PhD THESIS

FUNCTIONAL PROTEIN MICRODOMAINS IN THE PLASMA MEMBRANE OF T AND B CELLS: THEIR ROLE IN ANTIGEN-SPECIFIC T CELL ACTIVATION AND PROLIFERATION

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Summary

Experimental observations accumulated in the past decade led to the "membrane microdomain" concept depicting compartmentation/organization of membrane components into well-defined patterns. According to our recent knowledge lateral order of membrane lipids and proteins is a general phenomenon that may have fundamental importance in functions of the individual molecules as well as in that of the whole plasma membrane.

Non-random, yet dynamic, and eventually genetically determined distribution of cell surface proteins was first presumed by Damjanovich et al in 1981 and in the last three decades their existence was experimentally proved in many cases by them and other workgroups. On the basis of presently available data, non-random protein patterns exist at least two hierarchical levels in the cell membrane: in addition to protein clusters generated by the physical association/molecular proximity of the molecules (nanometer scale), accumulation of these clusters into larger islands at the submicrometer/micrometer scale can also be observed in many cases. While a lot of physical and chemical information are available about factors initiating and maintaining lateral heterogeneity of membrane lipids, exact mechanisms responsible for compartmentalization of membrane proteins in living cells are still unknown in most cases. Revealing these regulators can help to understand function of these protein patterns and also details of membrane-associated processes (e.g. signal transduction).

The main purpose of my work presented here was to characterize protein patterns formed by participation of the class I Major Histocompatibility Complex (MHC I) and interleukin-2 receptor (IL-2R) molecules and to reveal factors responsible for maintenance and regulation of protein clusters.

Introduction

MHC I glycoproteins, expressed by most nucleated cells, play a key role in the cellular immune response: peptides derived from endogenous (and sometimes from exogenous) antigens are recognized by $CD8^+$ T cells in association with MHC I molecules. It seems that correct folding and proper transport to the cell surface requires the MHC-encoded heavy chain (α-chain) to be non-covalently associated with the light chain (β2-microglobulin; β2m) and the antigenic peptide. At the surface of cells with defects in peptide transport/loading, a reduced expression and stability of class I MHC can be observed, while in most β2m-deficient human cells heavy chains don't reach the cell surface at all. On the other hand, β2m-free heavy chains (FHCs) were detected at the surface of numerous $\beta2m^+$ human and murine cell types. Appearance of free heavy chains in these cases

requires the cell surface expression of the intact β2m/HC heterodimers, since FHCs originate from such β2m-associated MHC I molecules. Although the exact functional role of these cell surface FHCs is still not clear, there are many data showing that free heavy chains are not simply the spoiled, functionally inactive versions of class I MHC molecules, but they do have biological role.

Clustering (self-association) of HLA-I glycoproteins as well as their hetero-associations with other membrane proteins (e.g. MHC II, ICAM-1) was observed earlier at the surface of several (mostly lymphoid) cell types by fluorescence resonance energy transfer (FRET) and lateral diffusion (Single Particle Tracking, SPT) measurements. Furthermore electron and scanning force microscopic experiments disclosed the non-random (clusterized) organization of HLA I molecules at a higher hierarchical level, as well: applying immunogold labeled MHC I molecules, their several hundred-nanometer size domains were observed. Since clusters of MHC molecules could be observed in "supramolecular activation clusters" within the immunological synapse (IS), it is reasonable to assume that these large MHC clusters at the contact of antigen presenting and T cells may influence the efficiency of T cell activation (antigen-presentation): their existence can promote IS formation and/or high local concentration of MHC I can significantly increase the avidity of APC -TC interaction.

Interleukin-2 (IL-2) is one of the most important humoral factors regulating peripheral homeostasis (proliferation, differentiation and effector function) of T lymphocytes. Interleukin-2 receptor (IL-2R) is composed of three subunits: the α-chain (CD25) is employed solely by IL-2 while the β- and γ_c-chains can be shared by other cytokines, too. Based on its subunit utilization and ligand binding affinity, several forms of the receptor complex can exist at the cell surface. Among them only the high affinity $\alpha\beta\gamma_c$ heterotrimer and the intermediate affinity $\beta\gamma_c$ heterodimer receptor complexes are capable for signal transduction, since heterodimerization of the intracellular domains of the β- and γ_c-subunits is essential for IL-2 signaling, The α-subunit regulates ligand binding affinity, its presence guarantees the specific, high affinity binding of IL-2 to its receptor. Although both forms can transmit signals, *in vivo* biological effects of IL-2 are mostly mediated by the high affinity complex. According to an earlier model, subunits of the IL-2R complex exist independently at the cell surface and formation of the high affinity complex requires IL-2 binding to the receptor α-chain. In contrast to this "sequential subunit-organization" (affinity conversion) model, recent FRET data suggested a 'preassembly' of the three IL-2R subunits, even in the absence of their relevant cytokine ligands in the plasma membrane of T lymphoma cells. Binding of the physiological ligands (IL-2, IL-7, IL-15) was reported to selectively modulate the mutual molecular proximities/interactions of the IL-2R α, β and γ_c chains. Our knowledge about factors playing role in formation of the heterotrimer complex and maintenance of the dynamic and ligand-dependent

