SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of biomolecular interactions in the regulation of protein phosphatases studied by surface plasmon resonance based binding technique

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2014

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The Examination takes place at the library of the Department of Physiology, Faculty of Medicine, University of Debrecen at 11:00 am, 16th October, 2014

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Introduction

The phosphorylation and dephosphorylation of proteins have important roles in the regulation of cellular processes. The extent of protein phosphorylation is determined by the activity ratio of the phosphorylating protein kinase and the dephosphorylating protein phosphatase. Many protein phosphatase types are identified and among them the phosphoserine/threonine specific protein phosphatase-1 (PP1) and -2A (PP2A) are thought to be responsible for the dephosphorylation of a great number of cellular phosphoproteins. The protein phosphatases are regulated by regulatory and inhibitory subunits, which determine their catalytic activity and substrate specificity.

Functions, structure and regulation of the PP1

The protein phosphatase-1 (PP1) is expressed in holoenzyme form in all eukaryotic cells and it plays important roles in the regulation of meiosis, cell proliferation, apoptosis, protein synthesis, methabolism, cytoskeletal arrangement as well as membrane receptores and channels. Three catalytic subunit (PP1c) isoforms are known in mammals: PP1cα, β/δ and γ. The active centre is at the intersection of the Y-shaped catalytic grooves, and is responsible for the enzyme activity. At the catalytic centre Fe^{2+} and Zn^{2+} ions are coordinated which activate a structural water molecule in order to facilitate the hydrolysis of the phospho-Ser/Thr side chain of the substrate. The catalytic groove is devided into three main parts: C-teminal, acidic and hydrophobic groove, which play important roles in the binding of the substrate.

The structure of the PP1 regulatory proteins is quite diverse, however, almost all of them contain a short sequence motif (K/R-x1-V/I-X2-F/W,

shortly RVxF), which ensures the specific binding to the catalytic subunit. The regulatory subunits of PP1 can be devided into two major groups: targeting and inhibitory proteins. The targeting subunits have PP1c- and subtrate-binding site and during their action they direct the holoenzyme to the substrate or to the appropriate subcellular compartment of the cell. In contrast, the inhibitor proteins have only PP1c-binding site, but not substrate-binding site, so these proteins can be the substrates (Rb, Nek2, AKAP149) and/or inhibitors of the enzyme. The inhibitors are generally 14-32 kDa heat stable molecules (I1, I2, CPI-17) and their binding to the PP1c regulate its catalytic activity. The activity of PP1 catalytic subunit (PP1c) can be inhibited by the cell cycle-dependent phosphorylation of its Thr320 side chain. Similar inhibition mechanisms may also occur via the tyrosine phosphorylation of PP2Ac.

The catalytic activity of PP1 can be regulated by membrane permeable toxins (okadaic acid, tautomycin, microcystin, cantharidin, calyculin A), which bind to the hydrophobic groove partially covering the substrate binding groove and in some cases the catalytic centre too, preventing the binding and hydrolysis of the substrate. These toxins influence the activity of PP2A, too. The inhibitory effect of tannins on the PP1 and PP2A activity has been previously proved, but the mechanism of inhibition is not known.

Functions, structure and regulation of PP2A

The PP2A is a multimeric enzyme, whose catalytic subunit (PP2Ac) retained its structure during the evolution. PP2A plays important roles in cell proliferation and apoptosis, cell motility, morphogenesis, cell cycle and in the regulation of many signal transduction pathways and it is considered as a tumor suppressor. The enzyme is heterotrimeric, which consists of a heterodimeric catalytic (C) and an A scaffold (or PR65) subunit as well as the

B regulatory subunit. Four classes of the regulatory B subunit (and many names) are known, these are: B (B55 or PR55), B' (B56 or PR61), B'' (PR48/PR72/PR130) and B''' (PR93/PR110) letters or they are represented by a wide variety of letter combinations. Each B subunit has 2-5 isoforms which give rise to the possible existence of many trimeric holoenzymes.

