Interaction of Arginine-Rich Peptides with Membrane-Associated Proteoglycans Is Crucial for Induction of Actin Organization and Macropinocytosis†

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Received June 26, 2006; Revised Manuscript Received September 20, 2006

ABSTRACT: Arginine-rich peptides, including octaarginine (R8), HIV-1 Tat, and branched-chain arginine-rich peptides, belong to one of the major classes of cell-permeable peptides which deliver various proteins and macromolecules to cells. The importance of the endocytic pathways has recently been demonstrated in the cellular uptake of these peptides. We have previously shown that macropinocytosis is one of the major pathways for cellular uptake and that organization of the F-actin accompanies this process. In this study, using proteoglycan-deficient CHO cells, we have demonstrated that the membrane-associated proteoglycans are indispensable for the induction of the actin organization and the macropinocytic uptake of the arginine-rich peptides. We have also demonstrated that the cellular uptake of the Tat peptide is highly dependent on heparan sulfate proteoglycan (HSPG), whereas the R8 peptide uptake is less dependent on HSPG. This suggests that the structure of the peptides may determine the specificity for HSPG, and that HSPG is not the sole receptor for macropinocytosis. Comparison of the HSPG specificity of the branched-chain arginine-rich peptides in cellular uptake has suggested that the charge density of the peptides may determine the specificity. The activation of the Rac protein and organization of the actin were observed within a few minutes after the peptide treatment. These data strongly suggest the possibility that the interaction of the arginine-rich peptides with the membrane-associated proteoglycans quickly activates the intracellular signals and induces actin organization and macropinocytotis.

Short basic peptide segments derived from HIV-1[1] Tat and Antennapedia homeodomain proteins translocate through cell membranes (*1*−*5*). These peptide segments bind to proteins resulting in the successful uptake of these exogenous molecules into cells, and the cellular responses (*3*, *5*). This suggests the possibility of the pharmaceutical and therapeutic application of this methodology. This efficient internalization pathway has been gaining substantial attraction for the rational design of highly efficient and effective delivery systems. Various arginine-rich peptides or guanidinium-rich derivatives have been shown to have a similar ability (*6*−*11*).

Endocytosis is one of the major pathways for the cellular uptake of proteins. However, the internalization of basic peptides has previously been shown to employ a system other than endocytosis. Endocytosis is an energy dependent pathway and usually suppressed at a low temperature. A highly efficient internalization of basic peptides, comparable to that at 37 °C, was observed even when the cells were treated with the peptides at 4 °C. Microscopic observation of a diffuse cytosolic distribution without punctate, endosome-like compartments and nuclear accumulation of the fluorescently labeled peptides also suggested this hypothesis. It has recently been reported that the fixation previously employed for the microscopic analysis of the peptide internalization can yield a considerable artifact (*12*). Using live cells without fixation has revealed the presence of endosome-like punctate structures in the cytosol, and a significant decrease in the cellular uptake efficiency at 4 °C. These facts strongly suggest active endocytosis of the arginine-rich peptides and their conjugates (*12*).

Macropinocytosis is one of the major pathways for the endocytic uptake of the arginine-rich peptides as well as their

† This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. T.T. is grateful for the JSPS Research Fellowship for Young Scientists.

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; HSPG, heparan sulfate proteoglycan; GAG, glycosaminoglycan; BSA, bovine serum albumin; GST-CRIB, Cdc42/Rac interactive binding motif protein tagged with glutathione S-transferase; CHO, Chinese hamster ovary; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle medium; α-MEM, α-minimum essential medium; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; Fmoc, 9-fluorenylmethyloxycarbonyl; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high-performance liquid chromatography; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi0612824 CCC: $37.00
Published on Web 12/16/2006

protein conjugate (*13*−*15*). Macropinocytosis is different from clathrin-mediated endocytosis which starts with the formation of concave clathrin-coated pits on the plasma membrane. Macropinocytosis involves an actin-driven membrane protrusion and the subsequent fusion with the plasma membrane that engulfs substantial amounts of extracellular fluid as macropinosomes (*16*−*19*). These processes cause the membrane ruffling. Membrane ruffles can be transiently induced in most cells by treatment with the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (*17*, *18*). We have already observed that these organizations of F-actin were induced in cells treated with oligoarginine peptides (*13*). Thus, we hypothesize that there are potential receptors for the arginine peptides that induce macropinocytosis together with F-actin organization.

It has been reported that membrane-associated proteoglycans, such as heparan sulfate proteoglycan (HSPG), play a crucial role in the cellular uptake of arginine-rich peptides (*20*−*24*) as well as the 86-residue full length HIV-1 Tat protein (*25*−*28*). A deficiency in HSPG often results in a decrease in the amount of these peptides taken up by the cells. HSPG has been suggested as a primary receptor for the cellular uptake of the arginine-rich peptides. However, the relationship between the interaction of the peptides with HSPG and other membrane-associated proteoglycans and the induction of macropinocytosis has not been established. In addition, the expression of HSPG differs in various tissues, including cancer cells, and thus, HSPG has been regarded as a promising target for drug delivery (*29*, *30*). Studies on the relationship of the macropinocytic uptake of arginine-rich peptides with HSPG should provide valuable information on the design of delivery systems using arginine-rich peptides.

In this report, we show that the structures and the charge densities of the arginine-rich peptides, including the branched-chain peptides, determine their cellular uptake dependency on the membrane-associated proteoglycans, especially HSPG. We also demonstrate that the treatment of the cells with arginine-rich peptides leads to the activation of the Rac protein to induce the F-actin organization and lamellipodia. Most importantly, we find that membrane-associated proteoglycans play a crucial role in the induction of the F-actin organization and macropinocytosis, suggesting that the membrane-associated proteoglycans function as potential receptors for the induction of macropinocytosis by the arginine-rich peptides.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Fluorescent Labeling. All of the peptides used were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis on a Rink amide resin as already reported (*13*). The amino acid derivatives and Rink amide resin were purchased from Novabiochem. Deprotection of the peptide and cleavage from the resin were conducted by treatment with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 3 h. Fluorescent labeling of the peptides was conducted by treatment with 1.5 equiv of Alexa 488 C_5 maleimide sodium salt (Molecular Probes) in a dimethylformamide/methanol mixture (1:1) for 1.5 h followed by reversed-phase high-performance liquid chromatography (HPLC) purification. The fidelity of the products was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). MALDI-TOFMS for R8-Alexa, Tat-Alexa, (R2)4-Alexa, (RG3R)4-Alexa, (R1)8-Alexa, R8 (nonlabeled, Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-amide), and Tat (nonlabeled, Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-amide): 2126.2 [expected for $(M + H)^+$ 2125.4]; 2520.7 [expected for $(M + H)^+$ 2520.9]; 2510.4 [expected for $(M + H)^+$ 2509.9]; 3194.2 [expected for $(M + H)^+$ 3194.6]; 3024.6 [expected for $(M + H)^+$ 3023.6], 1267.6 [expected for $(M + H)^+$ 1267.5]; 1718.8 [expected for $(M + H)^+$ 1719.1], respectively.

