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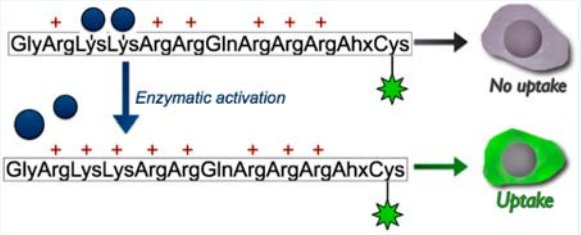
Enzyme-Activatable Cell-Penetrating Peptides through a Minimal Side Chain Modification

Saskia A. Bode,‡ Morten B. Hansen,‡ Roy A. J. F. Oerlemans, Jan C. M. van Hest, and Dennis W. P. M. Löwik*

Radboud University Nijmegen, Institute for Molecules and Materials, Bio-organic Chemistry, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

Supporting Information

ABSTRACT: Activatable cell-penetrating peptides are of great interest in drug delivery because of their enhanced selectivity which can be controlled by the external stimuli that trigger their activation. The use of a specific enzymatic reaction to trigger uptake of an inert peptide offers a relevant targeting strategy because the activation process takes place in a short time and only in areas where the specific cell surface enzyme is present. To this aim, the lysine side chain of Tat peptides was modified with an enzyme-cleavable domain of minimal size. This yielded blocked Tat-peptides which were inactive but that could be activated by coincubation with the selected enzymes.



The transport of numerous biologically relevant cargoes inside cells has been facilitated by a wide range of cell-penetrating peptides (CPPs).[1,2] One specifically well-studied class of these CPPs comprises the group of highly cationic peptides, such as the HIV-derived Tat-peptide and oligo-arginines. These charged CPPs have gained interest because their uptake into cells occurs both via endocytosis and, especially at higher peptide concentrations, via direct translocation through the plasma membrane.[3,4] Despite their promising characteristics and high potential for use in drug delivery, their pharmaceutical usage is still limited. This is mainly due to the lack of cell-type specificity for most CPPs and because their moment of uptake cannot be controlled. For this reason, much research is now focused on the development of strategies to add specificity to CPP-based delivery systems. Recently, several targeting strategies have been reported that utilize inert peptides of which their cell-penetrating ability can be induced by a specific change in the environment. This strategy is employed to circumvent nonspecific uptake before reaching the target. CPPs can now be activated by several triggers, such as a pH-change,[5,6] by exposure to UV-light,[7] or by enzymatic reactions.[4,8−11] Furthermore, cell-specific targeting peptides have been attached to CPPs to obtain fusogenic peptides with dual activity.[12−14] The pH-controlled activation of CPPs relies on the presence of an acidified microenvironment around the target tissue which is used to protonate a peptide to become a CPP, or to activate CPPs immobilized on a delivery vehicle. Although these strategies have shown great promise in efficient activation of CPP-based delivery vehicles, it necessitates a pH difference between the target and normal tissue, which only applies to a limited number of pathological conditions.[15] Activation through UV-light seems independent of the environmental characteristics of the target tissue; however, its usage for in vivo applications is limited. Fusogenic peptides that possess the ability to enter cells and that can be targeted to specific tissue allow delivery to the desired cell-types. However, nonspecific uptake at healthy tissue still occurs for these peptides. Activatable CPPs based on activation through a specific enzymatic reaction offer a particularly interesting alternative targeting strategy. In this regard, the specificity and efficiency originate from the activation process, which is catalyzed by specific cell-surface enzymes potentially leading to activation of CPPs in a very short time and in a designated area. As a result, the CPPs will be taken up primarily in areas where the specific surface enzymes are present. An elegant example of activation by enzymatic cleavage was reported by Tsien and co-workers.[11] A peptide hairpin was designed in which an oligoarginine CPP was attached to oligoglutamic acid through an enzyme-cleavable sequence. The anionic domain functioned to inhibit the activity of the CPP and enzymatic cleavage of the peptide linker led to release of the oligoarginine and thus to uptake.

