Cell. Mol. Life Sci. 63 (2006) 2682–2693
1420-682X/06/222682-12
DOI 10.1007/s00018-006-6346-6
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Cellular and Molecular Life Sciences

Research Article

Optimization of the cellular import of functionally active SH2-domain-interacting phosphopeptides

Á. Kertész [a], G. Váradi [b], G. K. Tóth [b], R. Fajka-Boja [c], É. Monostori [c] and G. Sármay [a, *]

[a] Department of Immunology, Eötvös Loránd University, Pázmány Péter s. 1c, 1117 Budapest (Hungary), Fax: +36 1 3812176, e-mail: sarmayg@elte.hu
[b] Department of Medical Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged (Hungary)
[c] Lymphocyte Signal Transduction Laboratory, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62. 6726 Szeged (Hungary)

Received 27 July 2006; received after revision 4 September 2006; accepted 18 September 2006
Online First 31 October 2006

Abstract. Phosphopeptides interacting with src homology 2 (SH2) domains can activate essential signaling enzymes *in vitro.* When delivered to cells, they may disrupt protein-protein interactions, thereby influencing intracellular signaling. We showed earlier that phosphopeptides corresponding to the inhibitory motif of Fcγ receptor IIb and a motif of the Grb2-associated binder 1 adaptor protein activate SH2-containing tyrosine phosphatase 2 *in vitro*. To study the *ex vivo* effects of these peptides, we have now compared different methods for peptide delivery: (i) permeabilization of the target cells and (ii) the use of cell-permeable vectors, which are potentially able to transport biologically active compounds into B cells. We found octanoyl-Arg_8 to be an optimal carrier for the delivery of phosphopeptides to the cells. With this strategy, the function of cell-permeable SHP-2-binding phosphopeptides was analyzed. These peptides modulated the protein phosphorylation in B cells in a dose- and time-dependent manner.

Keywords. Cell-permeable peptide, phosphopeptide, SH2 domain, signaling, tyrosine phosphorylation.

Phosphopeptides (PPs) representing functional sequences of intracellular signaling molecules offer excellent tools to study specific signal transduction events. PPs cannot penetrate the plasma membrane barrier without a membrane translocalization signal. For study of their effects on intracellular signaling, the cells must be permeabilized with membrane-disrupting, pore-forming agents, or the phosphotyrosine-containing motifs must be coupled to cell-membrane-permeable carrier peptide (CPP) vehicles, which then transport the peptides into the cells [1, 2]. The lysophosphatidylcholine (LPC)-mediated permeabilization of lymphocytes was previously optimized for study of the effects of PPs on tyrosine kinase activation [3]. A PP corresponding to the biphosphorylated immunoreceptor tyrosine-based activation motif (ITAM) was found to activate *lyn* and induce protein phosphorylation in LPC-permeabilized B cells [4], while ζ chain ITAM peptide triggered tyrosine phosphorylation of p56 *lck* and ZAP70 in permeabilized Jurkat T cells [5]. The inherent limitations of cell permeabilization by membrane disruption can be overcome by employing membrane-penetrating carrier peptides to deliver biologically active compounds such as PPs into the cell. In the past few years, several CPPs have been reported [1, 6–10]. The first molecules of this kind that were described were various sequences of viral proteins responsible for virus internalization. Arg-rich peptides, such as the basic peptide derived from the human immunodeficiency virus 1

* Corresponding author.

(HIV1) Tat protein [11, 12] and oligo-arginines comprised of six to eight Arg units have been reported to penetrate the plasma membrane, thereby delivering bioactive molecules into the cells [1, 7, 13]. *Ex vivo* characterization of the known CPPs is not complete. The aims of this study were to compare detergent and CPP- mediated PP delivery methods and to design, characterize and optimize the procedure of PP delivery in order to analyze the effects of these PPs on B cell signal transduction events.

The PP cargos to be transported into B cells were designed on the basis of the phosphorylated motifs of two important B cell regulatory molecules: (i) The phosphorylated form of immunoreceptor tyrosine-based inhibitory motif (ITIM) of FcγRIIb, a negative regulator of B cells, and (ii) a C-terminal phosphorylated motif of Grb2-associated binder 1 (Gab1) adaptor/scaffolding protein. FcγRIIb becomes tyrosine-phosphorylated upon its co-clustering with the B cell receptor complex (BCR). Subsequently, FcγRIIb recruits phosphatases carrying src homology (SH2) domains, such as protein tyrosine phosphatase (SHP-2) and phosphatidylinositol 5-phosphatase (SHIP) to the vicinity of the signaling complex, in this way terminating cell activation [14–19]. SHP-2 has a dual role in lymphocyte signaling: recruited by phosphorylated ITIM (pITIM), it facilitates the down-regulation of signals emanating from activatory receptors and, additionally, SHP-2 may play a positive role by activating extracellular-signal-regulated kinases (Erk1/2) via the interaction with the Gab1 adaptor/scaffolding protein [20]. Gab1 becomes phosphorylated upon ligation of antigen, cytokine and growth hormone receptors, and the C-terminal phosphorylated motifs of Gab1 then bind to and activate SHP-2, which in turn may dephosphorylate additional phosphotyrosine residues, leading to the release of the *ras* negative regulatory molecule, rasGAP, resulting in the full activation of Erk1/2 [21–23].

Tyrosine phosphorylation is involved in the modulation of the activities of target enzymes, and is also responsible for the generation of signaling networks [24–26]. Impaired signaling may lead to the development of various tumors or autoimmune diseases [27–30]. Since Gab1/SHP-2 interaction is essential for cell growth and transformation, and SHP-2 regulates signals leading to proliferation and cell survival, Gab1/SHP-2 interaction is an attractive target for therapy of malignant cell growth. Blockade of the interaction between a functionally relevant tyrosine-phosphorylated motif and the SH2 domain by cell-permeable PP or phosphopeptido-mimetics may disrupt signaling and inhibit cell activation [31–33]. In this paper we report that SHP-2-binding, cell-membrane-permeable PPs modulate intracellular protein phosphorylation in B cells in a dose- and time-dependent manner.

Materials and methods

Antibodies and reagents. Monoclonal human IgM μ-chain-specific antibody (BU1) was a kind gift from Prof. R. Jefferis (University of Birmingham, Birmingham, UK). Anti-SHP-2 monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.). PI3-K p85 specific antibody and anti-phosphotyrosine 4G10 were from UBI (Lake Placid, N.Y.) and anti-phosphoErk1/2 was from Cell Signaling Technologies (Beverly, Mass.). Enhanced chemiluminescence (ECL) reagent was purchased from Perbio UK Science (Tattenhal, UK) and other reagents were from Sigma.

Dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIEA) methanol (MeOH), trifluoroacetic acid (TFA) and HPLC-grade acetonitrile (MeCN) were purchased from Aldrich (Steinheim, Germany) and were used without further purification. Fluka (Buchs, Switzerland) supplied puriss grade dicyclohexylcarbodiimide (DCC). Protected amino acid derivatives were from Orpegen (Heidelberg, Germany). 1-Hydroxybenzotriazole (HOBt), p-cresol and dimethyl sulfide were purchased from Aldrich. Bodipy-FL was supplied by Molecular Probes (Carlsbad, Calif.). The different amidites were prepared in our own laboratories. Hydrogen fluoride (HF) was obtained from Gerling-Holz (Hamburg, Germany).

Peptide synthesis. The sequences of the chosen CPPs were synthesized by a solid-phase technique utilizing ^{t}Boc chemistry [34]. The side-chain-protecting groups were: Arg(Tos), Thr(Bzl), Ser(Bzl), Cys(Meb), Tyr(2BrZ) and Lys(2ClZ). The peptide chains were elongated on an MBHA resin (0.48 mmol/g) and the syntheses were carried out in an ABI 433A automatic machine. Couplings were performed with DCC and HOBt. Amino acid incorporation was monitored with the ninhydrin test [35]. The completed peptide resins were treated with liquid HF/dimethyl sulfide/p-cresol/p-thiocresol (86/6/4/2, vol/vol), at 0 °C, for 1 h. HF was removed and the resultant free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by reverse-phase HPLC (Shimadzu, Kyoto, Japan) using a Phenomenex (Torrance, Calif.) C-18 10 μ column (16 × 250 mm). The solvent system used was: 0.1% TFA in water, 0.1% TFA, 80% MeCN in water, gradient: 0%-0% B for 15 min, and then 0–15% in 60 min, at a flow rate of 3.5 ml/min, with detection at 220 nm. The appropriate fractions were pooled and lyophilized. The purified peptides were characterized by mass spectrometry with a Finnigan TSQ 7000 tandem quadrupole mass spectrometer equipped with an electrospray ion source.

Synthesis of fluorescent peptides. 2.4 μmol of the appropriate peptide was dissolved in phosphate buffer (pH

6.5, 100 mmol, 6 mol guanidine hydrochloride) together with 1 mg (2.4 μmol) Bodipy-FL (B 10250). After stirring of the solution for 1–2 h, the resulting construct was purified by HPLC.

PP synthesis. Peptide synthesis was carried out on Rink amide resin using the standard Fmoc protocol (DCC/HOBt coupling, ABI 433 synthetizer). The side protection used was Asp(OBu^t), Lys(Boc), Glu(OBu^t), Thr(Bu^t), Ser(Bu^t) and His(Trt). The hydroxy groups of the serine or tyrosine, which were the subject of phosphorylation, were unprotected. Phosphitylation was carried out in THF at room temperature for 30 min with 1*H*-tetrazole as activator and *O*-*tert*-butyl-*O*-β-cyanoethyl-*N,N*-diisopropyl phosphoramidite in the case of serine and di-*tert*-butyl-*N,N*-diisopropyl phosphoramidite in the case of tyrosine [36]. The subsequent oxidation was performed with 15% *tert*-butyl hydroperoxide in THF. Simultaneous cleavage of the β-cyanoethyl and Fmoc groups was performed with 20% DBU in DCM [37]. After the DBU cleavage, the peptide chain elongation was completed and the PP was detached from the resin with TFA/water (95/5) solution.

Conjugation of the PPs to CPPs. 8 μmol of the maleinimidocaproyl derivative of the appropriate PP was dissolved in phosphate buffer (pH 6.5, 100 mmol, 6 mol guanidine hydrochloride) together with 11.96 mg (8 μmol) of O-R8C, where the C-terminal cysteine amide moiety binds to the double bond of the PP via Michael addition. After stirring of the solution for 3–4 h, the resulting construct was purified by HPLC. Details of the MS and HPLC analysis of the synthesized cell-permeable peptides are given in Table 1.

Table 1. Calculated and found molecular masses of penetrating peptides, their Bodipy-FL-conjugated forms, their conjugates with PPs; and their HPLC retention time.

Code	Calc M	Found M	HPLC Rt
R8C	1368.68	1369.1	10.84 min[a]
P-R8C	1607.09	1607.5	7.90 min[b]
O-R8C	1495.09	1495.2	9.77 min[c]
Bodipy-O-R8C	1909.31	1909.2	8.08 min[d]
C-TP10	2284.76	2283.6	13.17 min[e]
Bodipy-C-TP10	2698.98	2698.4	9.423 min[f]
C-transportan	2943.48	2942.4	7.37 min[g]
Bodipy-C-transportan	3357.70	3357.6	12.16 min[f]
C-MPG-α	3047.83	3047.6	13.70 min[h]
Bodipy-C-MPG-α	3462.05	3463.4	8.567 min[i]
C-MPG-α-ox	3063.83	3063.0	8.834 min[h]
Bodipy-C-MPG-α-ox	3478.05	3477.0	8.281 min[j]
O-R8C-GDLD(Pe)	3289.95	3290.2	11.07 min[k]
O-R8C-RKHP(Pe)	5335.36	5333.7	8.85 min[l]

C stands for Cys, a maleinimidocaproyl linker.

HPLC conditions for the analytical control (column dimension 4 × 250 mm, flow rate 1.2 ml/min): [a] gradient 5% → 35% B in 20 min, [b] gradient 45% → 60% B in 15 min, [c] gradient 20% → 60% B in 20 min, [d] gradient 32% → 47% B in 15 min, [e] gradient 40% → 80% B in 20 min, [f] gradient 53% → 73% B in 20 min, [g] gradient 50% → 65% B in 15 min, [h] gradient 50% → 100% B in 25 min, [i] gradient 71% → 86% B in 15 min, [j] gradient 65% → 80% B in 15 min, [k] gradient 25% → 40% B in 15 min, [l] gradient 25% → 45% B in 20 min.

Cell culturing, stimulation of intact cells. BL41 Burkitt lymphoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10^5 U/l penicillin and 50 μg/l streptomycin at 37 °C in a 5% CO_2-containing atmosphere. The cells were washed twice in serum-free RPMI 1640 and incubated at 37 °C for 30 min. 5×10^7 cells/sample were stimulated with 2 μg/ml human μ-chain-specific monoclonal antibodies (BU1). After a 2-min stimulation at 37 °C, the cells were centrifuged for 20 s and immediately frozen in liquid nitrogen. BL41 cells were pretreated with the cell-membrane-permeable PPs for various periods of time and then stimulated with anti-μ.