equilibrium between receptor subunits are still rather incomplete. Forces initiating and stabilizing hetero-association of IL-2Rα and HLA I molecules observed at the surface of activated human peripheral lymphocytes and T lymphoma cells and physiological significance of this colocalization still remained to be elucidated.

Aims of the study

The aim of this study was to reveal and characterize factors involved in maintenance and regulation of supramolecular protein associations observed by physical methods at the surface of B and T limfoid cells of human origin. Our investigations were mainly focused on the cell surface organization of class I HLA molecules playing key role in antigen presentation and IL-2 receptor molecules essential in proliferation of T cells following antigen specific stimulation.

The following questions were studied:

- Is there any relationship between the microdomain structure (cholesterol level) of the plasma membrane and the oligomerization of HLA I molecules at the surface of B cells?

- What is the role of free ($\beta 2m^-$) heavy chains and the exogenous β2m concentration in the HLA I oligomerization at the surface of B cells? Do the exogenous β2m level or the degree of HLA I oligomerization influence the efficiency of T cell activation/antigen presentation?

- Can the existence of association motifs formed by the participation of IL-2R and HLA glycoproteins generalized at the surface of different T cells of leukemia/lymphoma origin? What kind of factors are involved in maintenance/stabilization of these association patterns?

- What causes the genetically non-determined assembly (compartmentalization) of IL-2R subunits in the plasma membrane of above-mentioned cell types?

- Are there any functional (e.g. signaling) consequences of membrane compartmentalization of IL-2R subunits?

Materials and Methods

Cell cultures

The following cell lines were used in our experiments: JY, EBV-transformed, human B lymphoblastoid cells; Kit225 K6, Kit225 IG3 and HUT102B2 cells of human T lymphoma/leukaemia origin.

Monoclonal antibodies

Monoclonal antibodies (mAbs) applied for specific labeling of cell surface proteins were purified by Protein A affinity chromatography from the supernatant of hybridoma cells in our department. For flow cytometric and fluorescence microscopic experiments mAbs or their Fab fragments were conjugated with fluorescent dyes.

Modification of membrane cholesterol content

Cholesterol content of the cell membrane was modified by treatment with methyl-β-cyclodextrin, phosphatydil-choline liposomes (depletion) or cholesteryl hemisuccinate (enrichment). The efficiency of treatments was tested by measuring steady-state fluorescence anisotropy of lipid probes incorporating into the plasma membrane of the cells.

Isolation of detergent resistant membrane (DRM) fraction

DRMs were isolated from the homogenisate of detergent treated cells by equilibrium saccharose- gradient centrifugation. Protein content of membrane fractions was analysed by SDS-PAGE and Western-blot methods.

Determination of protein expression

Expression level of cell surface proteins was determined by flow cytometric analysis of monoclonal antibody binding. Number of binding sites was estimated from the background corrected mean values of fluorescence intensity histograms of cells incubated with fluorescent antibodies in saturating concentration.

Fluorescence resonance energy transfer (FRET) experiments

Proteins were labeled by monoclonal antibodies or their Fab fragments conjugated with donor or acceptor fluorophores. Energy transfer efficiency (E) was expressed as percentage of the donor's excitation energy tunneled to the acceptor molecules. The mean values of the calculated energy transfer distribution curves were used as characteristic FRET efficiencies between the two labeled protein epitopes.

Occasionally FRET was also detected on donor- and double labeled cells by the microscopic photobleaching (pbFRET) technique. Here, minimum 5000 pixels of digital cell images were analyzed in terms of bleaching kinetics and the efficiency of FRET was calculated from the mean

bleaching time-constants of the donor dye measured on donor- and double labeled cells, respectively

Scanning microscopic measurements

Large-scale (>100 nm) organization of membrane components targeted by specific fluorescent labels was investigated either by confocal laser scanning or near field scanning optical microscopy.