Interestingly, in an alanine scan of Tat (RKKRRQRRR), Wender and co-workers have shown that substitution of either of the lysine residues by an alanine residue leads to a tremendous decrease in cellular uptake of the resulting peptide.[16] Jin et al. have recently used this finding to develop acid-activatable Tat peptides, which were obtained by amidation of the lysine residues to succinyl amides.[6] The resulting peptides were not able to enter cells, until they were exposed to an acidic pH, which led to hydrolysis of the succinyl amides.

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These reports inspired us to investigate if attachment of enzyme-cleavable sequences at the lysine side-chains may lead to an enzyme-triggered variant of this approach. Interesting model enzymes to validate such an enzyme-activatable strategy are two surface-expressed peptidase enzymes, aminopeptidase N (APN)[17] and dipeptidyl peptidase IV (DPPIV).[18] Expression of both enzymes is upregulated in multiple types of cancer cells.[19−21] Furthermore, APN serves as a receptor for several virus infections[22,23] and DPPIV is expressed on activated immune cells.[24] Additionally, these two peptidases exhibit interesting substrate selectivities as DPPIV preferentially cleaves Xaa-Pro dipeptides from the N-terminus of peptides,[25] whereas APN cleaves almost all unacylated amino acids.[26] Therefore, Tat peptides inactivated with sequences recognized by these enzymes might serve as a way to target these particular pathological conditions.

Here we report a simple yet efficient approach to temporarily inactivate a cell-penetrating peptide, Tat, by conjugating a shielding amino acid or dipeptide to one or both lysine side chains in its sequence. Subsequently, the blocked Tat peptide can be reactivated by cleaving off the inactivating amino acids by treatment with the appropriate enzyme (Figure 1). This approach offers a complementary strategy to previously reported systems based on enzymatic activation because it blocks the activity of the CPP as a result of a minimal modification of a peptide side chain in contrast to shielding via the peptide main chain. Our strategy presents activatable Tat peptides based on minimal modifications on the peptide sequence.

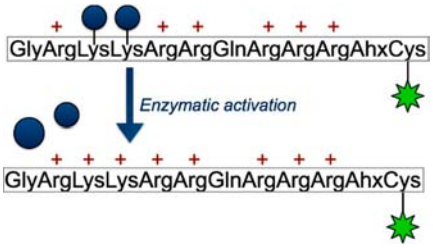

Figure 1. General concept of the enzyme-activatable Tat peptide. The positive charges from the lysine residues are shielded by inactivating modifications. These modifications can be cleaved off by treatment with the appropriate enzyme, yielding the active Tat peptide.

RESULTS AND DISCUSSION

Preparation of Enzyme-Activatable Tat-Peptides. We prepared various enzyme-activatable Tat-peptides by using two different shielding approaches on the lysine side chains. One involved coupling a single alanine residue to the side chains while the other made use of a glycine-proline dipeptide. Since the Tat-peptide contains two lysine residues, this modification was performed in three ways: by a single modification on the N-terminal or the C-terminal lysine or by a modification on both lysine residues. These modifications are denoted Tat-X(10) for coupling to the first lysine residue, Tat-X(01) for modification on the second lysine residue, and Tat-X(11) for a functionalization of both lysine residues, with X being an alanine residue or the glycine-proline dipeptide (Figure 2). Furthermore, all peptides were acetylated at the N-terminus to prevent degradation of the Tat peptide by peptidases. The alanine-blocked Tat peptides were selected because of their potential as a minimal modification to inhibit the activity of Tat, and because they were expected to be suitable substrates for APN, as this enzyme represents a broad substrate specificity. The glycine-proline dipeptide was selected because Mutter and co-workers previously reported the cleavage of arginine-proline from the N-terminus of an unnatural *O*-acyl isopeptide using DPPIV.[27] We however observed that introducing the dipeptide arginine-proline to the lysine side-chains in Tat did not inhibit the cellular uptake of the obtained peptide, which is likely to be caused by the guanidinium groups in the arginine side chains, which are known to enhance cellular uptake (data in SI). Therefore, the dipeptide sequence was modified to glycine-proline (GP), which was also expected to be a suitable substrate for DPPIV. To prepare the desired enzyme-activatable Tat-peptides, we made use of a methyltrityl (Mtt) protection group on the lysine residues. In this way, three Tat peptides were synthesized in which either one of the lysines or both lysine residues were protected with the Mtt-group. After acetylation of the N-terminus, the Mtt groups were selectively removed using a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and dichloromethane (DCM) in a ratio of TFA/TIS/DCM 1:2:97 (v:v:v). Subsequently, the lysine side chains were functionalized with alanine or the glycine-proline dipeptide employing standard solid phase peptide synthesis (SPPS). As both the alanine and the GP-dipeptide were Boc-protected at their N-terminus, cleavage of the peptide from the resin ensured that the side chain modifications had free N-termini. After global deprotection and cleavage from the resin, the C-terminal cysteine was used to conjugate fluorescein diacetate 5-maleimide to the peptides. This label allows for fluorescence-based techniques to analyze the ability of the peptides to enter cells.

