ELSEVIER

Biochimica et Biophysica Acta 1467 (2000) 165–176

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Deletion analogues of transportan

Ursel Soomets [a,c], Maria Lindgren [a], Xavier Gallet [b], Mattias Hällbrink [a], Anna Elmquist [a], Lajos Balaspiri [a], Matjaz Zorko [d], Margus Pooga [a,e], Robert Brasseur [b], Ülo Langel [a,*]

[a] *Department of Neurochemistry and Neurotoxicology, Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden*
[b] *Centre de Biophysique Moléculaire Numérique, Faculté Universitaire des Agronomiques, Passage des déportés 2, 5030 Gembloux, Belgium*
[c] *Department of Biochemistry, Tartu University, Ravila 19, 50411 Tartu, Estonia*
[d] *Institute of Biochemistry, Medical Faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*
[e] *Estonian Biocentre, Riia 23, 51010 Tartu, Estonia*

Received 14 October 1999; received in revised form 27 March 2000; accepted 5 April 2000

Abstract

Several shorter analogues of the cell penetrating peptide, transportan, have been synthesized in order to define the regions of the sequence, which are responsible for the membrane translocation property of the peptide. Penetration of the peptides into Bowes melanoma cells and the influence on GTPase activity in Rin m5F cellular membranes have been tested. The experimental data on cell penetration have been compared with molecular modeling of insertion of peptides into biological membranes. Omission of six amino acids from the N-terminus did not significantly impair the cell penetration of the peptide while deletions at the C-terminus or in the middle of the transportan sequence decreased or abolished the cellular uptake. Most transportan analogues exert an inhibitory effect on GTPase activity. Molecular modeling shows that insertion of the transportan analogues into the membrane differs for different peptides. Probably the length of the peptide as well as the location of aromatic and positively charged residues have major impact on the orientation of peptides in the membranes and thereby influence the cellular penetration. In summary, we have designed and characterized several novel short transportan analogues with similar cellular translocation properties to the parent peptide, but with reduced undesired cellular activity.

Keywords: Cell penetration; Transport peptide; Transportan peptide; Membrane interaction

1. Introduction

Today most approaches for delivering polar or large molecules such as peptides, proteins, antisense oligonucleotides etc. into cells use endocytosis, electroporation or microinjection techniques. These methods are limited by either in vitro applicability only or by low yield of cargo delivery and degradation of targeted molecules.

An alternative method for cellular delivery of bioactive cargoes is to use vector peptides i.e. peptides that interact with the plasma membrane and translocate into the cellular interior e.g. mastoparan [1], the fragments of antennapedia of *Drosophila* (penetratins) [2,3], transportan (TP) [4], Tat protein [5,6], herpes virus structural protein VP22 [7] etc. Trans-

* Corresponding author. Fax: +46-8-161371;
E-mail: ulo@neurochem.su.se

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00216-9

port peptides have been shown to deliver peptides [8], small proteins [9], DNA [10] oligomers and peptide nucleic acid (PNA) [11] oligomers over the plasma membrane by a non-endocytotic mechanism. However, the mechanism of penetration of these peptides and their constructs with large molecules still remains unknown.

TP, a representative of the cell-penetrating peptides, is a 27 amino acids long chimeric peptide containing the first 12 amino acids from the amino-terminal part of neuropeptide galanin and the 14 amino acid long wasp venom peptide, mastoparan, connected via a lysine residue [4]. It has been shown that TP rapidly enters cells by a receptor-independent mechanism [4]. Later TP has been shown to be able to deliver antisense PNA oligomers into cells [11]. TP contains the N-terminal part of the bioactive neuropeptide galanin and is therefore recognized by galanin receptors. Moreover, TP shows an inhibitory effect on basal GTPase activity in Bowes melanoma cell membranes, which is probably caused by the mastoparan part of the molecule. Although the inhibitory effect of TP is detectable at higher concentrations than commonly used in delivery experiments, this feature could be a drawback for carrier peptide. Therefore we have studied the structural requirements for the cell membrane penetrating activity of TP. We have synthesized nine novel truncated derivatives of TP aiming at minimizing the affinity for galanin receptors and the interaction with G-proteins and, if possible, to enhance cell penetration efficiency of the peptide. The resulting peptides have been tested for cellular uptake by Bowes melanoma cells by using indirect immunofluorescence and ^{125}I-labeled peptides. The effect of TP and its short analogues on the basal GTPase activity was studied in Rin m5F cell membranes.

The interaction of peptides with biological membranes was simulated by molecular modeling and the penetration as well as orientation of nine TP analogues in relation to the membrane surface was calculated [16]. The simulations reveal that the insertion and the relative orientation of short TP analogues in phospholipid membranes are remarkably different for different peptides.

2. Materials and methods

2.1. Peptide synthesis

The peptides were synthesized in a stepwise manner in a 0.1 mmol scale on a model 431A peptide synthesizer (Applied Biosystems, USA) using *t*-Boc strategy of solid-phase peptide synthesis. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole (HOBt) esters to a *p*-methylbenzylhydrylamine (MBHA) resin (Bachem, Switzerland) to obtain C-terminally amidated peptides. Biotin was coupled manually to the ε-amino group of Lys after orthogonal deprotection from the Fmoc-group, using a three-fold excess of HOBt and *o*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) activated biotin (Chemicon, Sweden) in DMF to the peptide on resin. Deprotection of the side chains from formyl and benzyl groups was carried out using the 'low TFMSA' method. The peptides were cleaved from the resin with liquid HF at 0°C for 30 min in the presence of *p*-cresol.

The purity of the peptides was $>98\%$ as demonstrated by HPLC on an analytical Nucleosil 120-3 C_{18} RP-HPLC column (0.4×10 cm). The molecular masses of the peptides were determined with a plasma desorption mass spectrometer (Bioion 20, Applied Biosystems, USA) and the calculated values were obtained in each case.