Cell permeabilization and stimulation of permeabilized cells. Cells were harvested by centrifugation, washed in RPMI 1640 without supplements and incubated in RPMI for 5 min at 37 °C. Cell pellets were resuspended in ice-cold permeabilization buffer [40 mM HEPES pH 7.4, 10 mM $MnCl_2$, 10 mM $Mg(OAc)_2$, 296 μM $CaCl_2$, 2 mM EGTA] and permeabilized with L-α-lysophosphatidyl choline at 50 μg/ml for 1 min on ice as previously described [3]. Permeability was assessed using trypan blue exclusion in each experiment.
Stimulation of permeable cells was initiated by adding peptides and/or anti-IgM for 3 min at 37 °C at the concentrations indicated for each experiment. The reaction was stopped with ice-cold 2× lysis buffer.

Preparation of detergent cell extracts, SDS-PAGE and Western blot. 2×10^6 cells/sample were solubilized in 0.1 ml lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaF, 10 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10% glycerol, 10 μg/ml aprotinin, 5μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride and 10 mM β-glycerophosphate. Samples were incubated on ice for 45 min and then centrifuged at 15,000 g for 20 min, and the postnuclear supernatants were used for further examinations. Cell extracts were diluted in 40 μl reducing SDS/PAGE sample buffer and boiled for 5 min at 95 °C. The samples were separated on 8.5% or 10% SDS/PAGE gels, and the proteins were then transferred to nitrocellulose membranes (BioRad), and consecutively probed

with various antibodies. Immunoblots were developed using HRPO-conjugated second antibodies, followed by ECL detection. Some membranes were stripped according to the manufacturer's instruction and reprobed with a different antibody.

Confocal microscopy. BL41 cells were incubated with 10 μM labeled CPP conjugates at 4 or 37 °C for 20 min. 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 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C18) was used to stain the cell membrane, while acidic lysosomes were detected with 100 nM Lyso-Tracker Red probe (Molecular Probes). The latter probes were excited by the 543-nm He-Ne laser line. The Pearson coefficient was calculated using Image J software and Image Correlator Plus plug in.

Flow cytometry. Cells were harvested and washed in PBS containing 1% FCS and 0.5 mg/ml NaN_3, and incubated for 5 to 60 min at 4 °C or at 37 °C (5×10^5 cells/sample) with different amounts of Bodipy-FL-conjugated cell-permeable carrier or PP constructs. Then, after the cells had been washed twice in the same buffer, the samples were analyzed on a FACSCalibur cytofluorimeter (Becton Dickinson, Franklin Lakes, N.J.) using CellQuest software. In some experiments, the cells were also stained with the DNA-intercalating dye propidium iodide to detect non-viable cells.

Phosphatase activity assay. Serial dilutions of BL41 cell lysate samples (equivalent to 10^5 cells) and of recombinant SHP-2 protein were prepared in 96-well plates and preincubated with 0.5 μM biotinylated PPs/well at 37 °C for 1 h in a phosphatase buffer containing 20 mM Hepes (pH 7.0), 5% glycerol, 0.05% Triton X-100, 2.5 mM $MgCl_2$, aprotinin (10 μg/ml) and leupeptin (10 μg/ml). 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 orthophenylene diamine as substrate. Optical densities in the wells were measured at 450 nm.

Results

The effects of pITIM and Gab1 peptides on protein tyrosine phosphorylation in LPC-permeabilized B cells. To study whether PPs (see list of PPs in Table 2) corresponding to the pITIM of FcγRIIb [AENTITY(p)SLLMHP] or to the SHP-2-binding motif of Gab1 [GDKQVEY(p)LDLDLD] (GD-LD) influence intracellular protein tyrosine phosphorylation, B cells were permeabilized with LPC and then treated with the PPs either in the presence or the absence of an anti-IgM stimulus. Anti-IgM triggered tyrosine phosphorylation in both LPC-permeabilized and non-permeabilized (control) (Fig. 1a) cells. Unphosphorylated ITIM, either alone or in combination with anti-IgM, had no effect on protein phosphorylation (Fig. 1a). In contrast, its phosphorylated counterpart, pITIM itself, induced tyrosine phosphorylation of proteins at 30, 50 and 66–70 kDa; this also occurred when pITIM was used in combination with anti-IgM (Fig. 1a). SHP-2 is activated by the simultaneous engagement of its two SH2 domains by a bisphosphorylated activation motif [2, 38]. Accordingly, we also tested the effects of a chimeric bisphosphorylated peptide consisting of pITIM and a phosphotyrosine-containing peptide of SHIP combined with a flexible linker [TEIINPNY(p)MGVG$(AG)_{15}$ AENTITY(p)SLLMHP, chimera] [39]. This chimeric peptide induced the tyrosine phosphorylation of proteins at 30, 50 and 66–70 kDa in a dose-dependent manner (Fig. 1b) and enhanced anti-IgM-triggered Tyr phosphorylation. The Gab1 PPs, GD-LD and another Gab1 motif [ELDENY(p)VPMNPN, EL-PN] reacting with the SH2 domain of the regulatory subunit of PI3-K, did not induce detectable tyrosine phosphorylation in LPC-permeabilized B cells (Fig. 1c). Our earlier peptide pull-down experiments followed by Western blot revealed that the major molecule interacting both with pITIM, the chimera and GD-LD PPs was SHP-2 [15, 39] indicating that these PPs may regulate the activity of SHP-2 phosphatase *ex vivo*.

Table 2. Sequences of PPs used in LPC-permeabilized cells.

Code	PPs used in LPC-permeabilized cells
pITIM	AENTITY(p)SLLMHP
pITIM chimera	TEIINPNY(p)MGVG$(AG)_{15}$ AENTITY(p)SLLMHP
EL-PN	ELDENY(p)VPMNPN
GD-LD	GDKQVEY(p)LDLDLD

Selection of an optimal cell-penetrating peptide for use as a vector for signaling peptides. To transport the PPs into the cells under more physiological condition, i.e. by cell-penetrating peptides, we first compared the effects of a series of CPPs. Several membrane-non-permeable peptides coupled covalently to CPP exhibited evidence of internalization and biological activity in cell cultures [1]. However, the various CPPs have not yet been compared in a homogenous system on the same target cell type. On the basis of known sequences, basic octa-arginin (R8) and its modified versions, palmitoyl-(P-R8) and octanoyl-octaarginine (O-R8), transportan, and a shorter version of