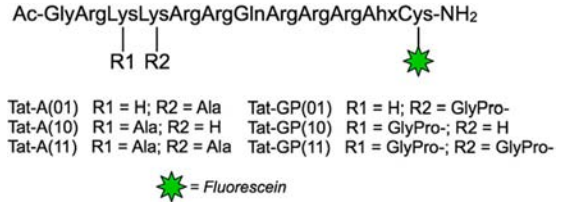

Figure 2. Peptide sequences with inactivating modifications on the lysine residues.

Uptake Studies on Inactivated Tat-Peptides. The uptake efficiency of the inactivated peptides at a concentration of 5 μM in Human Embryonic Kidney (HEK) cells was quantified by flow cytometry and visualized using confocal laser scanning microscopy (CLSM). Unmodified Tat peptide was also included and used as a reference. Flow cytometry (Figure 3) showed that the unmodified Tat was taken up extensively, which is in sharp contrast to the inactivated Tat peptides. It is important to note here that only internalized peptide is evaluated in this experiment, since the cells are washed with trypsin to remove any membrane-bound peptide. The Tat-A peptides almost completely lost their cell penetrating ability, and interestingly, the Tat-A peptides with a single modification (Tat-A(01) and Tat-A(10)) were inactivated slightly, yet significantly more efficiently than the Tat-A(11) peptide. Similar results were observed for the Tat-GP peptides, although these were taken up slightly more than the Tat-A peptides. This clearly shows the potential of (temporarily) blocking the activity of Tat by a side chain modification on the lysine residues, and that even a single amino acid residue is sufficient.

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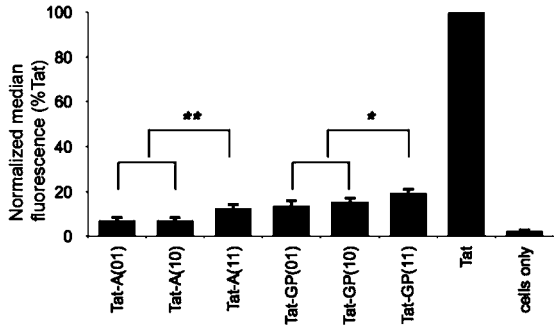

Figure 3. Flow cytometry results of uptake studies at 5 μM, with an incubation time of 30 min at 37 °C. No difference in uptake efficacy was observed for Tat(A(01) and Tat-A(10), but the uptake of Tat-A(11) was significantly higher (p = 0.0029). Similarly, no difference in uptake efficacy was observed between Tat-GP(01) and Tat-GP(10), but the uptake of Tat-GP(11) was slightly, but significantly higher (p = 0.012).