2.2. Iodination

Peptide iodination was carried out by the chloramine T method [12]. Na^{125}I (specific activity 16.1 mCi/mg, concentration 0.1 mCi/ml) was mixed with five equivalents of the peptide in phosphate buffer 50 mM, pH 7.4). The reaction was carried through for 2 min and stopped by adding an excess of sodium metabisulfite. The iodinated peptides were separated from free label by chromatography on a reversed phase (RP), column SEP-PAK 51910 (Millipore, USA) using a stepwise gradient of acetonitrile. The fractions containing the highest specific activity (eluted with 40% acetonitrile) were used for a cell penetration assay.

2.3. Cells

The human Bowes melanoma cell line (ATCC CRL-9607) was cultivated in minimal essential medium with Earl-salts (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 IU/ml penicillin and 5 μg/ml streptomycin in air with CO_2 enriched to 5% at 37°C. The cells were grown on round glass coverslips in a 24 well plate to approximately 50% confluence. Binding of ^{125}I-galanin to the Bowes cellular membranes was carried out as described earlier [4].

2.4. Cellular penetration of biotinyl-labeled peptides

The serum-containing medium on the cells was exchanged for a serum-free and the water solution of the biotinyl-peptides was added directly into the medium. The cells were then incubated for different time periods at 37°C and unbound peptide was removed by washing twice with PBS. The cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with cold methanol for 10 min at −20°C. Permeabilized cells were washed with PBS and incubated for 1 h in 5% (w/v) solution of bovine serum albumin in PBS in order to decrease unspecific binding. The peptides were visualized by staining with streptavidin-FITC (Amersham-Pharmacia Biotech, 1:100 dilution) in the same solution for 1 h at room temperature. The cell nuclei were stained with Hoechst 33258 (0.5 μg/ml) for 5 min, the coverslips were washed five times with PBS and mounted in 'Prolong' antifade reagent (Molecular Probes, USA). The images were obtained by Zeiss Axioplan 2 microscope (Carl Zeiss, Germany) equipped with a cooled digital CCD camera C4880 (Hamamatsu Photonics, Japan) or by a laser scanning confocal microscope (model LSM 510; Carl Zeiss, Sweden) equipped with an Ar laser (488 nm), a He/Ne laser (543 nm), a 63×1.4 oil immersion objective. The Ar and He/Ne lasers were operated at 8% and 60% power, respectively. Images were processed using Adobe Photoshop 3.0 software (Adobe Systems, CA, USA).

2.5. Cellular penetration of ^{125}I-labeled peptides

Bowes cells were grown to 70–80% confluence in a 75 cm^2 flask. The cells were scraped off and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 2 ml HEPES buffered Krebs Ringer solution (HKR) pH 7.4 (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM $MgCl_2$, 1.8 mM $CaCl_2$) with the addition of phosphate buffer and 1 mg/ml BSA. The cell suspension was aliquoted and the radiolabeled peptides were added to reach an activity of 1 000 000 cpm/tube. The suspensions were incubated by mild shaking in a water bath at 37°C up to 240 min, and centrifuged through a mixture of 40% dioctyl phtalate and 60% dibutyl phtalate to separate cell-bound and free radioactivity. The loosely bound ^{125}I-labeled peptides were detached from the cells by incubation in ice-cold 0.2 M acetic acid, 0.5 M NaCl (pH 2.5) for 5 min, in order to distinguish from internalized/membrane-inserted fraction of peptides. The acid-labile binding of labeled peptide comprised only about 2–3% of the total amount of cell associated peptide. This difference did not substantially change the kinetics of the uptake of radioactively labeled peptide and therefore in most experiments the step of washing of cells in acidic conditions was omitted. The fractions were counted in a γ-counter (Packard, Meriden, CT). Part of the cells was treated analogously with Na^{125}I instead of the peptide as a control in order to estimate the intactness of cells in suspension in the course of incubation.

2.6. Modeling of internalization of ^{125}I-peptides

In the simulation of uptake of TP analogues by Bowes cells we calculated the half-lives of internalization using the approximation that the internalization is a first order process in accordance with Eq. 1:

$$A \xrightarrow{k} B \quad (1)$$

where A and B are concentrations of ^{125}I-peptides outside and inside the cells, respectively, and k is the first-order rate constant of the internalization process. Curve fitting and calculations were performed using Prism computer program (GraphPad Software, USA)

2.7. Measurement of GTPase activity

Measurement of GTPase activity was performed radiometrically according to Cassel and Selinger [13], with the modifications suggested by McKenzie and co-workers [14]. To the diluted membranes the ice cold reaction cocktail containing ATP (1 mM), 5′-adenylyl imido-diphosphate (1 mM), ouabain (1 mM), phosphocreatine (10 mM), creatine phosphokinase (2.5 Units/ml), dithiothreitol (4 mM), $MgCl_2$ (5 mM), NaCl (100 mM), and trace amounts of [γ-^{32}P]GTP to yield 50 000–100 000 cpm in an aliquot of the reaction cocktail (with the addition of cold GTP to reach the required total concentration GTP of 0.5 μM) was added. Incubation medium was standard TE-buffer (10 mM Tris–HCl, 0.1 mM EDTA), pH 7.5. Background low-affinity hydrolysis of [γ-^{32}P]GTP was assessed by incubating parallel tubes in the presence of 100 μM GTP. Blank values were determined by the replacement of the Rin m5F membrane solution with assay buffer. GTPase reaction was started by transfer of the reaction mixtures to 30°C water-bath for 12 min. Unreacted GTP was removed by the 5% suspension of the activated charcoal in 20 mM H_3PO_4. The radioactivity of the yielding radioactive phosphate was determined in the LKB 1214 Rackbeta or in Packard 3255 liquid scintillation counter. The basal GTPase activity of the Rin m5F cellular membrane preparation was 1.9 pmol/min/mg protein.

2.8. Partition of ^{125}I-labeled TP and its analogues between octanol and water

^{125}I-labeled peptides were used to determine the partition coefficients for TP and its analogues in a water/octanol system. 20 nM water solution of a peptide and equal volume of water saturated 1-octanol were mixed and vortexed. The phases were separated by centrifugation for 5 min at $10\,000 \times g$. Samples were prepared in triplicates and were counted in a γ-counter.