The regulation of PP2A, similarly to PP1, occurs via regulatory protein interactions, or on the other hand can be accomplished through various posttranslational modifications. Growth factors, insulin receptor and tyrosine kinases may also be involved in the regulation of PP2A causing inactivation of the enzyme. The activity of PP2A holoenzymes is not influenced by inhibitor-1 (I1) and inhibitor-2 (I2) proteins, metal ions are not required to their activity, compared to the Mg^{2+}-dependent PP2C. The PP2A is inhibited by the traditional phosphatase inhibitors such as microcystin-LR (MC-LR), okadaic acid (OA) and calyculin A (CLA).

Myosin phosphatase: its structure and interaction with myosin substrate

Myosin phosphatase holoenzyme consists of PP1c, myosin targeting subunit MYPT1 and a 20 kDa protein of unknown function. The MYPT1 (Myosin Phosphatase Target subunit 1) regulatory subunit are first cloned from chicken gizzard and rat aorta library. First, a longer (133 kDa) and a shorter (110-130 kDa) MYPT1 isoform were identified. The differences in size reflect the presence or absence of the insert in the middle of the protein or leucine zipper motifs. The KVKF (35-38) PP1c-binding motif and the ankyrin repeats (39-296) can be found on the N-terminal fragment of MYPT1. Acidic (326-372), ionic (719-793, 814-848) and Ser/Thr (770-793) side chain rich regions and the central insert (517-553) can be further distiguished. The

leucine zipper motifs (1006-1030) at the C-terminal do not occur in all isoforms.

In the interaction of PP1c and MYPT1 attachment of PP1-binding motif is essential and it is followed by $MYPT1^{1-22}$ sequence engagement which facilitates the binding of ankyrin repeats containing $MYPT1^{10-296}$ as well as the acid $MYPT1^{304-511}$ region.

The N- and C-terminal regions of MYPT1 may play important roles in the interaction of MP with substrates. The phosphorylated light chain (P-MLC20) of the myosin substrate bound to the ankirin repeats of MYPT1 at the N-terminal region, while the heavy chain of the whole myosin interacts with the C-terminal part of MYPT1.

Interaction analysis of drugs and toxins with lipid micelles using surface plasmon resonance (SPR) based binding technique

Binding of drugs and toxins to proteins or lipid micelles covered sensor chip surface results in changes of the SPR resonance signals, which is suitable for the determination of the association-dissociation profiles of the interactions. The procedure can be automated and the interaction is followed in real time. The immobilized lipid bilayer can be used for the analysis of the different interactions of transmembrane-proteins as well as for investigation of the interactions of membrane-permeable molecules with micelles.

Identification of protein interacting partners from cell lysate

The SPR detection based measurement technique offers the possibility to isolate interacting partners of proteins from cell lysates, then the proteins in the recovered samples can be identified by immunological methods and/or mass spectrometry. Modern equipments (eg Biacore 3000) have an external

device, wherein more than three times of the sensor chip surface can be used for immobilization compared to the chip surface available in the integrated microfluidic system (IFC), ensuring a proportional increase in surface binding capacity.

Aims

The inhibitory effect of gallotannins for PP1 and PP2A activities have been shown earlier, but the mechanism and the phosphatase type specificity of inhibition are still not known. In the influence of membrane-permeable inhibitors on the cellular protein phosphatases the diffusion of the inhibitors through cell membrane may be a rate-limiting step. However, the methods used so far to assess the mechanism of action of these inhibitors have not provided data on the kinetics of these interactions. Previuos data suggest that the regulation of protein phosphatase catalytic subunits is accomplished via their interaction with various regulatory or inhibitory proteins, which may affect the direction and the rate of the cellular processes. The exploration of the above interactions with the currently available modern methods has not been fully developed yet.

Based on the above questions our research objectives have focused on studying the following issues:

1. Investigation of the inhibitory effect of polyphenols and their derivatives on the activity of PP1 and PP2A enzymes.
2. Determination of the EGCG and PGG binding sites in the structure of PP1 enzyme using SPR method.
3. Quantitative characterization of the interaction of phosphatase inhibitors and lipid micelles derived from different tissues in order to understand the mechanism of inhibition at the cell membrane level.
4. Development of a novel method based on SPR binding technique for the identification of protein phosphatase interacting partners from control and differenly treated cells.