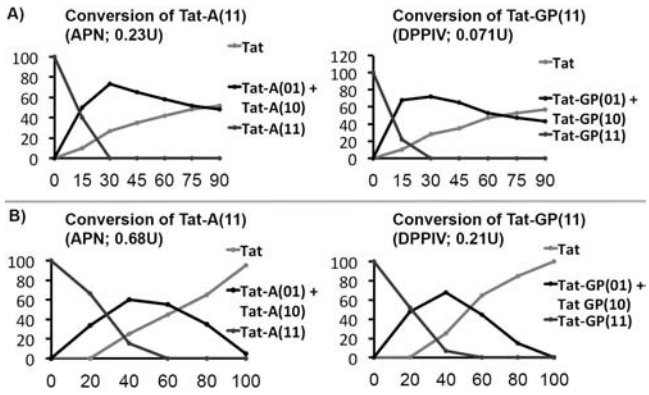

Figure 4. (A) Conversion of the Tat-A(11) and Tat-GP(11) peptides by APN and DPPIV, respectively, at low enzyme concentrations (0.23 U APN and 0.071 U DPPIV). (B) Enzymatic conversion of the peptides at the higher concentration of 0.68U APN and 0.21U DPPIV.

The inactivation strategy was furthermore studied by confocal laser scanning microscopy (CLSM) which allows for the visualization of the intracellular distribution of the peptides. The CLSM images showed a clear uptake by endocytosis (localized dots) for the unmodified Tat peptide, but hardly any intracellular fluorescence (smeared fluorescence of the cytosol) was observed for all of the inactivated Tat variants (SI Figures S4.1 and S4.2). These results agree with the flow cytometry experiments and furthermore suggest that at the concentration of 5 μM the inactivated peptides are not prone to membrane binding.

Enzymatic Activation of the Inactivated Tat-Peptides. To test whether the inactivated Tat peptides could be reactivated enzymatically, the peptides were treated with aminopeptidase (APN) and dipeptidyl peptidase (DPPIV) and the reaction mixture was analyzed by reversed phase HPLC. To study whether Tat itself withstands peptidase activity from APN and DPPIV, the acetylated Tat peptide was incubated with both enzymes. As expected, no conversion could be observed for either one of the enzymes. At low enzyme concentrations, no cross-reactivities were observed, meaning that the Tat-A peptides were only converted by APN, and not by DPPIV, and that the Tat-GP peptides could only be cleaved by DPPIV. However, at these low concentrations of enzyme, only a maximum conversion of 50% was achieved after 90 min (Figure 4A). In order to obtain full conversion within this time frame, the enzyme concentration was increased (Figure 4B). Under these conditions, we could keep the incubation time at 90 min, which is the preferred time-span for cellular uptake experiments, as longer incubation times could lead to less reliable results due to nonspecific binding of positively charged peptides to the plasma membrane and the possibility of subsequent internalization. Although we observed cross-reactivities of the enzymes at these concentrations, the unmodified Tat remained stable and therefore we decided to use the higher enzyme concentrations for further experiments.

Cellular Uptake of Reactivated Tat-Peptides. The potential toxicity of the Tat variants in the presence of the model enzymes was analyzed by means of a cell viability assay. For this purpose, HEK cells were incubated with the inactivated peptides at 5 μM together with APN or DPPIV. After 2 h, the cells were washed thoroughly and incubated with FCS-supplemented DMEM. The cells were allowed to rest for 4 h, after which the cell counting dye WST-8 was added and incubated with the cells for 3 h. In addition, a prolonged incubation time of 6 h of the peptides and enzymes with the cells was used. For these experiments, a survival value of 100% was defined for untreated HEK cells. Since both experiments showed no significant cell death, it was assured that at the desired incubation conditions for the cellular uptake studies the cells would remain viable (cell survival >90%, SI Figures S3.1 and S3.2) allowing us to proceed with analysis of the uptake efficiency of the reactivated Tat peptides.