Cell Culture. Chinese hamster ovary (CHO) cells (CHO-K1 cell lines: wild type; pgsA-745 (A-745) cell lines, all glycosaminoglycan deficient; pgsD-677 (D-677), heparan sulfate deficient) were purchased from the American Type Culture Collection (ATCC) and maintained in F-12 nutrient mixture (Ham's F-12) (Invitrogen) with 10% heat-inactivated fetal bovine serum. The 293T human renal epithelial cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The 293T cells stably overexpressing syndecan-2 (*31*) were maintained in the presence of 0.4 μg/mL puromycin. The human cervical cancer-derived HeLa cells were maintained in α-minimum essential medium (α-MEM) with 10% heat-inactivated calf serum. Cells were grown on 100 mm dishes and incubated at 37 °C under 5% CO_2 to approximately 70% confluence. A subculture was performed every 3−4 days.

Confocal Microscopy of Peptide Internalization. CHO cells (2×10^5) were plated on 35 mm glass-bottomed dishes (Iwaki) and cultured in Ham's F-12 with 10% heat-inactivated fetal bovine serum for 48 h. After complete adhesion, the culture medium was exchanged, and then the cells were incubated at 37 °C with fresh medium (200 μL) containing the fluorescently labeled peptides and washed with phosphate buffered saline (PBS) (×3). Distribution of the fluorescently labeled peptides was then analyzed using a confocal scanning laser microscope FV300 (Olympus) equipped with a 40× objective *without fixing* the cells to avoid artifactual localization of the internalized peptides (*12*, *13*).

Flow Cytometry. CHO cells (1.5×10^5) in fresh culture medium (1 mL) were plated into 24-well microplates (Iwaki) and cultured for 48 h in Ham's F-12 containing 10% heat-inactivated fetal bovine serum. After complete adhesion, the cells were incubated at 37 °C for 30 min with fresh medium (200 μL) containing peptides prior to washing with PBS. The cells were then treated with 0.01% trypsin in PBS (200 μL) at 37 °C for 10 min prior to the addition of PBS (200 μL). The cells were centrifuged at 2000 rpm (400*g*) for 5 min. After the supernate was removed, the cells were washed with 500 μL of PBS and centrifuged at 2000 rpm for 5 min. After this washing cycle was repeated, the cells were suspended in PBS (400 μL) and subjected to fluorescence analysis on a FACScalibur (BD Biosciences) flow cytometer using 488 nm laser excitation and a 515 to 545 nm emission filter. Three additional cell washes in the presence of 4 mg/mL heparin in PBS after the trypsinization did not yield any significant difference in the FACS analysis (data not shown). In addition, we confirmed that the cell surface proteoglycans

were effectively removed by the above trypsin treatment using the mouse IgM monoclonal antibody 10E4 raised against HSPG (Seikagaku) (*31*). CHO-K1 cells (1.5×10^5) were treated with 0.01% trypsin in PBS (200 μL) at 37 °C for 10 min prior to two washings with PBS. The cells were treated with the above anti-HSPG antibody (1 mg/mL, 2 μL) in PBS (200 μL) at 4 °C for 30 min prior to two washings with PBS. The cells were then incubated with the Alexa488-labeled secondary antibody against the mouse IgM μ chain (Alexa Fluor 488 goat anti-mouse IgM μ chain) (Molecular Probes) (2 mg/mL, 4 μL) in PBS (200 μL) at 4 °C for 30 min prior to three washings with PBS and then subjected to a FACS analysis. As a control, the cells were detached from the dishes by a treatment with 2 mM EDTA at 37 °C for 5 min instead of trypsin, and similarly analyzed as already stated. The amount of cellular HSPG after the above trypsin treatment was less than 5% in the EDTA-treated cells (Supporting Information Figure S1). We also confirmed that the fluorescence from the cells treated at 4 °C was as low as the autofluorescence level (see Supporting Information Figure S2). These data suggest that the surface adsorbed peptides are effectively removed by the above trypsin treatment. Analysis of the cellular uptake by 293T and the syndecan-overexpressing cells was similarly conducted using DMEM containing 10% heat-inactivated fetal bovine serum as the medium. For examination of the effect of the macropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (*13*, *15*−*17*) on the cellular uptake of the peptides, the cells were incubated in serum-free Ham's F-12 medium for 4 h prior to the pretreatment with EIPA (100 μM) in the medium at 37 °C for 10 min. The cells were then treated with the peptide (final concentration, 10 μM) in the presence of 100 μM EIPA for 30 min and analyzed by FACS as already described.

Immunoblotting of Rac1 Activation. CHO-K1 cells were seeded in 60 mm culture dishes at a density of 8×10^5 cells/dish, cultured for 24 h, and serum-starved in serum-free medium for 24 h. The cells were then stimulated with the R8 and Tat peptides (without fluorescent moieties) (10 μM), and EGF (400 ng/mL) for 5 min at 37 °C and lysed for 5 min with the ice-cold cell lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) containing 5 μg of the Cdc42/Rac interactive binding motif protein tagged with the glutathione S-transferase (GST-CRIB) (*32*). Cell lysates were then centrifuged for 5 min at 13400*g* at 4 °C, and the supernate was incubated with glutathione-Sepharose beads for 30 min at 4 °C. After the beads were washed with the cell lysis buffer, the bound proteins were eluted in the Laemmli sample buffer and separated by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 3% low fat milk in Tris-buffered saline and then incubated with a mouse monoclonal anti-Rac1 antibody (Transduction Laboratories) (1:1000 dilution). The Rac1 antibody was detected using a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Dako) and the ECL detection kit (Amersham Biosciences). A densitometry analysis was performed using NIH Image software, and the amount of GST-CRIB-bound Rac1 was normalized to the total amounts of Rac1 in the cell lysates. In the case of the HeLa cells, these cells were seeded in 60 mm culture dishes at a density of 6×10^5 cells/dish, cultured for 48 h, serum-starved in serum-free medium containing 1% bovine serum albumin (BSA) for 24 h, and then similarly treated with peptides.