Immunoprecipitation and cocapping experiments

Occasionally, colocalization of proteins was determined by immunoprecipitation or cocapping experiments also. In case of immunoprecipitation proteins precipitated by Protein G beads pre-coated with the appropriate antibodies were analyzed by SDS-PAGE and Western-blot methods. In cocapping experiments cells labeled with fluorescent monoclonal antibodies were incubated with anti-IgG. Then cells were fixed with formaldehyde, blocked with isotype control antibody and stained with fluorescent antibody against the other protein, on ice. The double-stained cells were analyzed for co-capping by fluorescence microscope.

Detection of IL-2 stimulated tyrosine-phosphorylation

Cells with or without IL-2 treatment were subjected to fixation and permeabilization and incubated with specific rabbit anti-phospho-STAT-3/STAT5 polyclonal antibodies. After incubation with an FITC-conjugated secondary anti-rabbit IgG, cells were analyzed by flow cytometry. Overall tyrosine phosphorylation pattern stimulated by IL-2 was assessed by immunoblotting, using anti-phosphotyrosine antibody conjugated with horse radish peroxidase.

Analysis of efficiency of antigen presentation

Activation of allospecific T cells was followed by flow cytometry, measuring cell surface expression of the T cell receptor (CD3) and CD69 molecules. Specific lysis of target cells (efficiency of antigen presentation) was determined by detecting luminescence intensity of Eu^{3+} released from target cells previously filled with Eu^{3+}-DTPA complex.

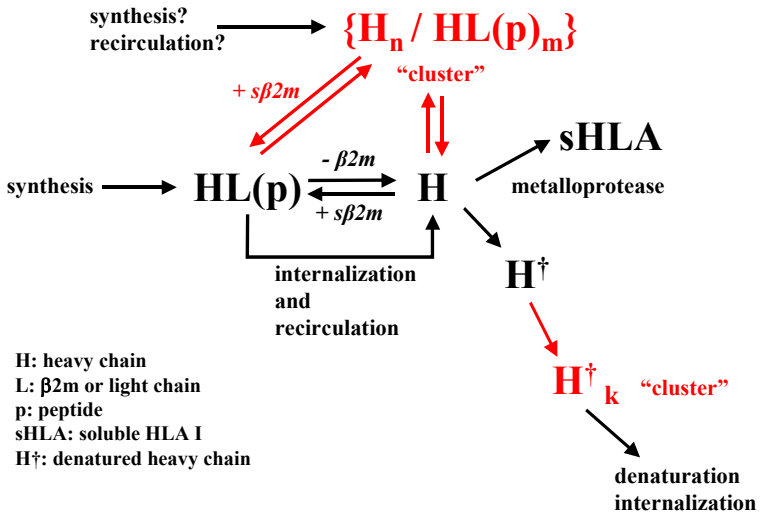
Summary of the results

(1) The cholesterol level of the plasma membrane (microdomain structure of the membrane) was shown to regulate both HLA I oligomerization and HLA I accessibility to antibodies at the surface of human B lymphoblasts.

(2) Flow cytometric and near field scanning microscopic measurements revealed participation of free ($\beta 2m^-$) HLA I heavy chains and intact HLA I heterodimers in the same protein patterns at the surface of JY human B cells. Concentration of β2-microglobulin available at the cell surface – presumably modifying equilibrium between different forms of HLA I heavy chains – plays an important role in controlling the extent of HLA I oligomerization.

(3) Applying allospecific T lymphocytes, it was shown that β2m level available at the surface of the antigen presenting cell – presumably through its role in controlling HLA I oligomerization (see previous paragraph) – has an important role in regulation of T cell activation and effector function.

(4) On the basis of our own results and the data available in the literature we propose the following model for the molecular organization of HLA I molecules at the surface of virus transformed and activated B cells:



(5) FRET experiments revealed existence of several "conservative" association motifs formed by the participation of IL-2R complex and HLA molecules in the plasma membrane of T cells of human lymphoma/leukemia origin. With the exception of cell specific

oligomerization of IL-2Rα, occurrence of these association motifs was not influenced by ligand binding of the IL-2 receptor.

(6) It was shown by physical and biochemical methods that at the surface of Kit225 K6 cells IL-2Rα is partially compartmented by lipid raft microdomains enriched in cholesterol and glycosphingolipids. FRET experiments have shown that integrity of lipid rafts is critical for stability of several association motifs formed by the participation of IL-2Rα. Abolition of lipid rafts decreased the efficiency of tyrosine phosphorylation induced by IL-2 stimulation, implying that these microdomains are essential in human T leukemia/lymphoma cells in formation and stabilization of the high affinity IL-2R complex. This idea was also supported by the analysis of detergent resistant fraction of cells: in addition to the α-chains, the signaling β- and γ_c-subunits were also identified in the DRM fraction. Our data also show that not all the surface CD25 molecules are located in lipid rafts; a significant portion of them is found in detergent soluble membrane fractions, i.e. they are distributed at the cell surface either randomly or in association with other microdomains (e-g. membrane domains accumulating transferrin receptors).