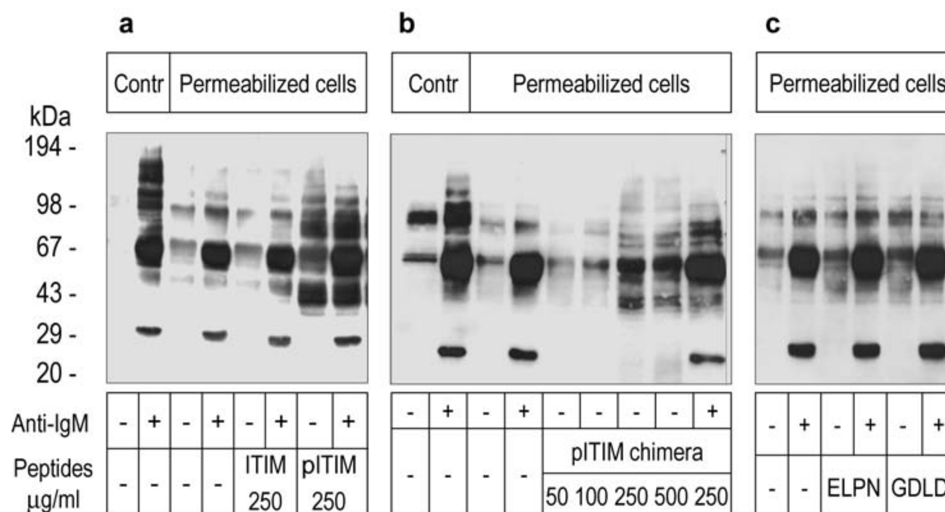

Figure 1. PP-induced protein tyrosine phosphorylation in LPC-permeabilized BL41 cells. (*a*) The permeabilized cells were treated with 250 µM ITIM, or pITIM peptides, alone, or before anti-IgM stimuli. (*b*) 50, 100, 250 and 500 µM pITIM chimera peptide composed of the SHIP phosphorylated motif and pITIM was added to the cells; last lane, 250 µM SHIP-pITIM chimera was added before anti-IgM. (*c*) 250 µM GD-LD or EL-PN PP was added with or without anti-IgM.

transportan (TP10), MPG and an oxidized form of MPG (MPGox), were synthesized and tested for cell penetration in the BL41 Burkitt lymphoma cell line (Table 3).

The CPPs were labeled with Bodipy FL N-(2-aminoethyl)maleinimide and their cellular uptake was followed by confocal microscopy. The lipophilic fluorescent probe, DiI-C18 was used to stain the cell membrane, while acidic lysosomes were labeled with a LysoTracker Red probe. All CPPs except MPGox penetrated the cell membrane and were detected in the cytoplasm as discrete granules after a 20-min incubation at 37 °C. MPGox displayed a very low uptake, and was not detectable with confocal microscopy under these conditions (Fig. 2). The correlation between the localization of the peptide-containing vesicles and acidic lysosomes was calculated using the Pearson coefficients. These values appeared to lie in the interval 0.4–0.5% for nearly all CPPs, indicating a partial co-localization; the only exception was MPGox, where it was 0.06%.

CPPs were further analyzed by flow cytometry. The time dependence of CPP uptake is depicted in Figure 3. All CPPs translocated into the cells within the first 5 min of incubation, and in most cases the penetration continued for up to 60 min. In the transportan- and TP10-treated samples, a cell group with a lower and a higher positivity was observed. The transport of CPPs was temperature independent, since all the CPPs penetrated equally well at 4 and 37 °C (data not shown).

Bodipy FL-conjugated CPPs (FL1) and propidium iodide staining (FL3) were applied simultaneously to detect dead cells among the peptide-positive population in order to determine the cytotoxicity of CPPs. All the CPPs except

Table 3. Sequences of cell-membrane-penetrating peptides.

Code	Cell-permeable peptides
R8	RRRRRRRR-C(BODIPY) (-NH2)
P-R8	palmitolyl -RRRRRRRR-C (-NH2)
O-R8	octanoyl -RRRRRRRR-C (BODIPY) (-NH2)
MPG	C (BODIPY)-GALFLAFLAAALSLMGLWSQPKKKRKV (-NH2)
MPGox	C (BODIPY)-GALFLAFLAAALSLM(O)GLWSQPKKKRKV (-NH2)
Transportan	C (BODIPY)- GWTLNSAGYLLGKINLKALAALAKKIL (-NH2)
TP10	C (BODIPY)-AGYLLKINLKALAALAKKIL (-NH2)
O-R8-GDLD(Pe)	octanoyl-RRRRRRRR-C(BODIPY) -**GD**KQVEY(p)LDLD**LD**(NH2)
O-R8-RKHP(Pe)	octanoyl-RRRRRRRR-C- **RK**KRISALPGPANPTNPDEADKVG- AENTITY(p)SLLM**HP**

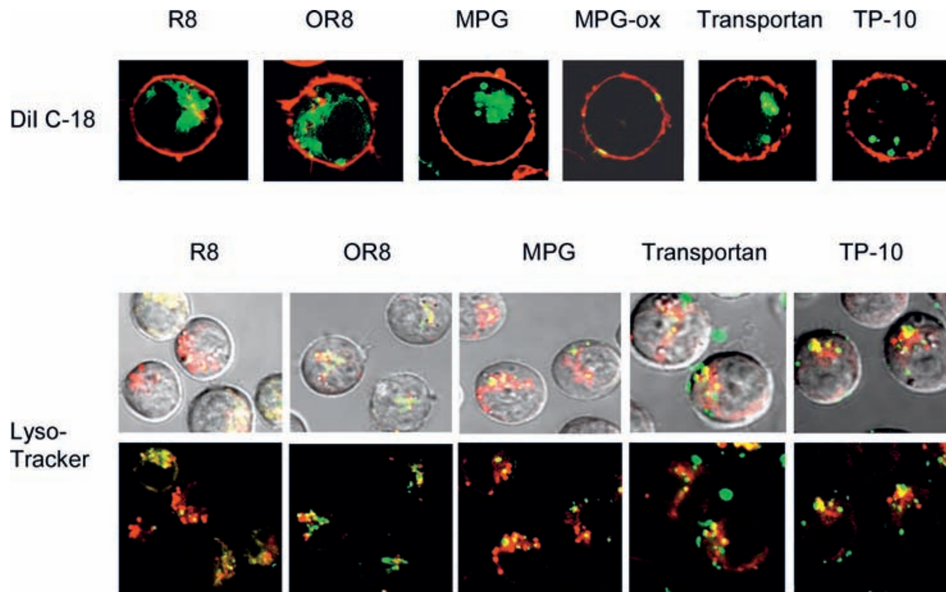

Figure 2. Confocal laser scanning microscopic analysis of the penetration of Bodipy Fl-labeled carrier peptide to BL41 cells. The cell membrane was stained with DiI-C18, and the acidic lysosomes were detected with LysoTracker Red. The cells were treated with the peptides at 37 °C for 20 min.

