High-throughput Enzyme-linked Immuno absorbent Assay (ELISA) for determination of Rho-kinase (ROK) activity

The invention is referring to an assay for measuring the activity of a ROK (Rho kinase) protein by means of a biotinylated peptide.

The invention pertains to an assay for identification of a compound that modulates (stimulates, diminishes, maintains) the activity of ROK protein wherein

a] a peptide that is capable of being phosphorylated by ROK protein is provided;

b] ROK protein is provided;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated, is provided;

d] a chemical compound is provided;

e] the peptide from a] is incubated with the ROK protein from b] as well as with the chemical compound from d] under conditions that allow phosphorylation of the peptide;

f] the antibody from c] is used after the incubation performed according to e] has been finished to detect phosphorylation of the peptide;

g] the result from f] is possibly compared to the result from an experiment wherein incubation of the peptide from a] the ROK protein from b] is performed without having chemical compound according to d] present.

Such an assay can easily be carried out in ELISA or RIA (Radio Immun Assay) format.

The peptide of step a] with respect to the assay as depicted before can consist of a size of between 15 to 25 amino acids. This peptide can comprise or consist of the following amino acid sequence: GGGGAKRRRLSSLRASTS. The peptide according to step a] of the assay can be used in a chemically modified form as e.g. being biotinylated or linked to a DNA or antibody.

The ROK protein of step b] with respect to the assay as depicted before can comprise the amino acid sequence of the according human protein or can be derived or taken from human biological material. Such a ROK protein can be manufactured by different techniques as e.g. by a procedure having biological material or processing steps of recombinant nature included. The assay of the invention as disclosed before can e.g. be used for a High Throughput Screening of a compound that modulates the activity of ROK protein.

The invention pertains also to a kit of parts comprising of

a] a peptide that is able of being phosphorylated by ROK protein;

b] ROK protein;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated;

d] possibly a second antibody that is capable of recognizing the antibody from c] and/or is linked to a marker system (e.g. alkaline phosphatase, ß-glucuronidase, peroxidase, other enzyme);

e] possibly reagents, buffers and/or equipment for realizing phosphorylation of the peptide from a] and/or a ELISA set up.

The peptide of step a] with respect to the kit of parts as mentioned before consists of a size of between 15 to 25 amino acids. This peptide can comprise or consist of the

following amino acid sequence: GGGGAKRRRLSSLRASTS. The peptide according to step a] of the assay can be used in a chemically modified form as e.g. being biotinylated or linked to a DNA or an antibody. The ROK protein of step b] with respect to the assay as depicted before can comprise the amino acid sequence of the according human protein or can be derived or taken from human biological material. Such a ROK protein can be manufactured by different techniques as e.g. by a procedure having biological material or processing steps of recombinant nature included.

The invention pertains further to the manufacturing of a kits of parts as mentioned before wherein

a] a peptide that is capable of being phosphorylated by ROK protein is provided;

b] ROK protein is provided;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated is provided;

d] possibly a second antibody that is capable of recognizing the antibody from c] and/or is linked to a marker system (e.g. alkaline phosphatase, ß-glucuronidase, peroxidase, other enzyme) is provided;

e] possibly reagents, buffers and/or equipment for realizing the phosphorylation of the peptide from a] and/or a ELISA set up are provided;

f] the components from a] to e] are packages accordingly and combined with a protocol mentioning therein amongst other the components of the kit of parts as well as the procedural steps of the resulting ROK protein assay.

The kit of parts as mentioned before can be used e.g. for determining the activity of a ROK protein, or for identifying of a compound that modulates the activity of a ROK protein, or for High-Throughput Screening.

The invention pertains further to a peptide comprising or consisting of the following amino acid sequence: GGGGAKRRRLSSLRASTS. Such a peptide can be chemically modified as e.g. biotinylated. The invention pertains also to the use of such a peptide for measuring the activity of a ROK protein.

A chemical compound in context of this invention shall mean any organic and/or carbohydratic compound that is either produced by chemical synthesis or isolated from a natural source. Such a compound may have a molecular weight of between 50 and 50 000 Dalton.

A protein shall be regarded as functional in context of this invention when it is in a condition to perform an activity in a biological context in particular as part of a living cell. Such an activity is detectable e.g. by an assay. A transporter is functional for example when this transporter moves a compound in particular the transporter's biological substrate from outside a cell into the inner compartment of this cell or vice versa. A biological substrate of an ion transporter protein consists e.g. of a monovalent and/or a divalent ion or other ions. The substrate of a glucose transporter protein is e.g. glucose. The substrate of a multiple drug resistance protein is e.g. a drug alone or conjugated to glutathione or gluconate.

The handling of proteins in context of this invention can be achieved by a person skilled in the art applying the according protocols from "Current Protocols in Protein Science" published by John Wiley & Sons (edited by: John E. Coligan, Ben M. Dunn, Hidde L., Ploegh, David W. Speicher, Paul T. Wingfield; 0-471-11184-8-Looseleaf; 0-471-14098-8-CDROM).