Materials and methods

Investigation of biomolecular interactions using SPR method

The surface plasmon resonance occurs in a thin conducting film between two media of different refractive index. In this method one interacting partner is immobilized on the surface of a sensor chip, while the other partner is flown over the surface. Binding of molecules to the immobilized ligand will change the conditions on the surface and this will cause a shift in the resonance signal, which is proportional to the amount and mass of the surface associated molecules. The sensorgram is suitable for the determination of kinetic parameters, like association and dissociation rate conatants (k_a, k_d), from which the equilibrium constants (K_A, K_D) can be calculated.

In our experiments CM5, SA and L1 sensor chips were used. The sensor chip CM5 contains carboxymethylated dextran matrix, which is suitable for covalent immobilization through amino, thiol or aldehyde groups. The covalently bound streptavidin modified SA sensor chip is useful for the immobilization of biotinylated proteins or molecules, while L1 is used for the attachment of lipid micelles.

Binding analysis of tannins to rPP1cδ by SPR method

Recombinant PP1cδ was immobilized to the surface of CM5 sensor chip through amino groups during the analysis. The tannin solutions were injected over the PP1c coated sensor chip surface in 70 µl volume for 7 min followed by a 6 min dissociation phase.

Two competition experiments were performed to determine the binding site of tannins, using OA and MC-LR as competing molecules, since their interaction mechanisms with the enzyme is already known. The

immobilized rPP1cδ was saturated with the solution of either 1 μM MC-LR or 5 μM OA, then at the same inhibitor concentration in case of OA 10 μM PGG (penta-O-galloyl-β-D-glucose) or 5 μM EGCG ((-)-epigallocatechin-3-gallate) solution was injected over the surface.

We designed a specific PP1c immobilization technique: biotin-MC-LR was immobilized to the streptavidin coated SA sensor chip surface then rPP1cδ in 1 μM concentration was injected to saturation over it. The biotin-MC-LR-rPP1c interaction is strong enough that the immobilized biotin-MC-LR keep the enzyme stable on the surface. PGG and EGCG in the above used concentration was injected over the biotin-MC-LR-rPP1cδ complex covered surface as well as Flag-MYPT1 in 1 μM concentration was used as control to demonstrate binding to different binding site of PP1c. The evaluation of the sensorgrams was carried out with BIAevaluation 3.1 softwere.

Preparation of lipid micelles

Lipid micelles were prepared from bovine brain, heart or liver total lipid extracts. First the lyophilized total lipid extracts were hydrated (~3 mg/ml) in the immobilization buffer (50 mM Hepes, 150 mM NaCl, pH 7.4) followed by four cycle of freezing (-80 °C), thawing (20 °C), and then vortexing. The size of the prepared lipid micelles was inhomogenoues, therefore homogenization was performed with repeated extrusion through polycarbonate filter with 100 nm pore diameter in an Avanti Mini-Extruder. After homogenization the lipid suspension was diluted in the immobilization buffer to 1 mg/ml concentration.

Immobilization of lipid micelles onto L1 sensor chip surface

The lipid micelle solutions prepared from brain, heart or liver total lipid extracts were immobilized to saturation in isolated flow cells at a flow rate of 2 µl/min. The average immobilization level of lipid micelles was 7500 ± 200 response unit (RU). The reference (lipid-free) and the three different composition lipid micelle covered surfaces were blocked with 0.1 mg/ml BSA solution.

Investigation of the membrane-association of protein phosphatase inhibitors using SPR method

The interaction between phosphatase inhibitors and lipid micelles from different tissues was investigated with SPR method. The membrane association and dissociation processes were performed at 20 °C as well as at physiological temperature (37 °C). The Kinject injection type was used and 120 s durations were chosen for both association and dissociation phases. In case of all inhibitors the injections were repeated with increasing sample concentration in successions. After each binding series, the sensor surfaces were regenerated and the sensor surfaces were recoated with fresh liposome solutions for the next binding series. The evaluation of the sensorgrams was carried out with BIAevaluation 3.1 softwere.