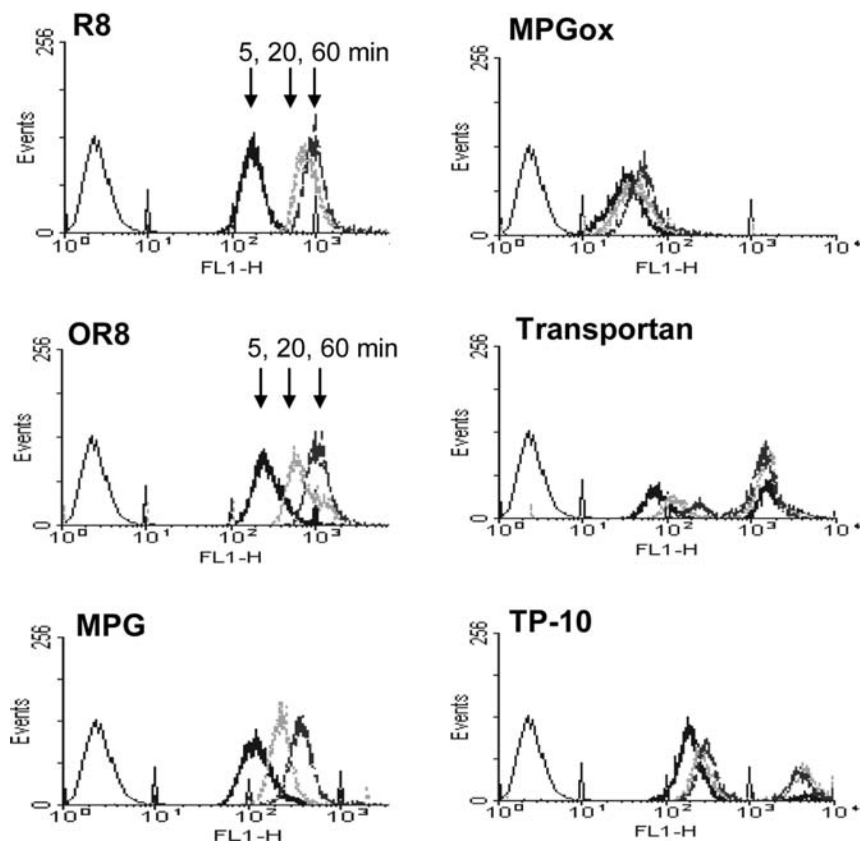

Figure 3. Time dependence of CPP uptake by BL41 cells at 37 °C. The cells were treated with the CPPs for 5, 20 and 60 min, washed and measured.

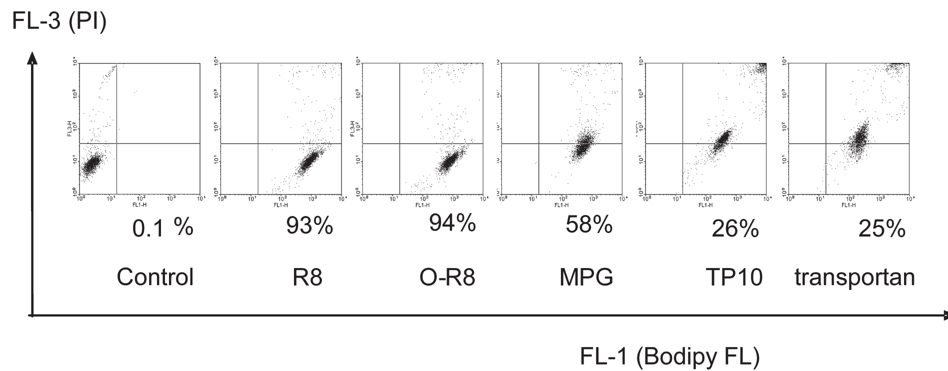

Figure 4. Uptake and cytotoxicity of cell-permeable peptides. BL41 cells were treated with Bodipy-FL-conjugated CPPs at 37 °C for 20 min, and then stained with propidium iodide. The numbers in the lower-right quadrants show the percentages of Bodipy-FL-positive cells.

MPGox stained the cells to an extent of over 90% (Fig. 4). MPG, TP10 and transportan were partially toxic to the B cells, since 42, 74 and 75% of the cells, respectively, died upon peptide treatment for 20 min at 37 °C. Most importantly, R8- and OR8-treated samples fully retained their viability (Fig. 4).

To test the effects of CPPs on cell viability, BL41 cells were further analyzed with respect to the concentration (10 and 50 µM CPPs) and time (1, 6 and 24 h) dependencies. As shown in Figure 5, all CPPs were toxic at 50 µM during co-culture with the cells for 24 h. R8 and O-R8 had no significant effect on the cell viability at 10 µM, even after treatment for 24 h. In contrast, MPG, MPGox and TP10 were significantly toxic in as short a treatment period as 6 h, and 80% of BL41 cells died after incubation for 24 h with P-R8 and transportan (Fig. 5). In view of the efficiency of the transport and the toxic effect of the CPPs on the cells, we selected O-R8 as a carrier for further experiments.

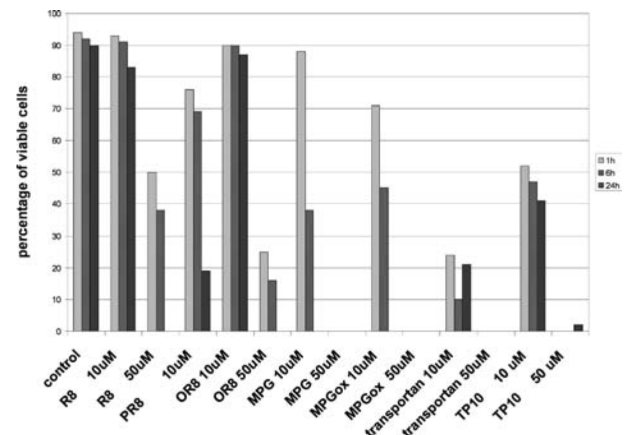

Figure 5. Cell viability in the presence of CPPs. BL41 cells were treated with various CPPs for 1, 6 and 24 h, and then stained with propidium iodide. The proportion of dead cells was calculated after flow cytometric analysis.