The uptake behavior of the blocked Tat peptides in HEK cells after in situ reactivation was studied using both flow cytometry (Figure 5A) and CLSM (Figure 5B). HEK cells were treated with 5 μM peptide in the presence of either APN (0.68 U) or DPPIV (0.21 U) and were incubated for 90 min at 37 °C. For the flow cytometry experiments, the cells were briefly washed after the incubation period with trypsin/EDTA to remove membrane-bound peptides, as trypsin leads to digestion of the membrane-bound peptides.[28] As a reference, unmodified Tat was included and the average median fluorescence of Tat was defined as 100%. Flow cytometry of cells treated with APN reactivated Tat-A peptides revealed an uptake efficiency up to 60% compared to unmodified Tat. Similar results were observed for the Tat-A peptides that were reactivated by DPPIV. The Tat-GP peptides were found to regain the ability to enter cells after reactivation with DPPIV up to an amount of ~75% compared to Tat. Treatment with APN was less efficient, yielding uptake efficiencies of ~60%. As expected from the HPLC data obtained during the enzymatic conversion of the inactivated Tat peptides (Figure 3), treatment with either APN or DPPIV did not affect the uptake of unmodified Tat itself. The same experiment was also performed with the lower enzyme concentration (0.23 U APN and 0.071 U DPPIV); however, this only showed uptake efficiencies up to 25% to 30% compared to unmodified Tat itself (Supporting Information Figure S5.1). These results again confirm the need to use the higher enzyme concentrations to achieve efficient in situ reactivation of the Tat peptides.

By means of CLSM we were able to study the cellular localization of the reactivated peptides after uptake by HEK cells. These experiments were, like the flow cytometry studies, performed with a peptide concentration of 5 μM and an incubation time of 90 min at 37 °C, using in situ enzymatic reactions with APN or DPPIV. Unmodified Tat as a reference was used and this peptide was also incubated in the presence of

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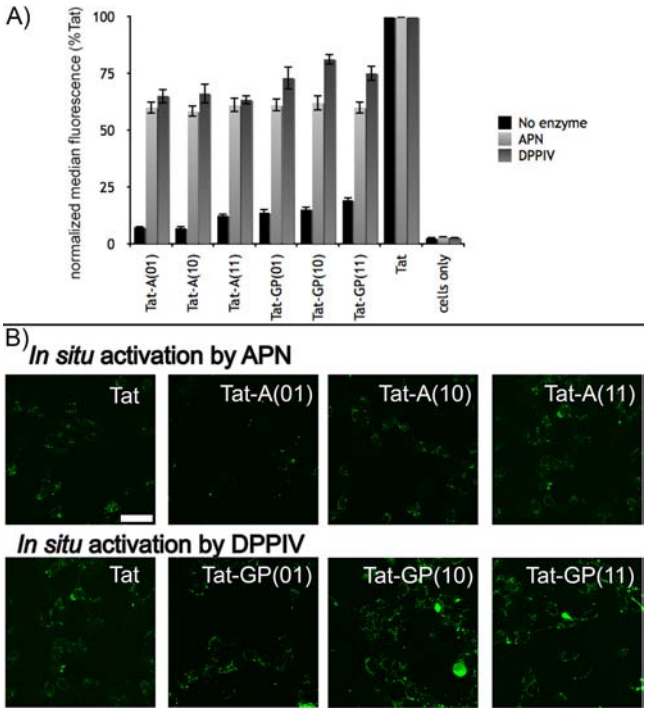

Figure 5. Cellular uptake studies at 5 μM Tat peptide concentrations in the presence of APN or DPPIV. All studies were conducted in the presence of serum. (A) Flow cytometry results of uptake studies conducted in the presence of APN or DPPIV. Unmodified Tat was also treated with the enzymes. The average median fluorescence intensity of Tat uptake was defined as 100%. (B) Confocal laser scanning microscopy images of the Tat-A series (top) which were coincubated with APN and (below) microscopy images of the Tat-GP series which were coincubated with DPPIV. Unmodified Tat was included and treated with APN or DPPIV as a positive control. Scale bar represents 50 μm.

the enzymes. We observed that all Tat variants entered HEK cells by endocytosis after reactivation, similarly to what we previously observed for the unmodified Tat peptide.