Cell cultures, incubation with PP inhibitors, UVA irradiation

B50 (neuroblastoma), H9C2 (cardiomyoblast), HepG2 (hepatocarcinoma) or human keratinocyte (HaCaT) cell lines were used. Cells were grown at 37 °C, 5% CO_2, 95% air and humidity over 80% in the incubator. D-MEM-based medium was used, which also contained 10% FBS, 2 mM glutamine and 100 U/ml penicillin/streptomycin and 500 U/mL

antibiotic-antimycotic. Prior to treatments with phosphatase inhibitors, cells were incubated in serum-free medium for 16 h. B50, H9C2 and HepG2 cells were treated with various concentrations of OA, TM (tautonycin), MC-LR, CA (cantharidin) and EGCG for 60 min. The influence of the inhibitors was investigated with phosphatase activity measurements. HaCaT cells were treated 100 nM CLA or 10 J/cm^2 UVA light in Bio-Sun UV irradiater chamber (Vilbert Lourmat, France). After lysing the cells followed by centrifugation the supernatants were used for the determination of phosphatase activity and for interaction analysis.

Western blot analysis

From cell lysates 30 μg (in case of phosphoproteins it was 50 μg) protein were loaded onto 10% SDS-polyacrylamide gel and subjected to SDS-PAGE, then transferred to nitrocellulose membrane which was blocked with 3% BSA solution. Membranes were probed for proteins of interest using primary antibodies in appropriate dilutions then incubated with HRP-conjugated secondary antibody. The immunoreactive bands were detected by ECL reagents.

Dot blot analysis

Samples obtained during the isolation of phosphatase interacting proteins were spotted directly onto nitrocellulose membrane, which was blocked after drying and incubated with the primary and secondary antibody solutions at the same dilutions as for Western blot and the spots were detected by ECL reagents.

Phosphatase activity measurement

Phosphatase activity was assayed with 1 μM ^{32}P-labelled 20 kDa myosin light chain (^{32}P-MLC20) substrate at 30 °C. The incubation time was 10 min in case of purified phosphatases and 1-2 min for cell lysates. After precipitation of proteins with trichloro-acetic acid and centrifugation the released $^{32}P_i$ was determined from the supernatant.

Transfection of siRNA

PP1c was silenced in HaCaT cells using panPP1 small interfering RNA (panPP1-siRNA), which was diluted in the appropriate DharmaFECT 2 transfection reagent containing serum-free D-MEM media. The mixture was added to HaCaT cells in 100 nM final concentration of siRNA and cells were incubated with the mixture in suspension for 30 min. Cells were plated into cell culture dishes and the same volume of 20% FBS containing D-MEM media was added and placed for 48 hours into the incubator.

Synthesis of biotin-microcystin-LR conjugate

First the N-methyldehydroalanine residue of MC-LR was derivatized with ethanedithiol (EDT) then iodoacetyl-LC-biotin was attached to it. The EDT-MC-LR was purified by C18 Sep-Pak cartridge then icubated in the dark with 6 mmol of iodoacetyl-LC-biotin/mmol EDT-MC-LR for 90 min at room temperature. Biotin-MC-LR was purified by preparative reverse phase HPLC. The conversion of MC-LR to biotin-MC-LR and the purity of the product were verified by mass spectrometry (MALDI-TOF-MS).

Immobilization of biotin-microcystin-LR on the SA Sensor Chip

Biotin-MC-LR was diluted in the running buffer (10 mM Hepes, 150 mM NaCl, pH=7.4) at approximately 10 μM concentration and injected at a

flow rate of 2 µl/min directly to streptavidin coated surface to reach saturation when the immobilized biotin-MC-LR corresponded to ~400 RU.