Delivery of PPs into BL41 cells by O-R8 carrier. To test whether the carrier CPPs are indeed capable of delivering PPs into cells, O-R8 was conjugated to the Gab1 PP, GD-LD, and the uptake of this construct by BL41 cells was then tested by means of confocal microscopy and flow cytometry. The intracellular localization of the construct was the same as shown for the carrier CPP (Fig. 2): the PP penetrated the plasma membrane and was detected in the cytoplasm as discrete vesicles, exhibiting a partial co-localization (Pearson coefficient: 0.45) with acidic lysosomes (Fig. 6). The time and temperature dependence of peptide delivery were tested by flow cytometry. The PP conjugate penetrated dose-dependently into the cells and was detected inside the cells after a 5-min incubation at either 4 or 37 °C (Fig. 7). Interestingly, the O-R8-GD-LD conjugate demonstrated maximal engulfment as early as 5 min after addition, in contrast with the CPP carrier without cargo, when this was increased up to 60 min. The

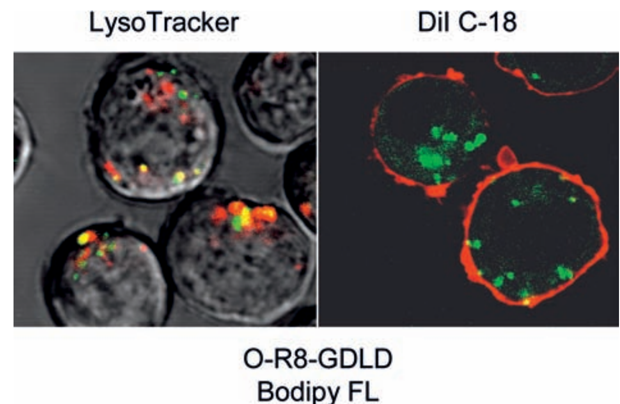

Figure 6. Confocal laser scanning microscopic analysis of the delivery of Bodipy-Fl-labeled GD-LD PP into BL41 cells by O-R8 CPP. The cells were treated with the conjugate at 37 °C for 20 min. The cell membrane was stained with DiI-C18; acidic lysosomes were detected by LysoTracker Red.

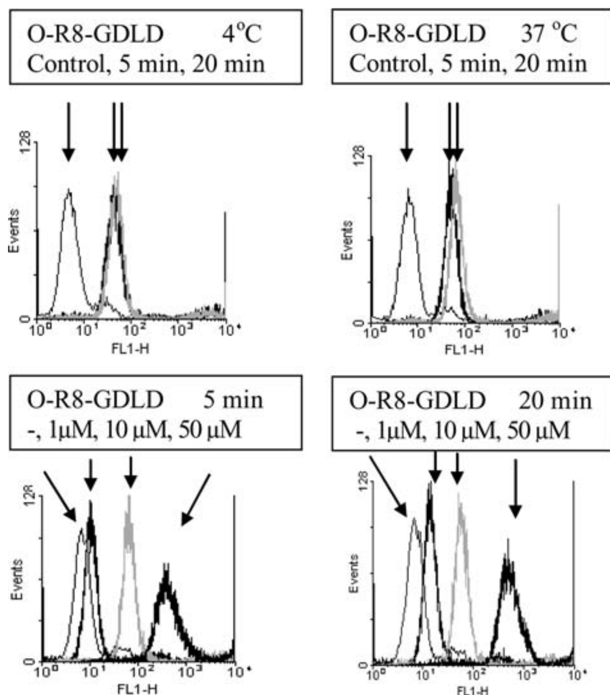

Figure 7. Time, temperature and dose dependencies of PP delivery into BL41 cells. The cells were treated for 5 or 20 min with 10 μM cell-permeable PP at 4 or 37 °C (upper panels). The cells were incubated in the presence of increasing doses of cell-permeable PP for 5 or 20 min (lower panels). The samples were analyzed by cytofluorometry.

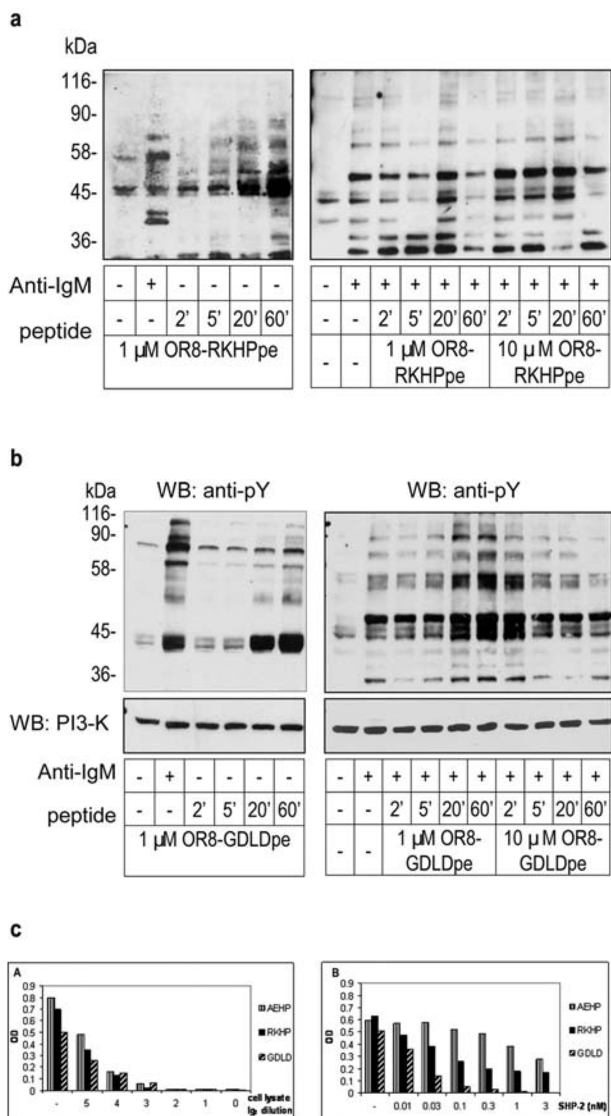

Figure 8. The effects of cell-permeable PPs on protein phosphorylation in BL41 cells. The cells were treated with O-R8-pITIM (*a*) or O-R8-GD-LD (*b*) at 37 °C for the indicated time period, and then anti-IgM was added for 2 min. The cells were spun down, instantly frozen in liquid nitrogen, then lysed and separated by SDS-PAGE. The samples were blotted into nitrocellulose membrane and analyzed by Western blot using phosphotyrosine-specific antibodies. Dephosphorylation of PPs by BL41 cell lysate samples and by recombinant SHP-2 protein (*c*). The biotinylated PPs were treated with various dilutions of cell lysate samples or recombinant SHP-2 protein for 1 h at 37 °C, then the samples were transferred onto a streptavidin-coated plate. After an overnight incubation at 4 °C, the remaining phosphotyrosine contents of the bound peptides were detected by ELISA using phosphotyrosine-specific antibodies.

temperature independence of peptide penetration indicated that the cell-permeable PP was not translocated to the B cells by endocytosis.