CONCLUSION

We have shown that it is possible to temporarily block the activity of a cell-penetrating peptide, Tat, by a minimal modification of the lysine side chains and restore its cell uptake potential upon enzymatic cleavage of the lysine modification. Importantly, our results show that only one lysine residue needs to be modified to obtain an almost complete loss in cellular uptake of Tat. Interestingly, we observed that this deactivation strategy works with no more than a single amino acid residue on a single lysine side chain. The inactivating groups on the lysine side chains could be enzymatically removed in situ using the model enzymes aminopeptidase N (APN) or dipeptidyl peptidase IV (DPPIV). This enzymatic activation of Tat has the potential to become a useful, physiologically benign strategy to control the activity of CPPs.

MATERIALS AND METHODS

General. NovaPEGRink amide resin was purchased from Novabiochem and Fmoc-L-amino acids from Bachem (Bubendorf, Switzerland) or Novabiochem (EEMBD Chemicals, Gibbstown, U.S.A.). Diisopropylcarbodiimide (DIPCDI) was obtained from Biosolve. Dipeptidyl peptidase IV (porcine kidney, EC 3.4.14.5) was from Sigma-Aldrich and aminopeptidase N (Rat, EC 3.4.11.2) from Calbiochem (Merck, Darmstadt, Germany). Fetal bovine serum (FBS) was obtained from Integro (Zaandam, The Netherlands), plain Dulbecco's Modified Eagle Medium (DMEM), and trypsin/EDTA were both from PAA Laboratories (Pasching, Austria). Human Embryonic Kidney cells were obtained from American Type Culture Collection (ATCC, Manassas, U.S.A.). All other chemicals were purchased from Baker, Fluka, or Sigma-Aldrich and were used as received.

Reversed Phase HPLC Peptide Purification and Liquid Chromatography Mass Spectrometry. Semipreparative HPLC was performed on a Shimadzu LC-20A Prominence system (Shimadzu,'s-Hertogenbosch, The Netherlands) equipped with a C18 ReproSil column, 250 × 10 mm, particle size 5 μm (Screening Devices, Amersfoort, The Netherlands). Elution of the peptides was achieved with a MeCN/water gradient containing 0.1% trifluoroacetic acid (5−100%, 1−50 min, flow 4 mL/min). The fractions were analyzed by liquid chromatography mass spectrometry using a Thermo Finnigan LCQ-Fleet ESI-ion trap (Thermo Fischer, Breda, The Netherlands) equipped with an Alltima C18 column, 2.1 × 150 mm, particle size 3 μm, or an Alltima C18 column, 2 × 50 mm (Alltech Applied Sciences BV, Breda, Netherlands), using the same gradient as used for HPLC (trifluoroacetic acid was replaced with formic acid) or a shorter gradient (3−100%, 3−10 min), respectively. The collected fractions containing the desired peptide were pooled, evaporated until the volume was halved, and lyophilized.

Synthesis of Mtt-Protected Tat-Peptides. The three Mtt-protected Tat peptides were synthesized on a NovaPEG Rink amide resin using a Labortec640 peptide synthesizer (Labortec, Bubendorf, Switzerland) and employing a standard Fmoc solid phase peptide synthesis (SPPS) protocol. The peptides had the general sequence Ac-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-(ε-Ahx)-Cys-NH_2. Either the first, the second, or both lysine residues were protected with a methyltrityl protecting group (Mtt) by incorporating the Fmoc-Lys(Mtt) amino acid instead of Fmoc-Lys(Boc). In brief, the resin was swollen in DMF for 20 min prior to use. The first and subsequent Fmoc protecting groups were removed by washing the resin with piperidine in DMF (20%, v/v) and then shaking for 25 min with another portion of piperidine in DMF. The desired sequence of amino acids was coupled to the resin using Fmoc-L-amino acids (3.0 equiv), diisopropylcarbodiimide (DIPCDI, 3.3 equiv), and *N*-hydroxybenzotriazole (HOBt, 3.6 equiv). Peptide couplings were monitored to completion using the Kaiser test.[29] After the final Fmoc removal, the N-terminus was acetylated by agitation in a mixture of pyridine (1 mL) and acetic anhydride (1 mL) in DMF for 10 min. Afterward, the resin was washed thoroughly with DMF, DCM, *i*-PrOH, DCM, and Et_2O and dried in vacuo. A small amount of the resin was used to verify the quality of the synthesized peptide and was suspended in trifluoroacetic acid/water/triisopropylsilane/thioanisole (90:5:2.5:2.5 v/v) and stirred for 8 h at rt. Then the resin was filtered off and the filtrate was precipitated from Et_2O, redissolved in water, and lyophilized yielding the crude peptide as a white powder. Liquid chromatography mass spectrometry was performed to verify the identity of the peptides (Supporting Information S1 and S7).