The handling of techniques concerning Molecular Biology as e.g. cloning, transforming of cells, sequencing, modifying promoters, expression proteins or others can be

achieved a the person skilled in the art by applying the according protocols from "Current Protocols in Molecular Biology" published by John Wiley & Sons (edited by: Fred M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A. Smith, Kevin Struhl; 0-471-50338-X-Looseleaf; 0-471-306614CDROM).

Biological material means any material containing genetic information and capable of reproducing itself or being reproduced in a biological system. Recombinant biological material is any biological material that was produced, has been changed or modified by means of recombinant techniques. Recombinant techniques are defined in textbooks as e.g. "Current Protocols in Molecular Biology, published by John Wiley & Sons; ISBN: 0471-50338-X-Looseleaf or 0-471-30661-4-CD-ROM".

The handling of biological cells can be achieved a the person skilled in the art by applying the according protocols from "Current Protocols in Cell Biology" published by John Wiley & Sons (edited by: Juan S. Bonifacino, Mary Dasso, Jennifer Lippincott-Schwartz, Joe B. Harford, Kenneth M. Yamada; 0-471-24108-3-Looseleaf; 0-471-24105-9-CDROM).

Examples

Rho-kinase (ROK) represents a highly validated target for cardiovascular diseases. Therefore, the search for specific ROK-inhibitors is the focus of many groups working in cardiovascular area. Here, we describe the development of an ELISA, specific for ROK-activity. The ROK-ELISA detects the amount of a ROK-phosphorylated peptide. ROK-Inhibitors inhibit ROK-activity and reduce thereby the amount of ROK-mediated peptide phosphorylation. ROK-ELISA was developed in a medium throughput 96-well format as well as in a high throughput 384-well format.

Protocol:

Stock solutions:

Biotinylated S6 peptide: store at –20°C in aliquots of 1 ml in Tris buffer 25 mM pH 8,0 (5 mg/ml)

recombinant active ROK: store at –20°C in aliquots of 10 µl (1 mg/ml)

ATP	100 mM	Sigma Cat.# A-6419 Lo# 29H7051	-20°C
Stop solution	3N HCL	Hydrochloride standard solution (Sigma)	RT
$MgCL_2$	1 M	$MgCl_2$ (Sigma)	RT
BSA	10 %	Sigma	-20°C
DTT	1 M	Sigma	-20°C
ODP-Peroxidase Substrate		Sigma	2-8°C
Superblock buffer	+ 0,2 % TWEEN 20	Superblock (Blocking buffer in TBS; Pierce Cat.# 37535), TWEEN 20	2-8°C
washing buffer (PBS)	PBS 1 x + 0,2 % TWEEN 20	PBS w/o $CaCl_2$ and $MgCl_2$, Gibco # 2006-01, TWEEN 20	2-8°C
reaction buffer	25 mM Tris pH 7,4; 0,02% BSA; 2mM DTT		use freshly prepared only

1) Coating of streptavidin coated wells with the biotinylated S6 peptide 0.5 ng/well for 2 hours or overnight at room temperature
2) triple wash out of excess S6 peptide with PBS 100 µl/well
3) Preparation of a 10 x ATP solution in the desired concentration in reaction buffer with $MgCl_2$ on ice
4) Dilute ROK in reaction buffer on ice, final concentration: 10ng/well
5) Prepare a 10 x substance solution in the desired concentration

ROK-ELISA 96-well format

Total volume = 100 µl

1) Preincubation of inhibitors (10 µl of a 10 x concentrated solution) in the desired final concentrations with 80 µl of ROK for 10 minutes
2) add 10 µl ATP of a 10 x concentrated ATP solution in the desired final concentration
3) incubate the plate for one hour at 30°C
4) Stop kinase reaction with 100 µl of a 500mM EDTA solution
5) remove supernatant of all wells
6) add 100 µl/well of primary monoclonal phosphospecific antibody (1:1000) and incubate for 15 minutes at room temperature
7) add 100 µl/well of secondary antibody (1:2000) and incubate for one hour at room temperature
8) remove supernatant and wash five times with PBS 200µl/well
9) add 100µl of substrate solution to all wells and incubate for 10 minutes at room temperature
10) add 100µl stop solution to all wells
11) measure OD at 492 nm

ROK-ELISA 384-well format

Total volume = 10µl

1) Preincubation of inhibitors (1µl of a 10 x concentrated solution) in the desired final concentrations with 8 µl of ROK for 10 minutes
2) add 1µl ATP of a 10 x concentrated ATP solution in the desired final concentration
3) incubate the plate for one hour at 30°C
4) Stop kinase reaction with 10µl of a 500mM EDTA solution
5) remove supernatant of all wells
6) add 10µl/well of primary monoclonal phosphospecific antibody (1:1000) and incubate for 15 minutes at room temperature

7) add 100µl/well of secondary antibody (1:2000) and incubate for one hour at room temperature
8) remove supernatant and wash five times with PBS 30µl/well
9) add 10µl of substrate solution to all wells and incubate for 10 minutes at room temperature
10) add 10µl stop solution to all wells
11) measure OD at 492 nm.