2.9. Membrane interaction modeling

The structure of each peptide (Table 1) was predicted using the Stereoalphabet algorithm and energy-refined with the Simplex procedure. This latter calculation was carried out in a medium of intermediate dielectric constant of a hydrophobic/hydrophilic interface [15]. The penetration and orientation of TP analogues in a biological membrane were tested using the Impala software [16]. Calculations were performed on RAMSES (rapid analysis master/salves extensible system), parallel hardware of 21 Tracor Europa Pentium Pro PC connected by a 100 Mb network and controlled by a HP Vectra VA Pentium Pro. The calculation software has been developed in our laboratory [16]. Molecular visualizations were performed using WinMGM 2.0 software [17] from Ab Initio Technology (Obernai, France). Non-peptidic parts of molecules (biotin, amide group at the C-terminal end) were added using the Hyperchem 5.0 software (Autodesk, Sausalito, Ca, USA).

3. Results

3.1. Design and synthesis of TP and its analogues

In order to obtain the shortest TP analogue with minimal biological activities, we synthesized three series of analogues differing in the positions of the deletions. In the first series we deleted amino acid residues from the N-terminus of TP by three residues at a time to yield a three, six or nine amino acid residues shorter peptide. The second series consists of peptides that lack amino acid residues at the N-terminus and in the middle of the sequence. In the third group, presented so far only by the peptide TP15, the amino acids are missing at the N- and C-terminus as well as in the middle of the TP sequence. All synthesized peptides were biotinylated at Lys(ε-NH) in the galanin-derived part of the molecule in order to facilitate determination of their intracellular localization by indirect immunofluorescence.

3.2. Penetration of TP analogues into Bowes melanoma cells

Most of the short TP analogues have retained the cell penetrating ability. However the new derivatives differ remarkably in the speed and efficiency of penetration as well as in cellular localization. Incubation of Bowes cells with biotinylated analogues of TP at

Table 1
Amino acid sequences of galanin, mastoparan (MP), TP and its deletion analogues

	Sequence	Length[a] (aa)	Cellular uptake[d]		α-helix (%)	Angle[b] (°)	Penetration[c] (Å)	Speed of uptake ($\tau_{0.5}$, min)	GTPase activity (IC_{50}, μM)	Partition water/ octanol
			0°C	37°C						
Galanin	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS[e]	30	−	+/−						88.8
MP	INLKALAALAKKIL[f]	14	+	+						nd
TP	GWTLNSAGYLLGK[g]INLKALAALAKKIL	27	+++	+++	32	65	−1.7	3.4	22	15.1
TP7	...LNSAGYLLGK[g]INLKALAALAKKIL	24	+++	+++	35	48	−9.1	4.3	nd	10.4
TP8	.........LLGK[g]INLKALAALAKKIL	18	−	+/−	41	50	−10.4	nd	nd	nd
TP9	GWTLNSAGYLLGK[g]..LKALAALAKKIL	25	+++	+++	46	55	−6.4	6.5	47	7.0
TP10	......AGYLLGK[g]INLKALAALAKKIL	21	+++	+++	37	34	−11.8	8.6	no effect	9.3
TP11	GWTLNS......K[g]INLKALAALAKKIL	21	+	+	38	65	−14.4	nd	nd	nd
TP12	...LNSAGYLLGK[g]..LKALAALAKKIL	22	++	++	50	30	−14.4	10.7	58	8.2
TP13	...LNSAGYLLGK[g]....ALAALAKKIL	20	−	+/−	29	70	−7.6	nd	nd	nd
TP14	......AGYLLGK[g]..LKALAALAKKIL	19	++	++	42.5	38	4.2	nd	69	7.3
TP15	...LNSAGYLLGK[g]..LKALAALAK...	19	−	+/−	37	51	−9.3	nd	nd	nd

Comparison of molecular modeling data and the experimental cellular uptake of the peptides.
[a]Number of amino acid residues.
[b]Calculated angle of the peptide in relation to the membrane surface.
[c]For the penetration, a value of zero is attributed to the center of the membrane. The penetration is the distance between the mass center of the peptide and this point (Å, Fig. 1). A negative value indicates that the mass center of the peptide lies in the first leaflet of the membrane and a positive value that the molecule reached the second leaflet.
[d]Detection of biotin by indirect immunofluorescence: '+++' denotes comparable, '++' or '+' decreased and '+/−' weak uptake in relation to that of transportan.
[e]Biotinyl-Gly-Gly-$N^{\varepsilon 25}$galanin(1–29).
[f]Biotinylated at the N-terminus.
[g]Biotinylated at ε-amino group of Lys.