Synthesis of Branched Tat-Peptides. The NovaPEG resin carrying the Mtt-protected Tat peptides was suspended in a mixture of TFA/TIS/DCM (1:2:97 v:v:v) for 20 min. A small

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amount of resin was then removed and to this 1−2 drops of neat TFA was added. If no bright orange color emerged, the resin was washed with DCM, MeOH, diisopropylamine (DIEA) in DMF (1% v/v), and finally with DMF. The resin was then ready for coupling with Boc-Ala to obtain the Tat-A peptides or with Fmoc-Pro and subsequently Boc-Gly to obtain the Tat-GP peptides, using standard SPPS protocols and resin cleavage procedures as described above. Liquid chromatography mass spectrometry was performed to verify the identity of the peptides (Supporting Information S1 and S7).

Solution Labeling of the Tat-Peptides. The peptides (25 μmol) were dissolved in a mixture of water−DMF (1:1, v/v, 6 mL) and to this triscarboxyethylphosphine (12 mg, 48 μmol) and fluorescein diacetate 5-maleimide (17 mg, 33 μmol) were added. The reaction mixture was stirred at 60 °C for 18 h. Next, the reaction mixture was concentrated in vacuo and redissolved in a water−formic acid (1:1 v/v) mixture and the product was purified using semipreparative HPLC yielding the fluorescein-labeled peptides. Identity of the peptides was confirmed by liquid chromatography mass spectrometry (Supporting Information S1 and S7).

Enzymatic Reactions. For the enzymatic reactions the peptides were dissolved in Tris buffer (200 mM; pH 7.4), in the case of APN and in Tris buffer (100 mM, pH 7.8) for DPPIV. The enzymes (APN: 3.75 μL for 0.23 U and 11.25 μL for 0.68; DPPIV (7.5 μL for 0.071 U and 22.5 μL for 0.21 U) were added to the peptide aliquots and analyzed by analytical HPLC at 15−20 min time intervals, with an isocratic gradient of 28% MeCN for Tat-A(11) and 36% MeCN for Tat-GP(11). The final peptide concentrations were 75 μM.

Cell Culture. HEK 293 cells were maintained in sterile conditions in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained on tissue culture plastic and kept at 37 °C in a humidified atmosphere of 7.5% CO_2. Cells were passaged every 2−3 days. Prior to cell viability assays or cellular uptake studies, cells within a confluent layer were detached using trypsin/EDTA. Cells were then resuspended in FBS-supplemented DMEM and the number of cells was counted using a standard inverted microscope and a cell counting chamber (Fuchs-Rosenthal).

Flow Cytometry. HEK cells were seeded in 24-well plates (Sarstedt, Numbrecht, Germany) one (80 000 cells/well) or two (40 000 cells/well) days prior to the experiment. On the day of the experiment, cells were incubated with the peptide solutions (5 μM) for 90 min at 37 °C in RPMI + 10% FBS. For the cellular uptake experiments with in situ activation using APN or DPPIV, the peptides were coincubated with APN (3.75 μL, 0.23 U for low enzyme concentrations or 11.25 μL, 0.68 U for high enzyme concentrations) or DPPIV (7.5 μL, 0.071 U for low enzyme concentrations or 22.5 μL, 0.21 U for high enzyme concentrations). After washing the cells with HBS buffer pH 7.4 (10 mM HEPES, 135 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.8 mM $CaCl_2$), cells were detached by trypsinisation for 5 min, spun down, and resuspended in 200 μL RPMI + 10% FCS. The fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium) and subsequently data was analyzed with the Summit software (Fort Collins, U.S.A.). Results were based on 10 000 gated cells.