Cell Ruffling Assay. The CHO cells were seeded into eight well Lab-Tek-II chamber slides (Nalge Nunc) at a density of 2.4×10^4 cells per well in Ham's F-12 containing 10% heat-inactivated calf serum for 48 h. The medium was replaced by fresh medium lacking serum, and the cells were incubated for 18 h. After two washings with PBS, the cells were treated with the R8 and Tat peptides (without fluorescent moieties) (10 μM) in Ham's F-12 for 2.5 min. The cells were then washed three times with PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, treated with 0.5% Triton X-100 in PBS at room temperature for 2 min, and then washed again with PBS. The cellular F-actin was stained with phalloidin-TRITC as already described (*13*) and observed using a confocal scanning laser FV300 microscope (Olympus) equipped with a 40× objective.

RESULTS

Effects of Membrane-Associated Proteoglycans on the Cellular Uptake of Arginine-Rich Peptides. HSPG has been reported to be important in the internalization of the arginine-rich peptides (*20*−*24*) as well as the full length Tat protein (86 residues) (*25*−*28*). Various arginine-rich peptides, including the linear and the branched-chain oligoarginine peptides, also translocate through biological membranes. However, detailed studies on the effect of membrane-associated proteoglycans for their cellular uptake of these oligoarginine peptides have not been reported. We have now studied octaarginine (R8) as a representative oligoarginine peptide using the wild-type Chinese hamster ovary CHO cell line, CHO-K1, and two deficient mutants in the proteoglycan biosynthesis, D-677 and A-745. D-677 has a single mutation that affects both the *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities necessary for the polymerization of the disaccharide chains of HSPG, and lacks HSPG. This mutant cell line produces approximately three times more chondroitin sulfate proteoglycan than the wild-type cells. The A-745 cell line lacks xylosyltransferase, an enzyme necessary for the initiation of the glycosaminoglycan (GAG) synthesis, and does not produce detectable levels of any proteoglycans (*26*). These cells were treated with Alexa488-labeled R8 and Tat peptides (Figure 1A) (10 μM each) at 37 °C for 30 min in Ham's F-12 containing 10% serum, washed, and trypsinized. The amount of the respective peptides taken up by these cells was then analyzed by FACS. The majority of the cell-surface adsorbed peptides are removed by trypsin treatment, and thus our data reflect the total cellular uptake of the peptides (the sum of endosome-trapped and cytosol-released peptides) (see Experimental Procedures). The amount of both peptides taken up by the cells was considerably lower in the A-745 cells (10−20%) compared with that in the CHO-K1 cells for these peptides (Figure 1B). In contrast to the uptake of the R8 peptide in the HSPG-deficient D-677 cells (76%), a significant decrease in the uptake was observed for the Tat peptide in the D-677 cells (34%), suggesting that the cellular uptake of the Tat peptides is highly dependent on HSPG. Similar observations