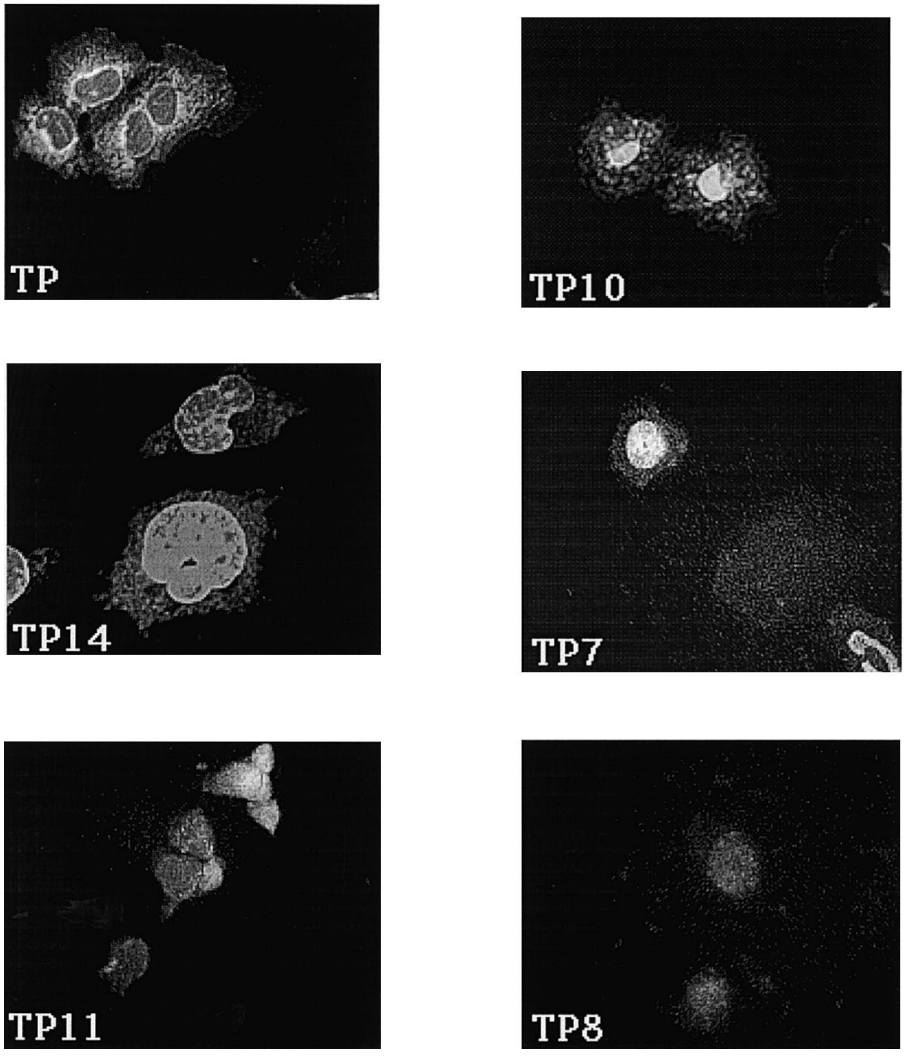

Fig. 1. Penetration of TP, TP10, TP14, TP7, TP11 and TP8 into Bowes melanoma cells visualized by staining with streptavidin-FITC. Cells were incubated with 10 μM biotinylated peptides at 37°C for 60 min.

0 or 37°C for 30 min revealed differences in internalization properties as demonstrated by indirect immunofluorescence (Fig. 1 and Table 1). Deletion of three or six residues from the N-terminus of TP (TP7 and TP10, respectively) or two residues from the middle (TP9) did not influence the penetration efficiency of the peptides, since they internalized to a comparable degree with TP at both temperatures tested. All three peptides could be detected all over the cytoplasm and nucleus of Bowes cells, accumulating mainly in the intracellular membranous structures (e.g. endoplasmic reticulum and Golgi complex) and nuclear envelope. However, the localization of these three peptides is not identical since the TP9 peptide showed strong staining of the nuclear envelope and distinct loci in the nucleus whereas TP7 and TP10 are distributed more evenly in the nucleus as judged by the diffuse staining.

Further shortening of the N-terminal part led to the loss of penetration ability, deletion of nine amino acid residues from the N-terminus (TP8) abolished the cellular penetration. Deletions from the N-terminus and/or from the middle of the TP molecule decreased (TP12, TP14, and TP11) or abolished (TP13) the internalization. Localization of peptides TP12, TP14 and TP11 in Bowes cells resembles to the cellular localization of TP9 i.e. peptides associate mainly with the plasma membrane, intracellular

membrane structures and are detectable in the nucleus.

Combination of deletions in the N- and C-termini and also in the middle of the sequence completely inactivated the peptide, TP15 did not internalize at all.

In order to compare the rate as well as the yield of internalization of different short analogues of TP, characteristic half times of internalization were calculated from Eq. 1. TP itself is the quickest penetrator with a half time of $\tau_{0.5} = 3.4$ min. Deletion analogues of TP show somewhat slower internalizations with characteristic half times: $\tau_{0.5} = 4.3$, 8.6, 6.5 and 10.7 min for TP7, TP10, TP9 and TP12, respectively.

3.3. Effects of TP analogues on GTPase activity in Rin m5F cell membranes

It has been shown that TP inhibits the basal activity of GTPase in Bowes cell membranes (IC_{50} of 21 μM) [4]. We have studied the influence of TP and its most efficiently penetrating analogues on the GTPase activity in Rin m5F cell membranes (Fig. 2) with the further aim to use the peptide that least affects GTPase activity as carriers of antisense peptide nucleic acid oligomers in this particular cell line. TP, TP9 and TP12 all inhibit basal GTPase activity in an analogous manner, IC_{50} values of 22, 47 and 58 μM, respectively. TP14 shows a biphasic effect, a small activation at low concentration, followed by inhibition with IC_{50} of 69 μM. The inhibitory effect of those peptides on the GTPase activity is probably caused by direct interaction of peptides with G-proteins as was previously shown for galanin(1-13)-mastoparan chimera, galparan [18]. These analogues of TP are mostly located in the membranous structures of the cell and may therefore change its physical properties and modulation of GTPase activity, this could also be caused by indirect i.e. membrane-mediated interaction of the peptides with G-proteins. Surprisingly, the peptide TP10 had no effect on the activity of the GTPases at least up to a 0.1 mM concentration.

3.4. Partition of ^{125}I-labeled analogues of TP between water and 1-octanol

The concentrations of short analogues of TP were found to be higher in water (c_1) than in octanol (c_2) in partition experiments. TP itself had the highest partition coefficient value of $P_{ow} = 15.1$, ($P = c_1/c_2$), and the coefficients for analogues were found to be between 7.0 and 10.4 (Table 1). Considering that $^{125}I^-$ ion from $Na^{125}I$ has a partition coefficient of about 500, these results indicated that TP and its shorter analogues are clearly lipophilic. The studied peptides may enter the plasma membranes and could be better membrane-entering compounds than TP itself, if the lipophilicity model can be taken as determinant of the cellular penetration properties of a peptide. However, the water/octanol system does not take all the aspects of peptide-lipid interaction into consideration and should be interpreted with caution.