Confocal Laser Scanning Microscopy. HEK cells were seeded in chambered coverslips (Nunc, Wiesbaden, Germany) at a density of 40 000 cells (1 day) or 20 000 cells per well (2 days prior to the experiment). Fluorescein was excited by an argon laser at 488 nm and emission was collected between 500 and 550 nm. Cells were incubated with the 5 μM peptide for 90 min at 37 °C. For the cellular uptake experiments with in situ activation using APN or DPPIV, the peptides were coincubated with APN (3.75 μL, 0.23 U for low enzyme concentrations or 11.25 μL, 0.68 U for high enzyme concentrations) or DPPIV (7.5 μL, 0.071 U for low enzyme concentrations or 22.5 μL, 0.21 U for high enzyme concentrations). Cells were washed twice after incubation with DMEM + 10% FCS and living cells were analyzed immediately by confocal microscopy using a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an HCX PL APO 63 × N.A. 1.2 water immersion lens. Fluorescein was excited by an argon laser at 488 nm and emission was collected between 500 and 550 nm.

Cell Viability Assay. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies). HEK cells were seeded in 96-well plates to obtain 40 000 cells/well the day of the experiment. On the day of the experiment, cells were incubated with 100 μL mixture of peptide and enzyme in DMEM for 120 min at 37 °C. A peptide concentration of 5 μM was used and this was coincubated with APN (3.75 μL, 0.23 U for low enzyme concentrations or 11.25 μL, 0.68 U for high enzyme concentrations) or DPPIV (7,5 μL, 0.071 U for low enzyme concentrations or 22.5 μL, 0.21 U for high enzyme concentrations). Cells were washed 3 times with DMEM + 10% FCS and maintained in this solution after the last washing step. After 4 h of incubation, the supernatant was removed and a solution of CCK-8 (10% in DMEM + 10% FCS) was added. After 3 h of incubation at 37 °C, the absorbance was measured at 490 nm using a microplate reader (Wallac Victor 1420 multilabel counter, PerkinElmer). The experiments were performed in triplicate and repeated twice independently. Mean values ± SEM are given in SI Figure S3.1 The same experiment was also performed using a prolonged incubation time of the peptides and enzymes of 6 h. After this incubation period, the cells were washed DMEM + 10% FCS and after the last washing step the solution of CCK-8 was added. After 3 h of incubation at 37 °C, the absorbance was measured at 490 nm using a microplate reader (Wallac Victor 1420 multilabel counter, PerkinElmer). The experiments were performed in triplicate and repeated twice independently. Mean values ± SEM are given in SI Figure S3.2.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional characterization (HPLC, Mass analysis) of the peptides, cell viability data, additional confocal microscopy images and analysis of reactivated Tat peptides at low enzyme concentration can be found in the electronic Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00066.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: d.lowik@science.ru.nl.

Author Contributions

‡S.A.B. and M.B.H. contributed equally.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

APN, amino peptidase N; CCK-8, Cell Counting Kit 8; CPP, cell-penetrating peptide; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DIPCIDI, diisopropylcarbodiimide; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N,N*-dimethylformamide; DPPIV, dipeptidyl peptidase IV; Et_2O, diethyl ether; FBS, Fetal Bovine Serum; FCS, Fetal Calf Serum; Fmoc, 9-fluorenylmethoxycarbonyl; HEK, Human Embryonic Kidney; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HOBt, *N*-hydroxybenzotriazole; *i*-PrOH, 2-Propanol; MeCN, acetonitrile; MeOH, methanol; Mtt, 4-methyltrityl; PBS, Phosphate Buffered Saline; RPMI, Roswell Park Memorial Institute medium; Tat, transactivator of transcription; TFA, trifluoroacetic acid; TIS, triisopropylsilane

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