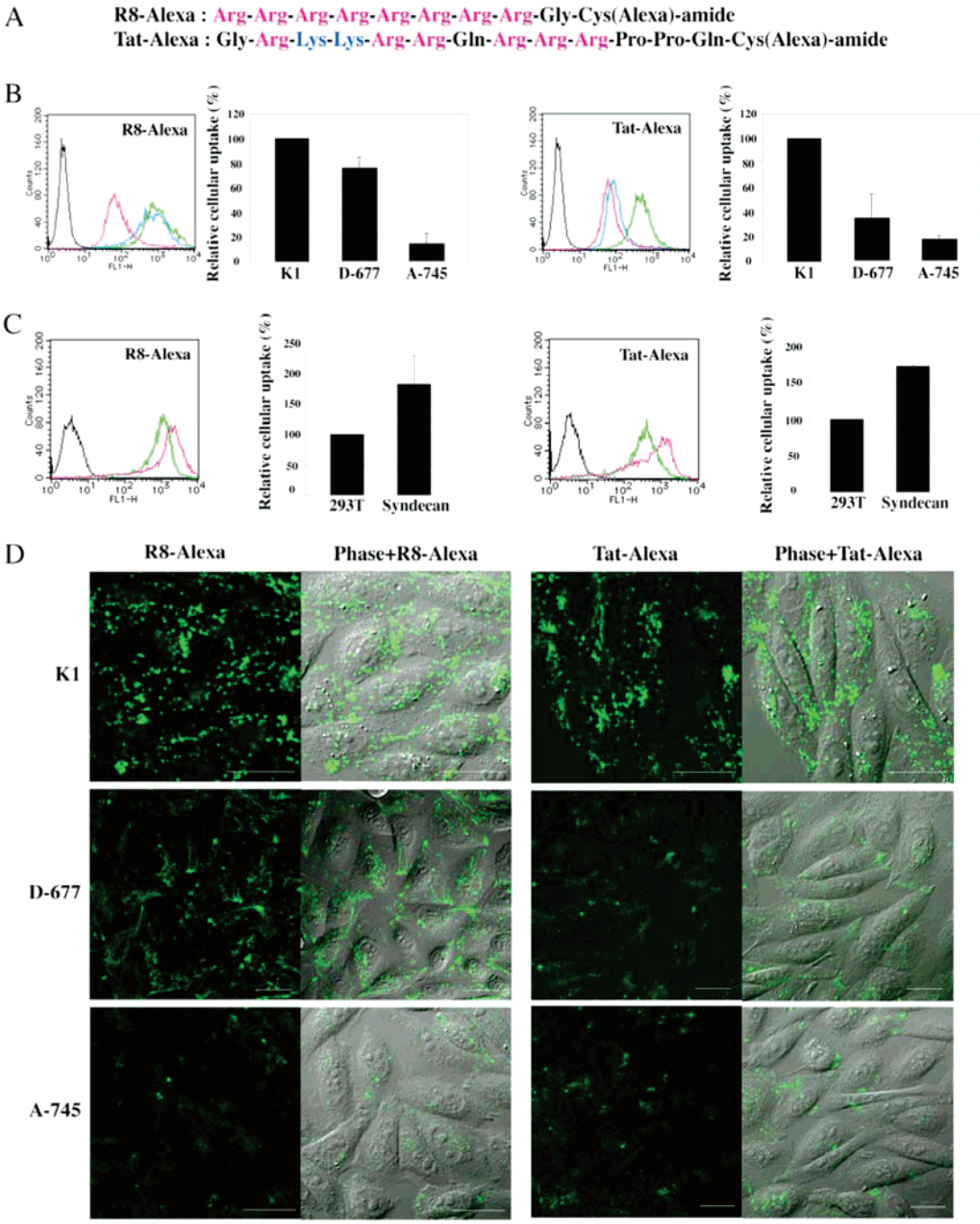

FIGURE 1: In contrast to the R8 peptide, the internalization of the Tat peptide is HSPG dependent. (A) Sequences of Alexa488-labeled R8 and Tat peptides. (B) FACS analysis of the proteoglycan-deficient cells treated with the Alexa488-labeled peptides (10 μM) at 37 °C for 30 min: green, CHO-K1 cells; red, A-745 cells; blue, D-677 cells. The amounts of the respective peptides taken up by these cells were normalized to the CHO-K1 cells as a standard. Error bars represent the mean ± standard deviation of 5 samples. (C) FACS analysis of the syndecan-2 overexpressing cells treated with Alexa488-labeled R8 and Tat peptides (10 μM) at 37 °C for 30 min: green, 293T cells; red, 293T-syndecan 2 cells. The results were normalized as the cellular uptake of the respective peptide by 293T cells as a standard. Error bars represent the mean ± standard deviation of 3 samples. (D) Confocal microscopy analysis of living CHO cells incubated with the Alexa488-labeled peptides (10 μM) at 37 °C for 30 min. Scale bar, 20 μm.

were previously observed in studies employing the fluorescein-labeled Tat peptide (*24*). On the other hand, the cellular uptake of the R8 peptide is not very highly dependent on HSPG as the Tat peptide. This suggests that the R8 peptide can be taken up by the cells by an alternative mechanism and/or via other proteoglycans.

Syndecan is one of the major classes of membrane-associated HSPGs. We next examined whether an increased cellular uptake of R8 and Tat is observed in 293T cells overexpressing syndecan-2 (*31*). The wild-type 293T cells and the mutant cell line overexpressing syndecan were treated with peptides (10 μM) for 30 min. Increases of 76% and 72% were observed for the uptake of the R8 and Tat peptides by the syndecan-2 overexpressing cells (Figure 1C), further supporting the importance of proteoglycan for the cellular uptake of the arginine-rich peptides.

Using confocal microscopy, we also studied the peptide internalization in a live control and mutant cells (Figure 1D). Since fixation was shown to cause significant artifacts on the cellular localization of peptides (*12*), and distribution of

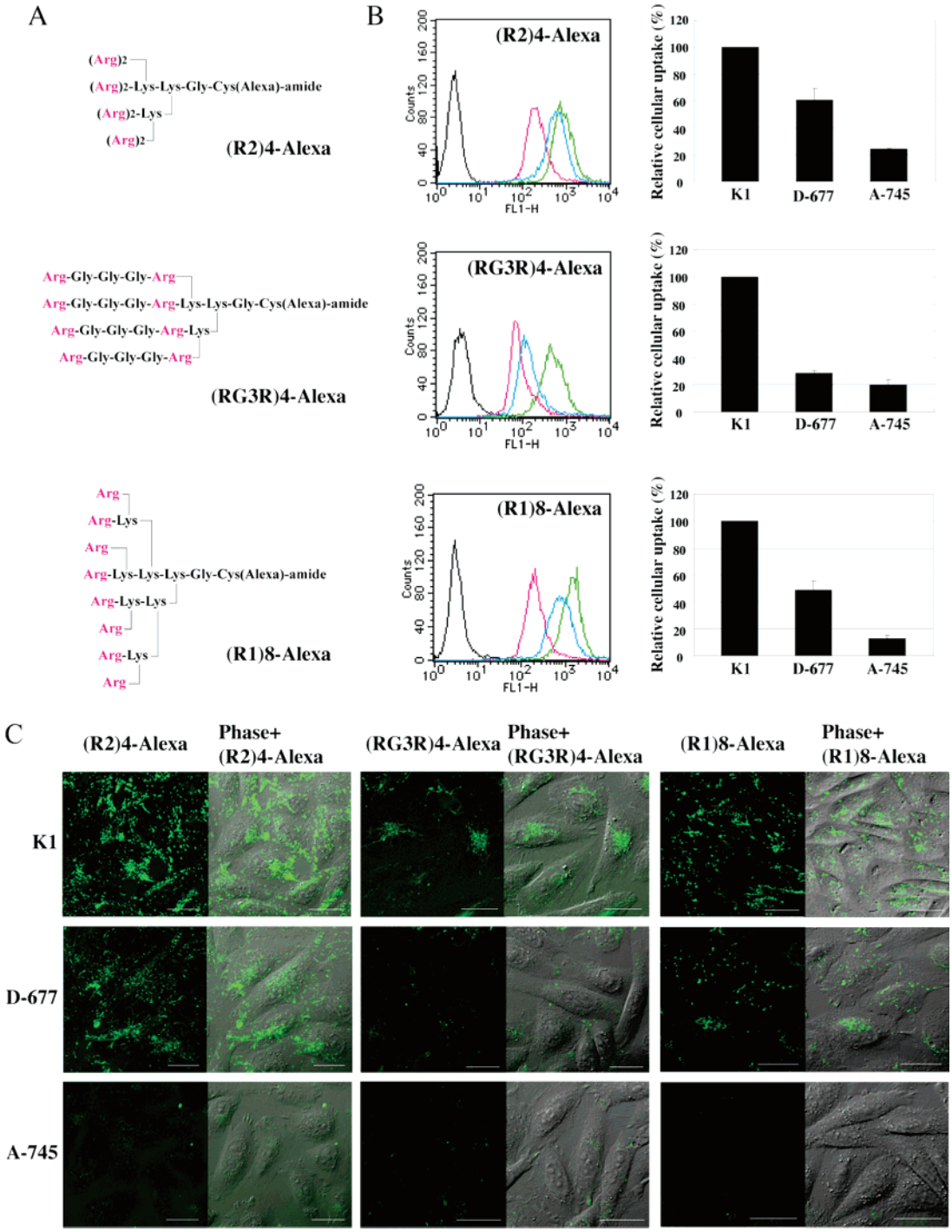

FIGURE 2: Comparison of branched-chain arginine-rich peptide uptake and their dependency on HSPG. (A) Structures of branched-chain arginine-rich peptides. (B) FACS analysis of the cells treated with (R2)4-Alexa, (RG3R)4-Alexa, and (R1)8-Alexa peptides (10 μM each) at 37 °C for 30 min: green, K1 cells; red, A-745 cells; blue, D-677 cells. The results were normalized as the cellular uptake of the respective peptides by CHO-K1 cells as a standard. Error bars represent the mean ± standard deviation of 3 samples. (C) Confocal microscopy analysis of living CHO cells incubated with branched-chain arginine-rich peptides (10 μM) at 37 °C for 30 min. Scale bar, 20 μm.