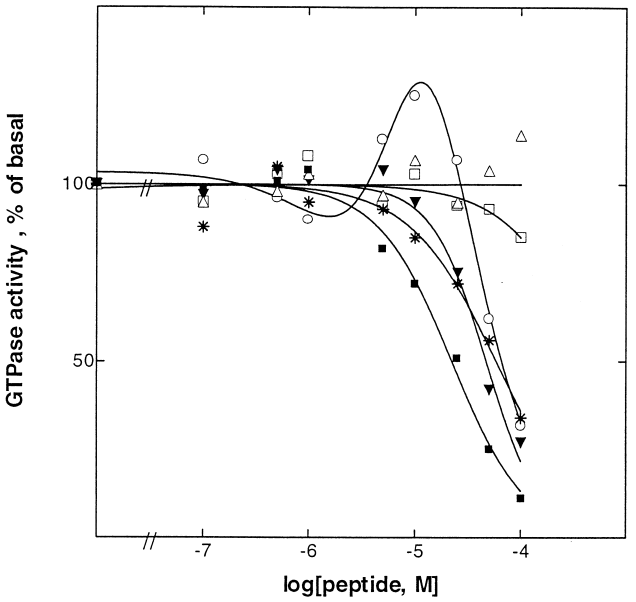

Fig. 2. GTPase activity in the presence of TP (■), TP10(△), TP9(▼), TP12(*) and TP14(○) in Rin m5F cell membranes. Points represent the mean values of three independent experiments. Curve for TP, TP10, TP9, TP12 was obtained by fitting the sigmoidal dose-response equation and for TP14 by fitting a double-phase sigmoidal dose-response equation to the experimental data. Concentration of GTP was 0.5 μM.

3.5. Molecular modeling of the interaction of TP analogues with membranes

The central parts of all the peptides adopt a α-helical structure (Table 1). This is most pronounced for the longer molecules, TP9 and TP7. The structure

of shorter peptides is less defined. The TP8 peptide (18 amino acids) has an almost entirely coiled conformation. In all cases, the N- and C-termini are in coiled structures, especially the N-terminal domains of TP15, TP10 and L266 peptides. Nevertheless, coiled conformation is stabilized in the TP9 peptide by 'π-stacking' interactions between the aromatic side chains of tyrosine and tryptophan residues. At the C-terminus of TP7, TP8 and TP14 peptides, the carbonyl group interacts with the amino group of the last but one lysine residue according to the specific sequence.

In previous studies, calculations showed that the [$N^{13\varepsilon}$-biotinyl]TP could span the membrane at an angle of 65° in relation to the surface plane. In this study we have analyzed cellular penetration of nine TP analogues. Molecular modeling shows that all these peptides are buried into the simulated biological membrane with different orientation. Therefore, we tried to group the results according to the angle and depth of penetration into the membrane (Table 1 and Fig. 3). This classification is tightly in correlation with the peptide sizes. The peptides TP7 and TP9 have rather similar orientation in the simulated membrane with TP. The main difference is the penetration depth: TP7 and TP9 peptides do not span the phospholipid bilayer although the molecules are entirely buried into the membrane. They both display oblique orientations relatively to the membrane plane with angles of 48 and 55°, respectively. The second group consists of peptides TP10, TP12 and TP14. They insert into the first leaflet only and lay more parallel in relation to the membrane surface (angles of 34, 30 and 38° respectively). The third subfamily contains the shortest peptides: TP11 (21 residues), TP13 peptides (20 residues), TP15 (19 residues) and TP8 (18 residues). The modeling predicts that the first two peptides exhibit almost perpendicular orientations in relation to the membrane surface (65 and 70°, respectively). The helical domain of the TP11 peptide is buried into the membrane whilst its coiled N-terminal part remains in the solution phase. The C-terminal part of TP13 peptide reaches the second leaflet while the biotinyl group and tyrosine residue stay in