Effects of O-R8-GD-LD and O-R8-pITIM on intracellular signaling. To test whether the SHP-2-binding cell-permeable PP decoy molecules influence the activity of SHP-2 *ex vivo,* BL41 cells were pretreated with O-R8-conjugated PPs for different intervals of time, the cells were then stimulated with anti-IgM, or left untreated, and the protein tyrosine phosphorylation was analyzed in the cell extracts by Western blot. A longer PP was used here than for LPC-permeabilized cells, containing pITIM (bold letters) completed with an N-flanking terminal sequence) [O-R8-RKKRISALPGPANPTNPDEADKVG-**AENTITY(p)SLLMHP,** RK-HP], in order to mimic better the intracellular sequence of FcγRIIB1. Figure 8a shows that RK-HP alone induced the tyrosine phosphorylation of several proteins in a dose- and time-dependent manner. RK-HP also enhanced the anti-IgM induced tyrosine phosphorylation of proteins at 40–43, 50 and 55 kDa. The enhancement was detected only at 20 min, and faded after incubation with the peptide for 60 min.

The cell-permeable Gab1 PP OR8-GD-LD alone induced the tyrosine phosphorylation of proteins at about 55, 68 and 98 kDa after incubation for 20 and 60 min, while pretreatment of the cells with O-R8-GD-LD for the same time resulted in a considerably elevated level of tyrosine phosphorylation of several proteins in anti-IgM stimulated samples (Fig. 8b). This effect was seen much earlier, after treatment for 2 min, at a higher dose (10 μM) of O-R8-GD-LD, and faded rapidly with time.

To check the possible fate of PPs within the cell, phosphatase activity assays were performed. PPs AE-HP, RK-HP and GD-LD were efficiently dephosphorylated *in vitro*, although to different extents, by both cell lysates and recombinant SHP-2, indicating that these PPs can indeed be dephosphorylated by intracellular phosphatases, and among these, by SHP-2 (Fig. 8c).

These data confirm that the O-R8 cell-permeable peptide is capable of delivering PPs into B cells and the CPP-PP conjugate can regulate intracellular signaling events.

Discussion

The interactions between SH2 domains and tyrosine-phosphorylated proteins are essential in signal transduction. Phosphotyrosine-containing motifs bind specifically to certain SH2 domains; they consequently generate signaling complexes and may regulate the activities of kinases and phosphatases [24, 25, 27, 28]. A number of attempts have been focused on interrupting the signaling cascade at the level of protein-protein interactions, such as those involving SH2 domains. Numerous synthetic PPs corresponding to the SH2-domain-binding sites are able to block the interactions of phosphorylated proteins with SH2-containing protein targets specifically *in vitro* [31, 40–45]. However, PPs are unable to penetrate the cell membrane as a consequence of the negative charge of the phosphate groups at physiological pH [46]. This problem can be overcome by permeabilization of the cell membrane or by applying membrane-permeable carrier peptides as delivery vectors. The specificity of the phosphotyrosine motifs for certain SH2 domains suggests that tyrosine-phosphorylated cell-permeable peptides can serve as models for the design of peptide-mimetic, small-molecule inhibitors, targeting protein interactions of SH2 domains [47].

SHP-2 tyrosine phosphatase is essential in signaling downstream of growth factor, cytokine and antigen receptors [20, 21]. It exerts both positive and negative regulatory effects on various signaling pathways operating in immune cells. The interactions of SHP-2 with Gab1/Gab2 adaptor/scaffolding molecules are indispensable for the full activation of Erk1/2 upon cytokine and antigen receptor signaling [48], but SHP-2 may also mediate the signal transduction of inhibitory receptors [49]. pITIMs of inhibitory receptors such as FcγRIIb [15, 18], programmed death 1 [50], the T cell inhibitory receptor, CTLA-4 [51], killer inhibitory receptors on natural killer cells [52, 53] and the platelet endothelial cell adhesion molecule-1 (CD31) [54, 55] all recruit SHP-2, beside other SH2-domain-containing phosphatases such as SHP-1 and SHIP. Various data suggest that SHP-2 is required for the functioning of most inhibitory receptors.

We earlier reported that the GD-LD motif of Gab1 and a chimeric bisPP consisting of the pITIM of FcγRIIb and a tyrosine-phosphorylated motif of SHIP bind to SHP-2 SH2 domains with a K_d = 1.5 nM and K_d = 9 nM, respectively, and activate the phosphatase *in vitro* [39]. Thus, our aim was to test whether these PPs have an *ex vivo* effect on BCR-mediated signaling when delivered into BL41 cells. We first compared the effects of pITIM, the chimera and Gab1 GD-LD and EL-PN peptides on the protein tyrosine phosphorylation signals in LPC- permeabilized B cells. pITIM and the biphosphorylated chimera peptide induced the tyrosine phosphorylation of several proteins and enhanced the BCR-stimulated phosphorylation signals, while GD-LD and EL-PN PPs had no detectable effect. The two peptides containing FcγRIIb sequences probably bind to SHP-2 and compete with the natural, intracellular phosphatase substrates, hence allowing phosphorylation. As expected, EL-PN, which does not bind to SHP-2, does not influence tyrosine phosphorylation events. Surprisingly, the Gab1 PP, GD-LD, does not modulate intracellular tyrosine phosphorylation, although it has previously been shown to bind to and activate SHP-2 [39]. A possible explanation is that a 3-min treatment period was too short for the peptide to exert its effect; alternatively, some other signaling molecule is required for the regulatory function of GD-LD, which is lost on permeabilization of the cells.

To turn to a more physiological PP delivery, our goal has been to find an optimal cell-permeable vector, which can translocate PPs into intact cells without causing a toxic effect. Cell-membrane permeable arginine- and lysine-rich peptides, such as the transactivation (Tat) domain from HIV1 and the homeodomain of the Antennapedia transcription factor from *Drosophila* (Penetratin) have been found to penetrate cell membranes directly [1, 2, 13]. Membrane-translocating sequences often have numerous positive charges. The minimal peptide of HIV1 Tat protein contains eight positive charges: six Arg and two Lys residues [2, 6]. Oligoarginines composed of more than six Arg residues have been found to penetrate cell membranes efficiently; furthermore, stearylation of oligoarginine increased the transfection efficiency 100-fold in COS-7 cells [56].