the fluorescently labeled peptides was then analyzed without fixing the cells. The punctate signals observed in the CHO-K1 cells suggest endosomal uptake of the peptides by the cells. On the other hand, a significant decreased amount of these punctate signals was observed in the A-745 cells. In the case of D-677 cells, punctate signals were predominantly observed only in the R8-treated cells. Significant increases in the amount of the punctate signals were also observed in the syndecan-2 overexpressing 293T cells compared with that in the wild-type 293T cells, supporting the results of the FACS analysis (data not shown). We concluded that proteoglycans mediate the endosomal peptide uptake that is peptide and proteoglycan type dependent.

We have previously reported that not only linear arginine-rich peptides but also the branched-chain peptides are internalized into cells, and that a cluster of arginines may be critical for their internalization (*7*). We also examined the role of the proteoglycans on the internalization of the branched-chain peptides. The Alexa488-labeled (R2)4, (RG3R)4, and (R1)8 peptides (Figure 2A) were selected as

examples of the branched-chain arginine-rich peptides. A significant decrease in the amount of the peptide taken up by the cells was observed in A-745 cells for all the peptides (Figure 2B). The cellular uptake by the A-745 cells of these peptides was less than 25% of that observed in CHO-K1 cells, confirming that proteoglycans play a crucial role in this process. The relative amount of the (RG3R)4 peptide taken up by the D-677 cells (28%) was considerably lower than that by the wild-type CHO-K1 cells and comparable to that observed in the A-745 cells (20%). On the other hand, the decrease in cellular uptake of the (R2)4 peptide by D-677 cells was much lower. A small but consistent decrease in the cellular uptake was also observed for the (R1)8 peptide compared to the other peptides. Therefore, the dependency on HSPG for cellular uptake of these peptides can be assigned (RG3R)4 > (R1)8 > (R2)4. These results suggest that the Tat and (RG3R)4 peptides can be internalized via similar mechanisms, whereas the uptake of (R1)8 and (R2)4 is internalized via a different mechanism, more akin to that observed for R8.

Similar results were observed in the confocal microscopic observations (Figure 2C). Punctate signals from the cells treated with these branched-chain peptides show endocytic cellular uptake of these peptides. The above results suggested that the cell-surface proteoglycans highly contribute to the cellular uptake of arginine-rich peptides and that the peptide structures may influence the interaction with HSPG.

Activation of Rac1 by Arginine-Rich Peptides. Macropinocytosis has been suggested in the cellular uptake of arginine peptides (*13*−*15*). We also reported that the organization of F-actin, which accompanies cellular uptake by macropinocytosis, is induced in HeLa cells by the arginine peptides (*13*). Rac has been reported to be involved in the F-actin organization, especially during the induction of lamellipodia (*33*, *34*). Rac is a member of the small molecular weight G protein Rho family and becomes active in its GTP-bound state (Rac-GTP) and inactive in its GDP-bound state (Rac-GDP). In the active state, Rac-GTP binds to various effector proteins by interacting with target motifs, such as the Cdc42/Rac interactive binding (CRIB) (*35*, *36*). We examined whether Rac1, a typical Rac protein, is activated by the arginine peptides. CHO-K1 cells were treated with the R8 or Tat peptide (10 μM) for 5 min. The cell lysates were then incubated with the CRIB motif protein which is tagged with GST (GST-CRIB) (*32*). Because the activated Rac1 (Rac1-GTP) can form a complex with GST-CRIB, this complex was isolated using a glutathione immobilized column, and the amount of the Rac1-GTP was analyzed by SDS−PAGE (Figure 3). The Rac1-GTP level increased 4.8- and 6.5-fold after treatment with the R8 and Tat peptides (10 μM), respectively. A 4.5-fold increase was obtained by treatment with the positive control, EGF (400 ng/mL). Therefore, the peptide treatment of the cells actually induces a significant activation of the Rac1 protein. Similarly, induction of Rac1 was also observed in HeLa cells by the treatment with these peptides; the Rac1-GTP level after treatment with the R8 and Tat peptides (10 μM) and EGF (400 ng/mL) was 3.1-, 6.5-, and 10.3-fold that of the control cells, respectively.

Effects of Proteoglycans on the Induction of Macropinocytosis and Actin Organization by Arginine-Rich Peptides. We next examined whether proteoglycans influence the induction of macropinocytosis. We examined the effects of 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) on the cellular uptake of the arginine-rich peptides using CHO-K1, D-677, and A-745 cells (Figure 4). EIPA has been reported as a potent inhibitor of macropinocytosis (*16*, *17*). EIPA is known not to affect the clathrin-mediated endocytic uptake of transferrin, but it effectively inhibits the macropinocytic uptake of dextran stimulated by the epidermal growth factor (EGF) or adenovirus (*17*). Cells were pretreated with EIPA (100 μM) at 37 °C for 10 min in a serum-free medium, and then treated with the respective peptides (10 μM each) in the continued presence of 100 μM EIPA for 30 min. After trypsinization, the amount of the internalized peptide was quantified by FACS analysis. In this experiment, the fluorescent intensity from the cells treated with the respective peptides was analyzed without normalization to gain a better understanding of the quantitative differences in the total cellular uptake of the respective peptides.