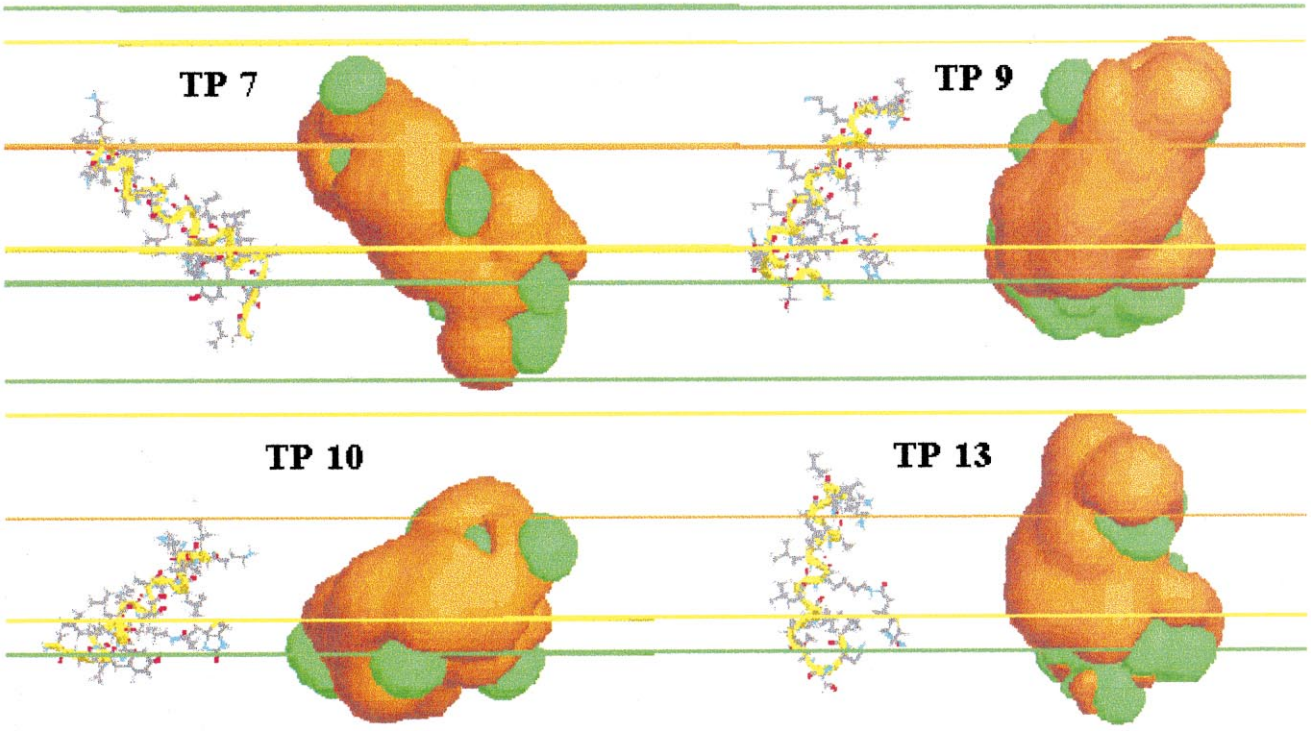

Fig. 3. Interaction of calculated peptides with the membrane model. The orange line denotes the center of the bilayer ($z=0$). For the yellow line $z=13.5$ Å and for the green line $z=18$ Å. The environment is completely hydrophilic between green and yellow lines and completely hydrophobic between two yellow lines. Orientation of TP7, TP9, TP10 and TP13 in relation to the membrane surface and calculations of MHP, around the peptides in the same orientations.

the peripheral polar area of the first leaflet. The shortest peptides (TP8 and TP15) display different oblique orientations (50 and 51°, respectively) but stay in the first leaflet because of their small size. The N-terminal part of TP8 peptide and the C-terminal part of TP15 peptide remain in the solution phase. In this meaning TP15 is exceptional as it is inserted into the membrane with the N-terminal part.

4. Discussion

TP is shown to penetrate into different cell types in a rapid and efficient way. The penetration is energy independent and not receptor-mediated [4]. TP is a ligand to galanin receptors (K_D = 17.4 nM in Bowes cell membranes) and also inhibits basal GTPase activity [4]. We have now synthesized nine shorter TP analogues to find a new transport peptide not recognized by galanin receptors and with less influence on the activity of cellular enzyme systems than the parent peptide, TP. We studied the cellular uptake of the peptides using the indirect immunofluorescence method.

Deletion of up to six amino acids from the N-terminus of TP (galanin part) did not impair the penetration ability of the peptides. These results, as well as the fact that the peptides are taken up at 4°C, show that the uptake of truncated TP analogues is not mediated by endocytosis of galanin receptors. It has been shown previously that Trp^2, Asn^5 and Tyr^9 are major pharmacophores in galanin(1-16) necessary for high affinity binding of this N-terminal fragment to the galanin receptor [19] (K_D = 3–7 nM for galanin(1–16), cf. review [20]). Furthermore, recently, different groups have shown that deletion of two first amino acids of galanin sequence results in an at least 1000-fold decrease in binding affinity of resulting galanin(3-29) to all three known different subtypes of galanin receptors (cf. review [21]). The K_D values for N-terminally truncated TP analogues in Bowes cell membranes were in range from 0.1 to 50 μM (data not shown), which is in accordance with above mentioned data. On the other hand, other processes than direct binding of the penetrating peptide to galanin receptors could cause the estimated apparent displacement of galanin from its receptors in Bowes cell membranes by short analogues of TP. The uncoupling of α-subunits of trimeric G-proteins from galanin receptors and/or shifting receptors into low-affinity state towards galanin is highly probable, since all these peptides (except TP10) influence the basal GTPase activity of Bowes or Rin m5F cell membranes. Moreover, the EC_{50} values of displacement of galanin from its type 1 receptors and inhibition of GTPase activity by TP are very close (17.4 and 21.1 μM, respectively) and may reflect the same process – interaction of peptide with G-proteins – in different experimental setups.

N-terminal deletion of nine amino acids of TP abolished the cellular uptake. The peptide TP11, where amino acids 7–12 were deleted, showed weak uptake at both temperatures used. Therefore it seems that for efficient uptake of TP derivatives, one of the necessary regions of the molecule is the C-terminus of the galanin part (middle part of TP).

It is known that mastoparan, the C-terminal part of TP, is penetrating into cell membranes where it creates short-living pores (cf. review [22]). An analogue of TP, TP9, where two amino acids in the N-terminus of mastoparan part are missing is taken up by Bowes cells as well as TP. Since we observed that truncation of the N-terminus of TP (TP7 and TP10) does not influence the cellular uptake, we introduced deletions by three (TP12) or six amino acids (TP14) into the N-terminus of TP9. The uptake of both peptides was significant, but somewhat lower than for TP9 confirming once more that the N-terminal part of TP is not the major determinant in penetration. In contrary, further shortening of mastoparan derived part either from the N- (TP13) or C-terminus (TP15) completely abolished the uptake. We conclude that two regions of TP molecule are necessary for internalization: mastoparan part or at least 10 of its amino acid residues and the C-terminal region of the galanin-derived part or a certain length of this region. We believe that the tyrosine moiety and the amphipathic helix of mastoparan are probably the motifs that are important for cellular uptake of TP analogues. All peptides that showed significant cellular uptake contained tyrosine and three positive charges in their sequences. Analogues without tyrosine or one positive charge in the C-terminal part did not internalize. The data about accumulation of short TP analogues obtained by indirect immunofluorescence are in good correlation with the rate

of internalization of these peptides estimated by using ^{125}I-labeled peptides (Table 1). Efficiently up taken peptides, TP and TP7 (+++ fluorescence intensity), penetrate into cells quickly, the characteristic times 3.4 and 4.3 min, respectively. Less efficient peptide TP12 (++) is internalizing at slower rate $\tau_{0.5} = 10.7$ min. The rate of internalization enables to distinguish the minute differences in the penetration abilities of efficiently penetrating peptides. The highest rate of penetration has the longest peptide-TP, shortening of the peptide causes slower internalization. The correlation between the length of the peptide and the rate of uptake is remarkable: longer peptides penetrate quicker. Truncation of the peptide in the same region led to proportional deceleration of internalization: TP, TP7 and TP10 ($\tau_{0.5} = 3.4$; 4.3 and 8.6 min, respectively). However, the length of the peptide is not the major determining factor for internalization, since peptide TP9 with two amino acids deletion penetrates into the cells slower than TP7, which has three amino acid residues deleted ($\tau_{0.5} = 6.5$ and 4.3 min, respectively). The same holds true for peptides TP12 and TP10 with five and six deleted amino acid residues, respectively, – shorter peptide penetrates quicker. Therefore the length of the peptide can not be considered the major determinant for the cell-penetration efficiency in the TP family of peptides.