Recovery of the surface bound components from the Integrated µ-Fluidic Cartridge (IFC)

Recombinant PP1cδ was flown over the microcystin coated and the control surface. After the saturation phase the surfaces were washed to stabilize resonance signals. The surface-bound PP1c was recovered with 0.5% trifluoro-acetic acid (TFA) solution, which was spotted directly to nitrocellulose membrane and analyzed with dot blot technique.

In further experiments the GST-coupled wild type MYPT1 (GST-MYPT1$^{1\text{-}1004}$), its PP1c-binding (His-MYPT1$^{1\text{-}633}$) or PP1c non-binding (GST-MYPT1$^{667\text{-}1004}$) fragments were injected over the biotin-MC-LR-PP1c surface, which was recovered and analyzed as described above.

Recovery of the surface bound proteins from the external unit

Biacore 3000 device has a specific external Surface Prep unit that allows more than three times (16 mm^2) larger sensor chip surface for immobilization than in the IFC unit (4×1.2 mm^2). Thus the amount of recovered proteins from the surface can be increased in Surface Prep unit as well as the IFC can be prevented from the contamination by cell extracts. The same adjustments for flow rate and injection time were used in these experiments than in the IFC. Cell lysates of untreated, PP1c depleted, CLA treated or UVA-irradiated HaCaT cells were injected over the biotin-MC-LR coated surface and the recovered proteins were spotted directly onto nitrocellulose membrane and analyzed with dot blot technique.

Investigation of the localization of PP1c and PP2Ac in cells using biotin-MC-LR

HaCaT cells were fixed, permeabilized then blocked. Coverslips were incubated with biotin-MC-LR then anti-PP1c(α/γ) or anti-PP2Ac antibodies. Alexa 488-conjugated Streptavidin (green) and Alexa 594-conjugated secondary antibody (red) were used for the detection. Finally coverslips were fixed with ProLong Gold Anitifade Reagent with DAPI to the glass slide and were imaged on a Zeiss LSM 510 confocal laser scanning microscope.

Results and discussions

The effect of tannins on PP1c and PP2Ac activity and investigation of their inhibitory mechanism

We analyzed the effect of PGG, EGCG and Aleppo tannin as well as their structural units on phosphatase activity in order to identify the tannin interacting surface in the PP1c structure. Our results suggest that PGG, EGCG and Aleppo tannin are effective inhibitors of protein phosphatases. They are partially selective inhibitors of PP1c, while their inhibitory effect on PP2Ac is weaker allowing the differentiation of PP1c and PP2Ac activities at certain tannin concentrations. We determined that the flavonol unit and the galloyl group of EGCG influence inhibitory effectiveness significantly, while the polyphenol ring and its hydroxil groups contribute to a lesser extent. Our SPR competition experiments prove that classical inhibitors (OA, MC-LR) which interact with the hydrophobic groove of PP1c prevent the binding of PGG and EGCG suggesting a common binding site of these inhibitors on PP1c. Based on the above the tannins form hydrophobic interaction with the hydrophobic groove in the vicinity of the PP1c active centre. The enzyme-tannin interaction is stabilized by hydrogen bonds between the hydroxil groups of EGCG and amino acid side chains of the enzyme. The effect of tannins on phosphatase activity is weaker (IC50 $\geq$ 0.2-0.47 μM) than that of classical phosphatase inhibitors (IC50 $\leq$ 0.1-0.05 nM). Our results imply that the phosphatase inhibitory effect of the analyzed tannins are caused by their interaction with the hydrophobic groove of PP1c thereby competing with the substrate. In contrast to the toxins tannins do not interact with either the active centre or any other substrate interacting surface (eg. acidic groove), which may explain their weaker inhibitory effect.