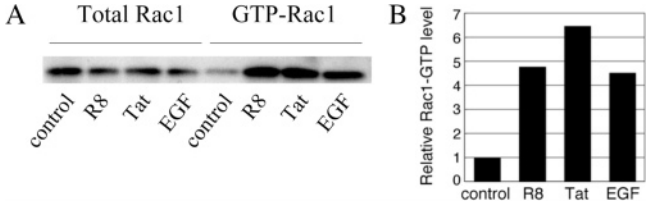

FIGURE 3: Activation of Rac1 by arginine-rich peptides. CHO-K1 cells were treated with the R8 peptide (10 μM), Tat peptide (10 μM), and EGF (400 ng/mL) for 5 min at 37 °C. The cell lysates were incubated with GST-CRIB, and the amounts of the GTP-bound Rac1 were determined by immunoblotting using a monoclonal anti-Rac1 antibody (A). Relative Rac1 activity was determined by the amount of GTP-bound Rac1 that was normalized to the amount of the total Rac1 in the cell lysates. The data were analyzed by NIH Image software, and the value from the control cells was arbitrarily set to 1. The average densitometric scan data of three samples are shown in B.

A 56% decrease in the R8 peptide uptake by the wild-type CHO-K1 cells was observed in the presence of EIPA (Figure 4), suggesting that macropinocytosis contributes to the cellular uptake. In the case of the HSPG-deficient the D-677 cells, cellular uptake of the peptide in the absence and presence of EIPA was 78% and 17% of that in the CHO K-1 cells incubated with the peptide in the absence of EIPA. This suggests the possible involvement of macropinocytosis for the cellular uptake of the R8 peptide, even in the absence of HSPG. Our data suggest that HSPG may not be the only proteoglycan that contributes to the induction of macropinocytosis. The total cellular uptake of the R8 peptide by the proteoglycan-deficient A-745 cells was only 23% of that by the CHO K-1 cells incubated with the peptide in the absence of EIPA; there was only a minimal EIPA effect on the peptide uptake. This suggests that macropinocytosis may not be induced by the peptide in the absence of proteoglycans.

In the absence of EIPA, the cellular uptake of the Tat peptide is highly dependent on HSPG, and the quantities of the internalized peptide in the HSPG-deficient D-677 cells is almost as low as that in the proteoglycan-deficient A-745 cells (Figure 4). Macropinocytosis may also be important for the Tat uptake (Figure 4). Treatment of the wild-type CHO-K1 cells with the EIPA produced a 40% decrease in the cellular uptake of the Tat peptide. However, identical treatment of the A-745 cells revealed no significant differences in the Tat uptake in either the presence or absence of

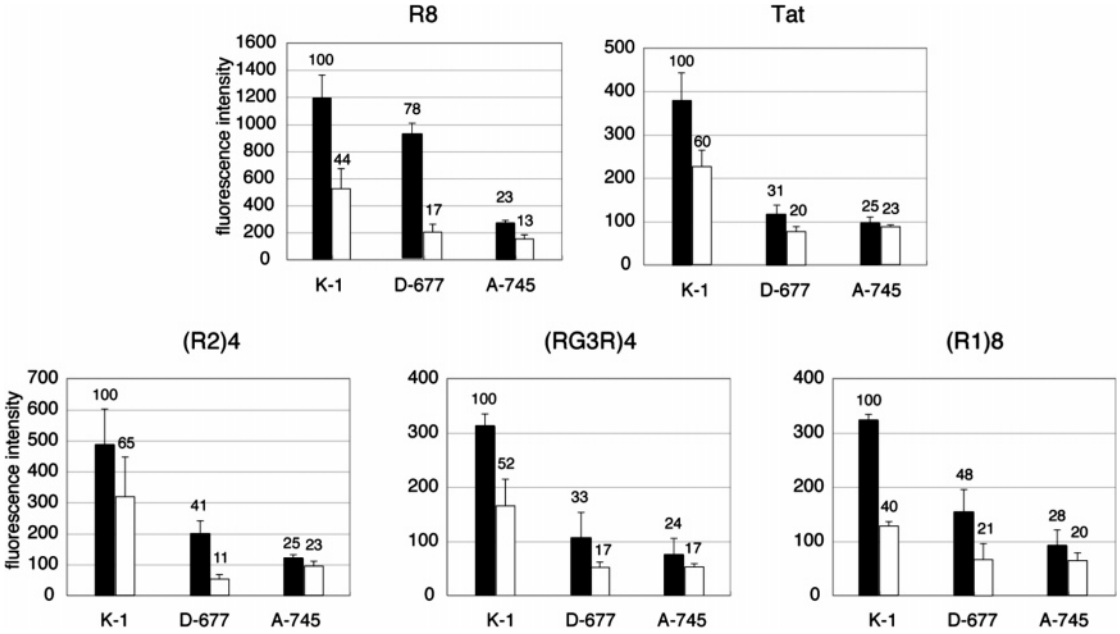

FIGURE 4: Effect of macropinocytosis inhibitor EIPA on cellular uptake of the arginine-rich peptides. The effect of the macropinocytosis inhibitor, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), on the cellular uptake of the arginine-rich peptides was examined by FACS analysis. CHO cells (K1, D677, and A745) were initially pretreated with EIPA (100 μM) at 37 °C for 10 min in a serum-free medium. The cells were then incubated with the medium containing the Alexa488-labeled peptides (final concentration, 10 μM) in the presence of 100 μM EIPA for 30 min, prior to the trypsinization and FACS analysis. Fluorescence intensity (arbitrary unit) corresponds to the cellular uptake of the peptides in the absence (closed columns) and presence (open columns) of EIPA. Cellular uptake of the respective peptides under the given conditions is also shown as a percentage of the values observed in the untreated CHO-K1 cells. Error bars represent the mean ± standard deviation of 3−4 samples.

EIPA. Only minimal effects were observed by EIPA treatment in the D-677 cells. The lower uptake of the Tat peptide by the D-677 cells compared with lower effects on the R8 peptide uptake, together with the effect of EIPA on the D-677 cells, suggests that HSPG is important for the macropinocytic uptake of the Tat peptide.

We have demonstrated that the HSPG dependency for the peptide uptake of the branched-chain peptides is in the order (RG3R)4 > (R1)8 > (R2)4; the method of internalization of (RG3R)4 seems closer to that of Tat whereas (R2)4 is more comparable to R8 (Figure 2B). In addition, the R8 and (R2)4 are more efficiently internalized, especially by the CHO K-1 and D-677 cells, than the other peptides (Figure 4). The effect of EIPA on the cellular uptake of these branched peptides follows the same pattern. During the uptake of (R2)4 peptide by CHO-K1 cells, the EIPA treatment resulted in a 35% decrease in the cellular uptake of the peptide compared with cells incubated in the absence of this drug (Figure 4). An identical EIPA treatment of the D-677 cells inhibited the peptide uptake by 73% compared to the untreated cells; however, EIPA did not significantly inhibit the peptide uptake in the A-745 cells. Alternatively, though a significant decrease was observed for the uptake of the (RG3R)4 peptide by the CHO-K1 cells, the effect of EIPA on the uptake by the D-677 as well as the A-745 cells was not significant. The above results demonstrate that the inhibition effect of EIPA on the cellular uptake of the respective peptides is similar to the amount of their cellular uptake by these proteoglyan-deficient cells. This strongly suggests that the interaction of the peptides with proteoglycans should induce macropinocytosis. In addition, the peptide structures determine the specificity for proteoglycans in the macropinocytic uptake.