Practical significance

Our results can contribute to understanding of molecular details of antigen presentation and T cell activation. According to our results and data available in the literature some of the protein patterns studied by us can also be found at the surface of activated peripheral lymphocytes from healthy donors while others are only characteristic for cells of lymphoma/leukemia origin. Comparing association patterns in the plasma membrane of cells of different origin, revealing similarities and differences in motifs existing at the surface of cells from healthy or sick patients or cells from another species can promote understanding their functional significance and molecular details of normal and pathological cell functioning (e.g. activation, cell transformation etc.), so it can have significance both in basic research and diagnosis or therapy of different immune diseases.

List of publications related to the thesis:

1. **Bodnár A.**, Jenei A., Bene L., Damjanovich S., Matkó J.: Modification of membrane cholesterol level affects expression and clustering of class I HLA molecules at the surface of JY human lymphoblasts. *Immunol Lett*. 1996, 54: 221-226. **IF: 1,546**
2. *Matkó J., *__Bodnár A.,__ Vereb G., Bene L., Vámosi G., Szentesi G., Szöllősi J., Gáspár R. Jr., Horejsi V., Waldmann T. A. and Damjanovich S.: GPI-microdomains (membrane rafts) and signaling of the multi-chain interleukin-2 receptor in human lymphoma/leukemia T cell lines. *Eur. J. Biochem.* 2002, 269: 1199-1208. **IF: 2,852**

   * Matkó J. and Bodnár A. are equally contributed.
3. **Bodnár A.**, Kwik J., Bacsó Z., Jenei A., Jovin T. M., Edidin M., Damjanovich S. and Matkó J.: Class I HLA oligomerization at the surface of B cells is controled by exogenous β2-microglobulin: implications in activation of cytotoxic effector T cells. (in preparation)

Other publications:

4. Penyige A., Matkó J., Deák E., **Bodnár A.** and Barabás G.: Depolarization of the membrane potential by beta-lactams as a signal to induce autolysis. *Biochem. Biophys. Res. Com.* 2002, 290: 1169-1175. **IF: 3,055**
5. Damjanovich S., Vámosi G., **Bodnár A.** and Bene L.: New trends in studying structure and function of biological membranes. (review) *Acta Physiol. Hung.* 2002 (accepted for publication)
6. Dornan S., Sebestyén Z., Gamble J., Nagy P., **Bodnár A.**, Alldridge L., Doe S., Holmes N., Goff L. K., Beverley P., Szöllősi J. and Alexander D. R.: Differential association of CD45 isoforms with CD4 and CD8 regulates the actions of specific pools of p56lck tyrosine kinase in T cell antigen receptor signal transduction. *J. Biol. Chem.* 2002, 277: 1912-1918. **IF: 7,368**
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8. Nagy P., Vámosi G., **Bodnár A.**, Lockett S. J., Szöllősi J.: Intensity-based energy transfer measurements in digital imaging microscopy. *Eur. Biophys. J.* 1998, 27: 377-389. **IF: 2,188**

9. Jenei A., Varga S., Bene L., Mátyus L., **Bodnár A.**, Bacsó Z., Pieri C., Gáspár R. jr, Farkas T. and Damjanovich S.: HLA class I and II antigens are partially co-clustered in the plasma membrane of human lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA,* 1997, 94: 7269-7274. **IF: 10,789**
10. Salga P., **Bodnár A.**, Damjanovich S. and Mátyus L.: Distance calibration of fluorescence energy transfer values on cell surfaces. *SPIE*, 1998, 3261: 244- 249.
11. Matkó J., Vereb G. and **Bodnár A.**: Lipid and protein mobility in the plasma membrane of cells: studies by fluorescence anisotropy and fluorescence photobleaching recovery (FPR). In: Practical guide to physical analysis of cell surface receptors. (ed. Krasznai Z. and Mátyus L.) Debrecen, 1998, pp. 92-109.
12. Bacsó Z., Bene L., **Bodnár A.**, Matkó J. and Damjanovich S.: A photobleaching energy transfer analysis of CD8/MHC-I and LFA-1/ICAM-1 interactions in CTL-target cell conjugates. *Immunol. Lett.* 1996, 54: 151-156. **IF: 1,546**
13. Matkó J., Mátyus L., Szöllősi J., Bene L., Jenei A., Nagy P., **Bodnár A.** and Damjanovich S.: Analysis of cell surface molecular distributions and cellular signaling by flow cytometry. *J. Fluoresc.* 1994, 4: 303-314. **IF: 0,771**