The IC50 values obtained for the phosphatase inhibition differ greatly in case of PP1c and PP2Ac. In the hydrophobic grooves of PP1c and PP2Ac the amino acid side chains, which are involved in the stabilization of the interaction and forming hydrogen bonds with the EGCG, are mostly similar. Even though, the position of EGCG in the structure of the two enzymes is different, but this still would not justify the different inhibitory efficiency. Based on the structural and modeling data we can suggest that the Ala182-Gly193 loop position in the PP2Ac different from the Arg187-Gly199 loop position in PP1c. This structural difference reduces the size of the hydrophobic groove in the PP2Ac, therefore EGCG could not enter easily to the groove. There is a barrier to carry out similar molecular modeling with PGG due to the higher rotational freedom of this molecule with the currently available programs. However, based on competition and NMR binding studies the main interaction of PGG with the hydrophobic groove of PP1c is assumed. These data imply that the specificity difference of PGG toward PP1 and PP2A may be attributed to similar structural features as in case of EGCG.

In summary, we identify tannins with various structures as a novel family of phosphatase inhibitors. Among these molecules PGG and EGCG proved to be as partially selective inhibitor of PP1c. They are less effective inhibitors than the natural toxins, but they are not toxic and after chemical modification of their relatively simple structure novel and more selective phosphatase inhibitors may be synthetized.

Investigation of membrane permeable properties of phosphatase inhibitors

The interaction between phosphatase inhibitors and cell membranes and their diffusion through the cell membrane into the cells may play an

important role in the phosphatase inhibitory effects. SPR detection based technique was used to study the membrane association and dissociation features of the inhibitors analyzing the interactions of inhibitors with lipid micelles characteristic for different tissues. We analyzed the association and dissociation properties of phosphatase inhibitors with micelles prepared from the total lipid extracts derived from different tissues. We determined the kinetic profile of the classical (OA, TM, MC-LR, CA, CsA) and the newly identified (EGCG) phosphatase inhibitors with lipid micelles derived from bovine brain, heart and liver tissues. Lipid micelles were immobilized stably on the sensor chip surface at 20 °C and their interaction with phosphatase inhibitors was found to be reversible. The dissociation of inhibitors from lipid micelles of different composition was very similar, but significant differences were observed in their association kinetics. We investigated the above interactions at 37 °C in order to characterize the lipid-inhibitor interactions at physiological temperature. There is difference in the association kinetics and the concentration dependence disappeared, and the dissociation surprisingly slowed down. It is assumed that phosphatase inhibitors (OA, TM, MC-LR) with hydrophobic character bind to the lipid micelle surface in the first step then incorporated into the bilayer structure and only slowly dissociate to the intracellular space. These properties may presumably explain the necessity of their longer incubation time for the effective inhibition exerted on intact cells. The phosphatase inhibitors (CA, EGCG) with partially hydrophilic character can not be sufficiently infiltrate into the lipid membrane, which reduces their permeation properties. The sensorgrams from the SPR experiments suggest that higher molecular weight inhibitors (OA, TM, MC-LR) with hydrophobic character get into the interior of the "more fluid" micelles in the association

phase at 37 °C and therefore retained and only dissociate slowly in the washing phase after injection. In contrast, the lower molecular weight inhibitors (CA, EGCG) embedded in the lipid membrane to a small extent and in the washing phase dissociate without significant restriction. The *in vitro* membrane association investigations show that the binding capacity of the lipid micelles from various tissues is different. The phosphatase inhibitors associate in the lowest extent to the brain lipids and in the greatest extent to the liver lipids. *In vivo* experiments show that the treatment of different cell lines with phosphatase inhibitors reduced the phosphatase activity the least on the neuroblastoma B50 cell line, which is in good agreement with the results of the *in vitro* binding data. We can assume according to both experiments that the saturation of lipid membranes has occurred at low inhibitor concentration and even a dramatic increase in the inhibitor concentration causes only slight change in the resonance signal or in the extent of inhibition. These data suggests that the diffusion of inhibitors into the intracellular space coupled with the inhibitor binding capacity of the membrane can be the rate-limiting step in the inhibitory process.