Macropinocytosis accompanies organization of the F-actin (*13*). We then examined whether the organization is observed in these proteoglycan-deficient CHO cells after incubations in R8 and Tat. The CHO-K1, D-677, and A-745 cells were treated with the respective peptides (10 μM), stained with the F-actin specific phalloidin-TRITC, and analyzed by confocal microscopy (Figure 5). Lamellipodia are thin, veil-like extensions at the edge of cells that contain a dynamic array of actin filaments. Although a very small amount of the lamellipodia-like structure was observed in the cells without the peptide treatment, a significant organization of F-actin, including lamellipodia-like structures (indicated by arrows in Figure 5), was observed in the CHO-K1 cells as early as 2.5 min after the treatment with these peptides. In contrast, neither a significant organization of the F-actin nor induction of the lamellipodia was observed for the A-745 cells. In the case of the D-677 cells, a significant induction of lamellipodia-like structures was observed by the R8 treatment, whereas a smaller degree of induction was observed in the Tat-treated cells (Figure 5). These results strongly suggest that cell-surface proteoglycans have an important biological role in the induction of macropinocytosis by the arginine-rich peptides. In addition, the lamellipodia formation in the HSPG-deficient D-677 cells supports the assumption that HSPG would not be a specific primary receptor for the induction of macropinocytosis by these peptides.

DISCUSSION

We have previously demonstrated that the cellular uptake of arginine-rich peptides, including the R8 and HIV-1 Tat peptides, is dependent on the cell-surface proteoglycans (*20*). We have also demonstrated that significant amounts of the

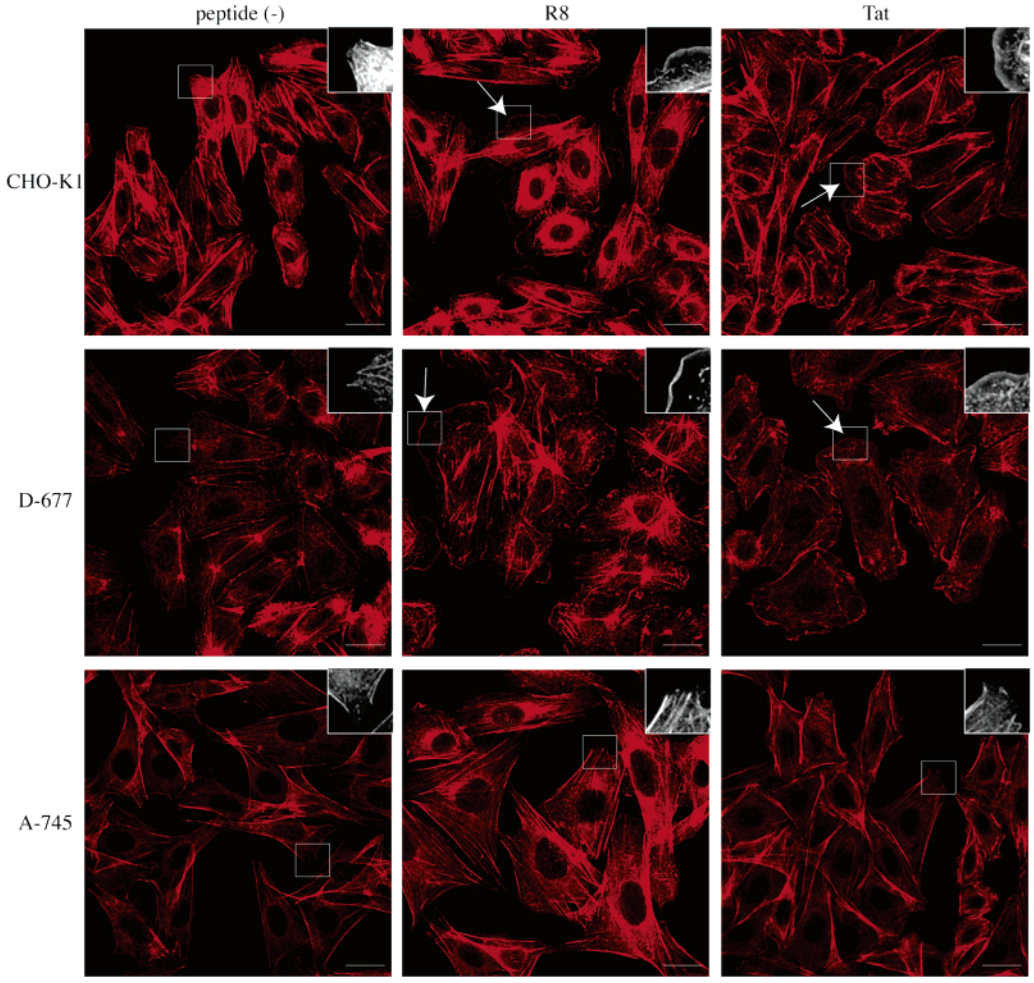

FIGURE 5: Absence of lamellipodia formation by the treatment of the R8 and Tat peptides in glycosaminoglycan deficient A745 cells. The CHO-K1, D-677, and A-475 cells were incubated in serum-free conditions for 18 h. After washing the cells twice with PBS, the cells were treated with the respective peptides (10 μM) in Ham's F-12 medium lacking serum for 2.5 min at 37 °C. After fixation of the cells using 4% paraformamide, the cells were stained with Phalloidin-TRITC. The arrows indicate the induced lamellipodia. Insets show images at $\times 2$ magnification of boxed regions. Scale bar, 20 μm.

peptides are taken up by macropinocytosis (*13*). However, the relationship between the proteoglycans and the induction of macropinocytosis has not been elucidated. In this paper, we demonstrated that the cell-surface proteoglycans are required for the induction of macropinocytosis as demonstrated with the proteoglycan-deficient A-745 cells. A few minutes after treatment of the cells with arginine-rich peptides, activation of the Rac protein and organization of the actin were observed. This suggests that a quick cellular response is induced by these peptides.

