B cell signal modulating effect of cell membrane permeable phosphopeptides based on the sequences of Gab1 adaptor protein

Ph.D. Thesis

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INTRODUCTION

Reversible phosphorylation of protein tyrosine residues play critical role in signaling cascades triggered by various receptors. These activation events controlled by the opposing activities of protein tyrosine kinases (PTKs) and phosphatases (PTPs) eventually lead to diverse cellular response such as proliferation, survival, apoptosis, differentiation or migration. Adaptor proteins play important regulatory role in downstream signal transduction from the cell membrane towards the nucleus, providing a surface to form multimolecular signaling complex. Grb2-associated binder 1 (Gab1) protein is involved in the signal transduction pathways of growth factors, cytokines, and antigen receptors. Gab1 adaptor protein has several tyrosine residues, which are phosphorylated upon ligand mediated tyrosine kinase activation and binds signaling molecules with SH2 domains, such as SHP-2 tyrosine phosphatase and phosphatidyl inositol 3-kinase (PI 3-K). Gab1/SHP-2 contact is essential for cell growth, in addition SHP-2 and PI3-K regulate signals leading to proliferation and cell survival. Malfunction of SHP2 or PI3-K due to the oncogenic mutation of these genes may cause undesirable cell proliferation and transformation. Accordingly Gab1/SHP-2 and

Gab1/PI3-K interactions might be attractive targets for the therapy of malignant cell growth.

We hypothesized that synthetic phosphopeptides associated with endogenous SH2 domains may disrupt the protein-protein interactions, thus modulating the cell activation. Our main goal to design and analysis cell membrane permeable phosphopeptides based on the tyrosine containing motif of Gab1 that would selectively regulate SHP2 and PI3-K mediated B cell responses.

OBJECTIVES

1. To selection of the optimal cell-penetrating peptide (CPP) for use as a vector for bioactive phosphopeptides.
2. To make clear the penetrating mechanism and intracellular localization of CPP and CPP- phosphopeptide construction.
3. To study the effect of Gab1 derived synthetic phosphopeptides on SHP2 enzime activity.
4. To evaluate the connected SH2 domain containing proteins with Gab1 phosphotyrosine motifs representing GDLDpe and ELPNpe peptides.
5. To investigate protein interactions modulating capacity of penetrated phosphopeptides based on the SH2 domain-phosphotyrosyne connection.
6. To study the influence of penetrated CPP-phosphopeptides on the phosphorilation process in resting and activated B cells.
7. To study the regulatory role of SHP2 and PI3-K on Erk and Act activation in B lymphocyte.
8. To examine the effect of OR8-GDLDpe and OR8-ELPNpe on the SHP2 and PI3-K mediated signaling pathway in B cells.

METHODES

Cell culturing

Burkitt lymphoma cell line BL41 was used in most of the experiment. BL41 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

FACS analysis

Cells were incubated on different temperature and for different time interval with Bodipy-FL-conjugated cellpermeable carrier or PP constructs. The sampleswere analyzed on a FACS Calibur cytofluorimeter (BectonDickinson) using CellQuest software.

Confocal microscopy

$2x10^6$ BL41 cells were incubated with Bodipy-FL-labeled cell permeable peptides. Cellular uptake was followed by confocal laser scanning microscopy, using the 488-nm line of an argon ion laser (Olympus FluoView 500). The lipophilic fluorescent probe (DiI-C18) was used to stain the cell membrane, while acidic lysosomes were detected with LysoTracker Red probe The latter probes were excited by the 543-nm He-Ne laser line.

Stimulation of intact cells, preparation of detergent cell extracts, SDS-PAGE and Western blot.

BL41cells were pretreated with the cell-membrane-permeable PPs and then stimulated with anti-μ. After stimulation the cells were centrifuged immediately and frozen in liquid nitrogen. Cell pellets were resuspended in lysis buffer. Following centrifugation the postnuclear supernatants were used for further examinations. Cell extracts were diluted in reducing SDS/PAGE sample buffer and boiled for 5 min at 95 °C. The samples were separated on SDS/PAGE gels, and the proteins were then transferred to nitrocellulose membranes, and consecutively probed with various antibodies. Immunoblots were developed using HRPO-conjugated second antibodies, followed by ECL detection.