There is no correlation between the apparent dissociation constants ($K_{D(app)}$) and activity measurements. In case of OA the calculated dissociation constant shows high affinity ($K_{D(app)}$) to the liposomes, in contrast, caused only a slight decrease in activity even in the highest concentration. The $K_{D(app)}$ value for TM was high, however the effect on the phosphatase activity at low concentrations was significant. The CA binds with high affinity to the lipid micelles, whereas inhibition of phosphatase activity was no significant or only very slight. These findings suggest that the inhibitory effect depends on not only the strength of the interaction between the lipids and toxins but other

factors, too. The MC-LR is able to get into the hepatocytes via bile acid transporter. The investigated HepG2 cell line does not contain this transporter, so it is not surprising that the inhibition of MC-LR in case of all three cell lines are similar. These latter findings are also consistent with previous data demonstrating that the MC-LR can diffuse into the cells at higher concentration even in the absence of bile acid transporters and inhibits phosphatase activity.

We can conclude based on our measurements that SPR detection based measurements are suitable for characterization of the interaction between complex composition lipid micelles and phosphatase inhibitors. The binding of inhibitors is affected by the lipid composition of micelles. The phosphatase activity measurements performed on B50, H9C2 and HepG2 cell lines demonstrate that the development of the inhibiton of cellular phosphatases depends on the type of cells, which may presumably reflect the different lipid composition of membranes.

Isolation and identification of protein phosphatase interacting partners from HaCaT cell lysate

The interaction of protein phosphatase catalytic subunits with regulatory and inhibitory proteins as well as substrates plays an important role in the regulation of cellular processes by phosphorylation. The SPR based technique is theoretically suitable for isolation of protein complexes from small amount of cell lysate, and the mass spectrometric analysis of these recovered samples can provide much faster results on the possible protein interactions than the conventional methods. A sensor chip surface immobilized "capture" molecule, which forms a stable interaction with the target protein(s), is needed for the isolation of these protein complexes coupled with a sample

recovery procedure. The isolation of microcystin-sensitive protein phosphatases (PP1, PP2A, PP2B, PP4-7) and their interacting proteins is fast and requires minimal sample volume with the use of the method developed in our experiments. MC-LR was biotinylated and immobilized on the sensor chip surface covalently modified with streptevidin. The MC-LR-biotin-streptavidin interaction is strong enough ($K_D \sim 10^{-15}$) to keep the captured phosphatases and their interacting partners isolated from the cell lysate on the surface. In addition, the MC-LR-biotin-streptavidin interaction is not affected by the procedure involved in the recovery process, so the created surface is suitable for isolation of phosphatases and their interacting partners several times. In the first step the method was tested with the recombinant PP1c, one of its regulatory subunit (MYPT1), and its PP1c-binding and non-binding fragments. We found that the biotin-MC-LR can stably bind rPP1c, the resulting complex interacts with the full-length regulatory subunit and its PP1c interacting N-terminal fragment but not with the non-binding C-teminal one.

In normal and PP1c-depleted as well as CLA treated keratinocyte cell lysates the changes in the amount of PP1c as well as the phosphorylation level of MYPT1 interacting protein could be detected after application of the above isolation technique followed by detection of the proteins in the recovered samples by dot blots. The dot blot analysis generally does not provide quantitative measure, but the presented experiments proved that the concentration of proteins focused on in the recovered sample reflects the changes of their amount in the cell. The quantitative changes of PP1c, PP2Ac and their regulatory and inhibitory proteins were analyzed in untreated and UVA-irradiated HaCaT cells. Our results show that the amount of phosphatase catalytic subunits and regulatory proteins in the recovered samples reduced

after UVA irradiation. Phosphatase activity confirmed that the UV treatment reduced the phosphatase activity in keratinocyte cells nearly 40%, however, the Western blot identified no change in the amount of proteins in the cell lysate. It is assumed that the effect of UVA irradiation generated reactive oxygen species in the cells which initiated oxidation of the metal ions and/or cysteine side chains in PP1c and PP2Ac. These modifications may cause changes in the structure of PP1c and PP2Ac accompanied by decreased activity and altered ability to associate with inhibitors (such as MC-LR) and the regulatory proteins, too.

