2017)

dTRABI analysis. New 3D analysis system based on radial-aperture-based intensity estimation (TRABI; Franke *et al.*, 2017)

4   RESULTS:

LAT versions lacking palmitoylation exhibited trapping in the intracellular structures.

Other transmembrane adaptor proteins (PAG, NTAL) were trapped partially but the sorting to plasma membrane was not blocked even in absentia of palmitoylation.

After addition of highly glycosylated extracellular domain totally suppress the impact of palmitoylation on LAT mutant localisation.

Presence of ER-exit amino acid motifs were found in the C-terminal intracellular domain of LAT and PAG, which significantly increased the release of these proteins. The effect was presented using the minimal version C-terminal end connected directly to the TMD of LAT.

The positive rule inside was confirmed using an artificial TRAP-like protein with TMD swapped for LW19 peptide. Protein carrying the same charge on both ends was trapped in the intracellular compartments significantly. The difference was suppressed in the presence of longer TMD.

LAT with extended TMD by adding six amino acids (LLLLLL or PILAML) did not overcome the essential role of palmitoylation in the LAT surface targeting.

Accumulation of these variants in lysosomes was exhibited.

A kink in the TMD of LAT around position of Pro8, Gly12 and Pro17 residues was characterised using all-atom MD simulations.

The replacement of these conserved kink-forming amino acids for helix supporting residues (P8A, G12L, P17A and P8,17A) did not prevent the sorting of LAT mutants to the plasma membrane *in vivo*.

The same partial trapping of proteins in the Golgi aparratus as observed for LAT-WT was demonstrated.

Transfection with these LAT forms showed good calcium response to the activation stimulus indicating proper function of the mutants.

The partially restoration of the surface localisation of non-palmitoylated LAT-P8A, LAT-G12L and LAT-P17A was demonstrated, however the plasma membrane sorting of these variants was incomplete.

None of the non-palmitoylated LAT-P8A, LAT-G12L, LAT-P17A and LAT-CS mutants was able to respond to the stimulus, irrespective of their surface localisation.

No differences in the nanoscopic organisation of palmitoylated LAT-P8A compared to LAT-WT were detected using super-resolution microscopy.

Non-palmitoylated version of LAT-P8A formed patchy structures on the T-cell surface, but these cells were almost unable to make any contact with the imaging surface.

The biggest source and influence of the contaminants during sample preparation for SMLM was determined.

New sample preparation preserving the nano-topography of the cell membrane was introduced.

Using newly adapted system, samples were prepared for adjusted 3D dTRABI

image analysis. Qauntitative analysis was improved for elucidating the localisation of CD4 and CD45 molecules.

Segregation of these two molecules on the molecular level was determined.

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       Contribution of the applicant in this project: development and standardisation of sample preparation procedure, sample preparation for all SMLM methods, SOFI measurements and analysis, interpretation of the measured data.

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       Contribution of the applicant to this project: characterization of the colocalisation of prepared mutants with markers of intracellular compartments in living cells, live cell imaging of LAT versions with extended TMD and technical support and advice for the super-resolution microscopy measurements.