Scheller et al. [23] have studied the correlation between helix parameters, charge and molecular size with the efficiency of uptake of α-helical model peptides into endothelial cells. They found that only the amphipathicity of the α-helix of the peptide is important for the internalization and that the minimal length of the peptide corresponds to four complete helix turns (16 amino acids). We used the prediction program Agadir [24] to calculate the extent of α-helicity in TP derived peptide sequences. Only 29–50% of the peptide molecule is predicted to be in the α-helical structure that is located mostly in the middle of the peptide chain. Helical wheel projections reveal that all peptides, except TP15, have an amphipathic structure, but there is no obvious correlation between the internalization and amphipathicity.

TP and most of its penetrating analogues inhibit the basal GTPase activity of Rin m5F membranes. It is known that one building block of TP, mastoparan activates and galanin does not affect GTPase activity [18,25]. The inhibition of GTPase activity by most TP-derived peptides is similar to galparan, the precursor peptide of TP. Galparan is suggested to bind with the C-terminus (mastoparan moiety) to the mastoparan binding site of the G-protein and with the N-terminus to the allosteric site of G_α subunit [18]. Wagner et al. [26] have suggested a structure for peptide inhibitors of GTPase activity consisting of a hydrophobic tail, which is covalently attached to an amphiphilic moiety. All TP analogues, that inhibit the GTPase activity, are composed of the amphipathic helix extended with the hydrophobic tail of galanin in the N-terminus, which is in good accordance with the above-mentioned model. TP14, in contrast, has completely different biphasic effect: a weak activation and subsequent strong inhibition of basal GTPase activity. We have shown a similar pattern of GTPase activity modulation in Rin m5F cell membranes for the chimeric peptides M375 and M391 that consist of vasopressin receptor V_1 antagonist and mastoparan connected directly or via 6-aminohexanoic acid [27]. Furthermore, we demonstrated that these chimeric peptides stimulate GTPase activity of $G_{\alpha i}/G_{\alpha o}$ and inhibit $G_{\alpha s}$ protein [27]. The only exception in this series of TP analogues is TP10 that has no effect on GTPase activity in Rin m5F cell membranes.

In the peptide TP10 six amino acid residues from TP's N-terminus are missing and the mastoparan-derived part is left intact. The absence of effect on GTPase activity was unexpected. In fact, TP10 does not fit very well into Wagner's model for GTPase inhibitors of peptide character. TP10 has an amphiphilic moiety-mastoparan part but the long hydrophobic tail that is present in other derivatives of TP is missing or at least substantially shortened, what could explain the absence of the inhibitory effect. On the other hand, TP10 can be interpreted as N-terminally prolonged mastoparan, where seven additional amino acids interfere in manifestation of GTPase activating property of mastoparan. The data, however, are too limited to draw any clear conclusion about the mechanism involved in inhibition of GTPases by TP analogues and comprehensive study is necessary to unravel the question.

Molecular modeling analysis shows that all TP analogues have stable positions when inserted into the membrane, but their orientations in relation to

the membrane surface differ (Table 1 and Fig. 3). Two predicted parameters can be correlated with the ability of the peptides to traverse phospholipid membranes. First, an oblique orientation relative to the membrane plane seems to be important, since peptides with more parallel orientation in relation to the membrane (angle less than 40–45°) are efficiently internalizing. In contrast, short peptides (22 amino acids or less) with more perpendicular orientation are poorly internalizing (TP11) if at all (TP13 and TP15).

TP and its derivatives of 24 or 25 amino acids, i.e. long analogues penetrate into the cells most efficiently. It seems that the increased length of the peptide facilitates penetration into the hydrophobic core. Shorter peptides (less than 21 residues) may not reach the second leaflet, and are therefore too short to span the lipid bilayer.

The difficulties in finding unambiguous correlation between the characteristics obtained from molecular modeling of interaction of peptides with membrane and experimental data on cell penetration of TP and its analogues may be caused by plausible multimerization of these peptides. All analogues of TP form dimers and higher oligomers in the presence of SDS micelles [4] and could multimerize in the membranes or interact with biological membranes as multimers for efficient membrane penetration as is suggested for the antennapedia derived peptide [10].

Molecular modeling data show that the C-terminal part of TP analogues is buried into the membrane, only TP15 is an exception and has N-terminal in the membrane. Both lysine residues of the C-terminus of the longest peptides (TP, TP7 and TP9) may reach the opposite side of the membrane. Consequently, aromatic residues of the N-terminal and the biotinyl group remain anchored in the first phospholipid polar head area. According to the calculation of the molecular hydrophobic potentials (MHP, [28]) the N-terminal part is hydrophilic due to the presence of threonine, serine or aromatic residues (Fig. 3). According to the calculations, TP15 has an inverted orientation, although this is the sole peptide that has a charged hydrophilic lysine residue in the C-terminal part. All other TP derivatives, in comparison, have additional residues Lys-Ile-Leu in the C-terminus. We conclude that the most important features, which determine the orientation of cell penetrating peptides in the membrane, are the peptide length, but also the location of aromatic and lysine residues in the terminal regions of the sequence.

In conclusion, we have synthesized several shorter TP analogues that retain the efficient cell penetration property of the parent compound. One of these, TP10, does not modulate the basal GTPase activity even at very high concentrations nor is it recognized by galanin receptors. These features make TP10 a promising candidate for a new generation of transport peptides with significantly less potential side effects.

Acknowledgements

This study was supported by the European Community Biotechnology programs, BI 104-98-0227, PL 960466, by EU MAST III CT97-0156, by the Swedish Research Councils (TFR and NFR), by the Swedish Institute (Visby program), by Wenner-Gren Center, Stockholm, Sweden (stipend to M.Z.), and by Estonian Science Foundation (ETF 4007). RB is Directeur de Recherche FNRS (Fonds National de la Recherche Scientifique). X.G. is supported by the 'Interuniversity Poles of Attraction Programme-Belgian State, Prime Minister's Office-Federal Office for Scientific, Technical and Cultural Affairs' contract No. P.4/03.