Mass spectrometric analysis of the recovered samples did not provide reasonable results. The immobilized biotin-MC-LR captures not only PP1c and PP2Ac, but many other MC-LR interacting protein phosphatases. Therefore, the biotin-MC-LR binding capacity is shared between these enzymes. On the other hand, the amount of phosphatase interacting proteins also reduced in the recovered sample by the fact that the large complexes formed cover many potential unoccupied MC-LR molecules on the surface, thus sterically inhibiting further binding proteins to the immobilized capture molecules. Therefore, the binding capacity of MC-LR is only partially used and the amount of the bound proteins does not reach the level necessary for mass spectrometry analysis.

Our results indicate that the method developed by us is able to detect the changed association ability of PP1c and PP2Ac to MC-LR immobilized on the chip surface or to interacting proteins in cells after physiological or pathological challenges. The method can also be used to investigate posttranslational modifications of PP1c and PP2Ac or their regulatory proteins, and may provide preliminary results for the composition of

phosphatase complexes. At present, the above technique is limited to identifying known phosphatase interacting proteins that are needed to be detected by a specific antibody. In further development of the method we focus on the possibility that the biotin-MC-LR surface will not be damaged during the experiments, so larger sample amounts required for MS-analysis could be obtained by repeating the binding-recovery cycles several times.

Summary of the novel results

1. We showed that tannin derivatives such as EGCG and PGG inhibit effectively the activity of PP1 and PP2A and they are partially selective inhibitors of PP1. Their inhibitory effect was exerted by their binding to the substrate binding hydrophobic groove of PP1c as determined by SPR competition experiments. Our results suggest that the tannin components represents a new and effective family of phosphatase inhibitory compounds, which are less toxic than the natural phosphatase inhibitory toxins and their chemical modification may be promising to develop selective inhibitors for the distinct phosphatase types.

2. The membrane association of inhibitors is fast at physiological temperature and depends on the composition of the lipids; their dissociation is slow, presumably due to the intercalation of the inhibitors in lipid micelles. It is established that the interaction characteristics of phosphatase inhibitors to membrane lipids influence their inhibitory effects in a cell type dependent mannar.

3. The biotinylated form of phosphatase inhibitory microcystin-LR on the surface of streptavidin coupled sensor chip was used as capturing molecule to isolate PP1c and PP2Ac as well as their interacting proteins from HaCaT cell lyaste by SPR method. The immunological analysis of the recovered samples proved that the method is suitable for the detection of PP1c and PP2Ac as well as their interacting proteins and for the changes of their phosphorylation state in normal and phosphatase inhibitor treated or UVA-light irradiated HaCaT cells.

Register number: DEENKÉTK/180/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Bálint Bécsi
Neptun ID: P8Q2RR
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10034521

List of publications related to the dissertation

1. **Bécsi, B.**, Kiss, A., Erdődi, F.: Interaction of protein phosphatase inhibitors with membrane lipids assessed by surface plasmon resonance based binding technique.
Chem. Phys. Lipids. 183C, 68-76, 2014.
DOI: http://dx.doi.org/10.1016/j.chemphyslip.2014.05.009
IF:2.147 (2012)

2. **Bécsi, B.**, Dedinszki, D., Gyémánt, G., Máthé, C., Vasas, G., Lontay, B., Erdődi, F.: Identification of protein phosphatase interacting proteins from normal and UVA-irradiated HaCaT cell lysates by surface plasmon resonance based binding technique using biotin-microcystin-LR as phosphatase capturing molecule.
J. Photochem. Photobiol. B. 138C, 240-248, 2014.
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3. Kiss, A., **Bécsi, B.**, Kolozsvári, B., Komáromi, I., Kövér, K.E., Erdődi, F.: Epigallocatechin-3-gallate and penta-O-galloyl-β-D-glucose inhibit protein phosphatase-1.
FEBS J. 280 (2), 612-626, 2013.
DOI: http://dx.doi.org/10.1111/j.1742-4658.2012.08498.x
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Total IF of journals (all publications): 36.228
Total IF of journals (publications related to the dissertation): 9.507

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

24 July, 2014