Another group of cell-permeable peptides do not contain arginine. The best characterized representative of this group is transportan. This is a 27-amino-acid chimeric peptide consisting of a combination of an N-terminal fragment of a neuropeptide, galanin, and the membrane-interacting peptide of the wasp venom, mastoparan [57]. MPG, a recently developed peptide-based gene delivery system, derived from the fusion peptide domain of the HIV1 gp41 protein and the nuclear localization sequence of the SV40 large T antigen, has been reported to form non-covalent complexes with nucleic acids and deliver them into a large panel of cell lines [11, 58].

These previous studies led us to compare Arg-containing and non-containing peptide delivery vectors in B cells; additionally, we synthesized and tested new CPPs, such as palmitoylated and octanoylated octa-arginine, and an oxidized form of MPG. The latter was a side-product of the synthesis of MPG sequences, and displayed surprising chromatographic behaviors, suggesting a drastic three-dimensional structure change after the modification of the methionine residue.

The B cells were highly permeable for most of these vectors. Octa-arginine and octanoyl-octa-arginine penetrated all BL41 cells and also primary B cells (data not shown), and the cell viability was over 80% after incubation for 24 h. R8 was also tested after conjugation to a longer fatty acid chain (palmitolyl-R8), but this construct was surprisingly highly toxic for BL41 cells. MPG and transportan (likewise penetrating all cells) drastically decreased the cell viability in a time- and concentration-dependent manner. MPGox did not penetrate the cells, probably due to the drastic conformational change indicated by various physicochemical methods [our unpublished data]. Transportan exerted the highest toxic effect: 80% of BL41 cells were dead after incubation with this peptide for 1 h. TP10 containing only the minimum sequence of transportan, which was reported to be still active in membrane translocation [59], was less toxic than transportan. Since the CPPs used here in BL41 cells were previously applied as efficient vectors in different cell types and showed no toxic effect, these data together suggested that the optimal targeting vector had to be chosen circumspectly for various types of cells.

CPPs can translocate into cells by different mechanisms [7, 60, 61]. Penetratin and R8 peptides have been shown to have distinct internalization mechanisms in HeLa cells. R8 is taken up by macropinocytosis, while penetratin translocation is less sensitive to the inhibitors of macropinocytosis and cytochalasin B, an inhibitor of actin rearrangement [60]. Other investigators have shown that Arg-rich peptides can be internalized even at 4 °C, suggesting that the transportation cannot be explained by typical endocytosis [13]. An alternative mechanism for peptide internalization is that the CPPs, containing many positive charges, may interact with the negatively charged heads of membrane phospholipids, which may induce a transient formation of inverted micelles that would engulf the CPP and its cargo. The micelles may reopen inside the cells, releasing the peptides into the cytoplasm [9, 10, 61]. Some micelles may also fuse with the lysosomes. The third model is based on the interaction of hydrophobic residues of CPPs with membrane lipids, forming a channel through oligomerization. This would allow the efficient passage of hydrophilic molecules, but pore formation would also be a threat for cell survival [9]. As all the CPPs that we have used are internalized at 4 °C as efficiently as at 37 °C and are detected as distinct vesicles in the cytoplasm of BL41 cells, we suggest that the non-toxic carriers R8 and O-R8 are internalized by inverted micelle formation. The toxic effect of the other CPPs (P-R8, MPG and transportan) might be explained by their capacity to form pores through the cell membrane, which BL41 cells are not able to repair.

To check that the CPP (O-R8), which is optimal for BL41 cells, can deliver biologically active PPs into the cells, O-R8 was coupled to the SH2-binding PPs, an extended ITIM of FcγRIIb and the Gab1 motif. These PP conjugates are internalized in a similar way as the carrier and are located in the cytoplasm after just 5 min. The translocation is dose dependent, temperature independent and, interestingly, the internalization of O-R8-GD-LD does not increase further after a longer incubation time, as is the case for the O-R8 carrier. This suggests that the translocation of O-R8-GDLD is rapid and irreversible, and may be mediated by a slightly different mechanism compared with the lone vector.

One of the major points of this paper involves determination of the effects of signaling PPs, once they have been delivered into the cells. The tyrosine phosphorylation events were therefore studied using CPP-PP conjugates. In activated lymphocytes, SHP-2 is recruited to the phosphorylated scaffolding/adaptor molecule Gab1 and/or Gab2; consequently, their two N-terminal phosphorylated motifs activate the phosphatase [20, 38]. We have previously reported that the most efficient activator of SHP-2 is the PP GD-LD of Gab1. Upon co-clustering of BCR and FcγRIIb, the ITIM of the latter becomes phosphorylated and recruits SHP-2 and SHIP. SHIP is also a target of tyrosine kinases and may co-operate with pITIM in activating SHP-2 [15, 17, 30, 39]. When delivered to BL41 cells by O-R8, both the GD-LD and the pITIM-containing peptides modulate the phosphorylation of certain intracellular proteins. These data indicate that these decoy peptides may interact with SHP-2 and activate the phosphatase, which in turn may dephosphorylate, for example, inhibitory tyrosyl residues on src kinases or on its negative regulators, such as PAG/CPB [21], resulting in kinase activation and the phosphorylation of target proteins. In the presence of anti-IgM stimuli, GD-LD has a biphasic effect. During the early activation signal, the PP may compete with the natural SHP-2 substrates, while later it may operate in the manner described above and induce tyrosine kinase activation. This effect may be lost due to the inactivation of the PP by SHP-2.

In conclusion, we have shown that CPPs effectively mediate the delivery of functional signaling peptides into living cells. Finding the optimal CPP vector for different types of cells is essential for delivery of the peptide or secondary modified peptide cargo into the cells. Hence, we have shown that PPs that interact with SHP-2 are delivered into B cells by O-R8, and such conjugates can modulate the events leading to signal transduction. Fur-

ther investigations are underway to clarify the functional consequences of this finding. The cell- permeable PPs might be utilized for the design of small molecular inhibitors, targeting SHP-2 molecular interactions and intervening in malignant cell growth.

Acknowledgements. The authors are grateful for support from the National Research and Development Fund (NKFP-1A/040/2004); Economic Competitiveness Operative Program, GVOP 3.1.1.2004-05-0183/3.0; OMFB-00541/2004 and Asboth Oszkar grant XTTP-SRT1 from the National Office for Research and Technology and the Hungarian National Science and Research Development Foundation (OTKA) T048848 and K60760. We thank Dr. H. Gu (Boston, Ma.) for the generous gift of the expression vector pGEX-3T containing the cDNA of SHP-2.

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