References

[1] T. Higashhijima, J. Burnier, E.M. Ross, Regulation of G*i* and G*o* by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity, J. Biol. Chem. 265 (1990) 14176–14186.

[2] F. Perez, A. Joliot, E. Bloch-Gallego, A. Zahraoui, A. Triller, A. Prochiantz, Antennapedia homeobox as a signal for the cellular internalization and nuclear addressing of a small exogenous peptide, J. Cell. Sci. 102 (1992) 392–396.

[3] E. Bloch-Gallego, I. Le Roux, A.H. Joliot, M. Volovitch, C.E. Henderson, A. Prochiantz, Antennapedia homeobox peptide enhances growth and branching of embryonic chicken motoneurons in vitro, J. Cell. Biol. 120 (1993) 485–492.

[4] M. Pooga, M. Hällbrink, M. Zorko, Ü. Langel, Cell penetration by transportan, FASEB J. 12 (1998) 67–77.

[5] I. Choudhury, J. Wang, A.B. Rabson, S. Stein, S. Pooyan, S. Stein, M.J. Leibowitz, Inhibition of HIV-1 replication by a Tat RNA-binding domain peptide analog, J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 17 (1998) 104–111.

[6] J.-M. Péloponèse Jr., Y. Collette, C. Grégoire, C. Bailly, D. Campèse, E.F. Meurs, D. Olive, E.P. Loret, Full peptide synthesis, purification, and characterisation of six Tat variants, J. Biol. Chem. 274 (1999) 11473–11478.
[7] G. Elliott, P. O'Hare, Intracellular trafficking and protein delivery by a herpesvirus structural protein, Cell 88 (1997) 223–233.
[8] L. Theodore, D. Derossi, G. Chassaing, B. Llirbat, M. Kubes, P. Jordan, H. Chneiweiss, P. Godement, A. Prochiantz, Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse, J. Neurosci. 15 (1995) 7158–7167.
[9] M. Rojas, J.P. Donahue, Z. Tan, Y.Z. Lin, Genetic engineering of proteins with cell membrane permeability, Nat. Biotechnol. 16 (1998) 370–375.
[10] A. Prochiantz, Getting hydrophilic compounds into cells: lessons from homeopeptides, Curr. Opin. Neurobiol. 6 (1996) 629–634.
[11] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.-X. Hao, X.-J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, Ü. Langel, Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo, Nature Biotechnol. 16 (1998) 857–861.
[12] J.R. Gavin, J. Roth, P. Jen, P. Freychet, Insulin receptors in human circulating cells and fibroblasts, Proc. Natl. Acad. Sci. USA 69 (1972) 747–751.
[13] D. Cassel, Z. Selinger, Catecholamine stimulated GTPase activity in turkey erythrocyte membranes, Biochim. Biophys. Acta 452 (1976) 538–551.
[14] F.R. McKenzie, in: G. Milligan, (Ed.), Signal transduction. A practical approach, Oxford University Press, Oxford, 1992, pp. 31–56.
[15] R. Brasseur, J.M. Ruysschaert, Conformation and mode of organisation of amphiphilic membrane components: a conformational analysis, Biochem. J. 238 (1986) 1–11.
[16] P. Ducarme, M. Rahman, R. Brasseur, IMPALA a simple restraint field to stimulate the biological membrane in molecular structure studies, Proteins 30 (1998) 357–371.
[17] M. Rahman, R. Brasseur, WinMGM: a fast CPK molecular graphics program for analysing molecular structure, J. Mol. Graph. 12 (1994) 212–218.
[18] M. Zorko, M. Pooga, K. Saar, K. Rezaei, Ü. Langel, Differential regulation of GTPase activity by mastoparan and galparan, Arch. Biochem. Biophys. 349 (1998) 321–328.
[19] T. Land, Ü. Langel, M. Löw, M. Berthold, A. Undén, T. Bartfai, Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor, Int. J. Pept. Protein Res. 38 (1991) 267–272.
[20] T. Bartfai, T. Hökfelt, Ü Langel, Galanin – a neuroendocrine peptide, Crit. Rev. Neurobiol. 7 (1993) 229–274.
[21] S. Wang, E.M. Parker, Galanin receptor subtypes as potential therapeutic targets, Exp. Opin. Ther. Pat. 8 (1998) 1225–1235.
[22] U. Soomets, M. Hällbrink, M. Zorko, Ü. Langel, From galanin and mastoparan to galparan and transportan, Curr. Top. Pept. Protein Res. 2 (1997) 83–113.
[23] A. Scheller, J. Oehlke, B. Wiesner, M. Dathe, E. Krause, M. Beyermann, M. Melzig, M. Bienert, Structural requirements for cellular uptake of α-helical amphipathic peptides, J. Pept. Sci. 5 (1999) 185–194.
[24] V. Muñoz, L. Serrano, Development of the multiple sequence approximation within the Agadir model of α-helix formation. Comparison with Zimm–Bragg and Lifson–Roig formalisms, Biopolymers 41 (1997) 495–509.
[25] T. Higashijima, S. Uzu, T. Nakajima, E.M. Ross, Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins), J. Biol. Chem. 263 (1988) 6491–6494.
[26] T. Wagner, C. Oppi, G.P. Tocchini Valentini, Differential regulation of G protein α-subunit GTPase activity by peptides derived from the third cytoplasmic loop of the alpha adrenergic receptor, FEBS Lett. 365 (1995) 13–17.
[27] M. Hällbrink, K. Saar, C.-G. Östenson, U. Soomets, S. Efendic, J. Howl, M. Wheatley, M. Zorko, Ü. Langel, Effects of vasopressin-mastoparan chimeric peptides on insulin release and G-protein activity, Regul. Pept. 82 (1999) 45–51.
[28] R. Brasseur, Differentation of lipid-associating helices by use of three-dimensional molecular hydrophobicity potential calculation, J. Biol. Chem. 266 (1991) 16120–16127.