Phosphatase activity assay

Serial dilutions of recombinantSHP-2 protein were prepared in 96-well plates and preincubated with biotinylated PPs in phosphatase buffer. The samples were harvested and transferred onto an ELISA plate precoated with avidin, and were further incubated at 4 °C overnight. After several rinsings, the plates were developed with phosphotyrosine-specific antibodies followed by peroxidase-conjugated anti-mouse IgG

and tetramethyl-benzidine as substrate. Optical densities in the wells were measured at 450 nm.

RESULTS

The CPPs were labeled with Bodipy-FL dye and their cellular uptake was followed by confocal microscopy. Considerable amount of CPPs were detected in acidic vesicules while another part CPPs were localised in cytoplasm. CPPs translocated into the cells within the first 5 min. of incubation a temperature independent and dose dependent manner. R8 and OR8 vectors showed the highest penetration efficiency and least cytotoxicity, therefore we selected O-R8 as a carrier for further experiments.

OR8 conjugated to the Gab1 PPs showed lower penetration efficiency but even less cytotoxicity in contrast with the CPPs analysed by flow cytometry.

OR8-GDLDpe and OR8-ELPNpe associated to the corresponding SH2-domain-binding sites of endogenous SHP2 and PI3-K after cellular uptake in BL41 cells. In the OR8-GDLDpe pretreated Bl41 cells diminished the strength of Gab1-SHP2 interaction triggerd by anti BCR stimuli, consequently phosphopeptide interfered with tyrosine phosphorylated Gab1 binding to SH2-domain of SHP2.

The in vitro experimental results showed that GDLDpe efficiently activated recombinant SHP-2 phosphatase, otherwise both of GDLDpe and ELPNpe peptides proved to be a substrate of the enzyme.

We have shown that OR8-GDLDpe and OR8-ELPNpe modulated intracellular protein phosphorylation on different manner in resting B cells. The OR8-GDLDpe treatment of BL41 cells modulated tyrosine phosphorylation in a dose- and time-dependent manner, while OR8-ELPNpe totally blocked tyrosine phosphorylation of a protein at 50 kDa.
Or8-GDLDpe pretreating inhibited the BCR induced Erk phosphorylation in BL41 cell line, while O8-ELPNpe alike reduced both of Erk and Akt activation induced by BCR crosslinking.

These data suggests that cell penetrating phosphopeptides dose- and time-dependent manner can modify SHP2 and PI3-K mediated signalling pathways, acordingly may affect on cell proliferation and apoptosis.

CONCLUSIOS

1. Octanoyl-Arg8 based carrier peptides suitable for the delivery of phosphopeptides into living cells. The CPP-phosphopeptide constructions are less cytotoxicity but penetrate with lower efficiency than CPPs alone.
2. The synthetic phosphopeptides associate specifically with the corresponding SH2 domaines of endogenous SHP2 and PI3-K. The Gab1 627Y representing, SHP2 connecting GDLDpe peptid in addition to bind PLCγ.
3. The GDLDpe affectively modify the SHP2 enzyme activity in the in vitro experiment and both phosphopeptides may play a role as alternative enzyme substrates.
4. The SHP2 binding OR8-GDLDpe inhibits the BCR induced Gab1-SHP2 interaction by competed for SH2 domains of the phosphatese.
5. OR8-GDLDpe and PI3-K binding OR8-ELPNpe peptides modulate the basic phosphorylation level of intracelular proteins on a different manner in BL41 cells.
6. The cell membrane permeable phosphopeptides a dose- and time-dependent manner modulet the SHP2 and PI3-K mediated signaling cascades triggered by BCR engagement with an even unknown mechanism.

PUBLICATIONS

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