Material

ROK (ROKα/ROCK-II (active recombinant aa 11-550 or longer),
Biotinylated S6 peptide (Biotin-GGGGARRRLSSLRASTS),
Streptavidin coated 96- or 384-well plates (e.g. Roche StreptaWell, High Bind (transparent) 1989685/1989677, Roche Diagnostics, Mannheim),
Monoclonal phosphospecific antibody from Transduction Labs (clone 22a, BD Biosciences, Heidelberg), goat-anti-mouse IgG-HRP 200µg/0,5ml (Santa Cruz Cat# sc-2005).

Description of Figures:

Fig.: 1 depicts the amino acid sequence according to SEQ ID NO. 1.
Fig.: 2 represents a typical layout for the ROK ELISA
Row H 1-6 serves as control wells for kinase reaction. Wells of row H1-3 were without kinase and with ATP and represent the minimal signal. Wells of row H4-6 were with kinase and with ATP and represent the maximal signal. Wells of row H7-12 served for a concentration relationship for the reference compound. All other wells were used to obtain a concentration relationship for the compound of interest. Please note the pale color in wells of row E1-7 indicating that a highly potent inhibitor inhibited ROK mediated substrate phosphorylation over the whole concentration range used. In the case of IC50 estimation with single point determination at the same time 14 compounds plus one reference compound can be tested in the 96-well format.
Fig.: 3 and 4 A and B illustrate results (% inhibition and IC50 calculation) from the ROK ELISA

Claims

1. Assay for identification of a compound that modulates the activity of Rho kinase (ROK) protein wherein

a] a peptide that is capable of being phosphorylated by ROK protein is provided;

b] ROK protein is provided;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated, is provided;

d] a chemical compound is provided;

e] the peptide from a] is incubated with the ROK protein from b] as well as with the chemical compound from d] under conditions that allow phosphorylation of the peptide;

f] the antibody from c] is used after the incubation performed according to e] has been finished to detect phosphorylation of the peptide;

g] the result from f] is possibly compared to the result from an experiment wherein incubation of the peptide from a], the ROK protein from b] is performed without having chemical compound according to d] present.

2. Assay as claimed in claim 1, wherein the incubation according to steps e], f] and g] is performed as ELISA assay.

3. Assay as claimed in claim 1, wherein the incubation according to steps e], f] and g] is performed as RIA assay.

4. Assay as claimed in claims 1 to 3 wherein the peptide according to step a] has a size of 15 to 25 amino acids.

5. Assay as claimed in claim 1 to 4 wherein the peptide according to step a] has the following amino acid sequence: GGGGAKRRRLSSLRASTS.

6. Assay as claimed in claim 5 wherein the peptide is biotinylated.

7. Assay as claimed in one or several of claims 1 to 6 wherein the ROK protein is from human.

8. Assay as claimed in one or several of claims 1 to 7 wherein the ROK protein has been manufactured recombinantly.

9. Use of an assay according to one or several of claims 1 to 8 for High-Through-Screening of a compound that modulates the activity of ROK protein.

10. Kit of parts comprising of

a] a peptide that is able of being phosphorylated by ROK protein;

b] ROK protein;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated;

d] possibly a second antibody that is capable of recognizing the antibody from c] and/or is linked to a marker system;

e] possibly reagents, buffers and/or equipment for realizing phosphorylation of the peptide from a] and/or a ELISA set up.

11. Kit of parts as claimed in claim 10 wherein the peptide from a] has a size of 15 to 25 amino acids.

12. Kit of parts as claimed in claim 10 or 11 wherein the peptide from a] has the following amino acid sequence: GGGGAKRRRLSSLRASTS.

13. Kit of parts as claimed in claim 12 wherein the peptide is biotinylated.

14. Kit as claimed in one or several of claims 10 to 13 wherein the ROK protein is from human.

15. Kit as claimed in one or several of claims 10 to 14 wherein the ROK protein has been manufactured recombinantly.

16. Manufacturing of a Kit of parts according to claims 10 to 15 wherein

a] a peptide that is capable of being phosphorylated by ROK protein is provided;

b] ROK protein is provided;

c] an antibody that is capable of recognizing the peptide from a] if this peptide is phosphorylated is provided;

d] possibly a second antibody that is capable of recognizing the antibody from c] and/or is linked to a marker system is provided;

e] possibly reagents, buffers and/or equipment for realizing the phosphorylation of the peptide from a] and/or a ELISA set up are provided;

f] the components from a] to e] are packaged accordingly and combined with a protocol mentioning therein the components of the kit of parts as well as the procedural steps of the resulting ROK protein assay.

17. Use of a kit of parts according to claims 10 to 16 for determining the activity of a ROK protein.

18. Use of a kit of parts according to claims 10 to 16 for identifying of a compound that modulates the activity of a ROK protein.

19. Use of a kit of parts according to claims 10 to 16 for High Throughput Screening.

20. Peptide consisting of the following amino acid sequence: GGGGAKRRRLSSLRASTS.

21. Peptide of claims 21 that is biotinylated.

22. Use of a peptide according to claims 20 and 21 for measuring the activity of a ROK protein.