HSPG has been suggested as the cellular receptor of the Tat peptide as well as for the full-length Tat protein (86 residues) (*20*−*28*). However, we have demonstrated here that the structures of the arginine-rich peptides determine this dependency. The branched-chain (RG3R)4 peptide also shows a high specificity for HSPG. Although proteoglycans play a crucial role in the cellular uptake of the R8, (R2)4, and (R1)8 peptides, uptake of these peptides appears less dependent on HSPG. The precise reason to explain these differences in HSPG specificity is not clear at this stage, but one possible reason could be the differences in the charge density. The former group contains other amino acids intervening between the arginine residues, whereas the latter consists of only arginines. It has been reported that the sulfuric acid content of HSPG is higher than other membrane-associated proteoglycans, such as chondroitin sulfate (*37*). The Tat and (RG3R)4 peptides, which have a smaller charge density than R8, can interact with HSPG, but may fail to interact with proteoglycans having a smaller negative charge density. The peptides in the latter group have a higher positive charge density and thus may be able to interact with other proteoglycans than HSPG to initiate cellular uptake. An alternative explanation, but maybe less plausible, is that the Tat and (RG3R)4 have conformations that promote the interaction with HSPG. A further study is necessary to clarify this mechanism. However, the above results strongly suggest that HSPG is not the only receptor to induce macropinocytic uptake of R8 and other oligoarginine peptides. Lamellipodia formation and induction of macropinocytosis in D-677 cells by the R8 peptide treatment even in the absence of HSPG further support this idea of their receptors.

In this study, we have shown that the Rac protein, which induces membrane ruffles that are enriched with actin filaments around the boundary of cells, was actually activated as early as 5 min after the treatment of the cells with the Tat and R8 peptides. This correlates with the lamellipodia formation observed 2.5 min after the peptide treatment. These results indicate that certain rapid cellular signals are transmitted by the interaction of these peptides with the cells to induce actin organization and macropinocytosis.

How can the peptide signals be transmitted to Rac to induce the eventual actin organization and macropinocytosis? It is known that an interaction with HSPG is critical for activation of certain receptors by growth factors, such as the fibroblast growth factor (FGF) (*38*). One possible idea is that the arginine-rich peptides may activate the receptors of bioactive proteins, including growth factors such as FGF, in collaboration with proteoglycans. These peptides likely do not have a specificity for these receptors, since there are no consensus sequences among the arginine-rich peptides except that they have clusters of arginines or guanidinium moieties in their molecules. It has been suggested that the full-length Tat protein (86 residues) could activate a vascular endothelial growth factor (VEGF) receptor (*39*), although the Tat peptide is not a high-affinity ligand for the receptor (*40*). However, there remains the possibility that these arginine peptides activate a receptor in the concentration ranges of nonspecific interactions. A 10 μM R8 peptide and 10 nM EGF induce a similar actin organization, in which there is a 3-order of concentration difference (*13*). In addition, there is a report on the induction of actin organization by the full length Tat protein at the concentration of ~5 nM (*41*). However, the Tat and R8 peptides do not influence actin at this concentration (data not shown). It seems likely that domains other than the basic domain in the Tat protein would contribute to the specificity or give a high binding affinity. This also suggests that the biological effects induced by the Tat and other arginine-rich peptides are not necessarily identical to those of the full-length Tat protein.

As an alternative possibility, arginine-rich peptides may compete with the binding of the bioactive proteins by proteoglycans. This competition may lead to a release of the matrix-trapped proteins that interact with their specific receptors. It has also been proposed that the interaction of extracellular ligands with syndecan leads the multimerization of syndecan to induce actin polymerization (*42*), and arginine-rich peptides might have a similar effect on the signal transduction via syndecan multimerization.

Interestingly, lamellipodia formation was also observed in the Tat-treated D-677 cells whose cellular uptake of these peptides is considerably lower than that observed in the CHO-K1 cells. Further studies are necessary to determine why this is the case. We can, however, hypothesize that the cellular uptake of the arginine-rich peptides would be determined by the balance between the extent of their ability to induce macropinocytosis and their affinity to the plasma membrane. Assuming that the Tat peptide can still bind proteoglycans other than HSPG even with a lower affinity, the higher ability of Tat, compared to R8, to activate Rac1 as shown in this report may yield lamellipodia formation to some extent. However, this may not be reflected in the cellular uptake level due to its lower affinity to D-677 cells. A similar idea would be applicable to analyze the cellular uptake by the wild-type CHO-K1 cells. The Tat peptide can activate the Rac1 more effectively than the R8 peptide whereas the more positively charged R8 peptide would yield a significantly higher cellular uptake than the Tat peptide. Therefore, not only the ability to induce macropinocytosis but also their affinity to the plasma membrane should be taken into account in order to consider the efficacy of the peptides for cellular uptake.

In conclusion, this report highlights the possibility that the binding of arginine-rich peptides with the cell surface proteoglycans activates intracellular signals that lead to the actin organization and macropinocytosis. This binding step is required for the induction of the macropinocytic uptake of the peptides. EIPA is an inhibitor of the Na^+/H^+ antiporter required for macropinocytosis, and further work is necessary for the effect of the disruption of ionic equilibrium operated by EIPA on the internalization of the peptides. However, the results obtained in this study provide novel insights into the internalization mechanisms of the arginine-rich peptides and are useful for the design and development of more sophisticated delivery systems using peptide vectors.

SUPPORTING INFORMATION AVAILABLE

HPLC retention times for the synthetic peptides and FACS analysis of the cellular fluorescence/HSPG after trypsin treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994) Tat-mediated delivery of heterologous proteins into cells, *Proc. Natl. Acad. Sci. U.S.A. 91*, 664−668.
2. Vives, E., Brodin, P., and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, *J. Biol. Chem. 272*, 16010−16017.
3. Wadia, J. S., and Dowdy, S. F. (2005) Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer, *Adv. Drug Delivery Rev. 57*, 579−596.
4. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes, *J. Biol. Chem. 269*, 10444−10450.
5. Joliot, A., and Prochiantz, A. (2004) Transduction peptides: from technology to physiology, *Nat. Cell Biol. 6*, 189−196.
6. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery, *J. Biol. Chem. 276*, 5836−5840.
7. Futaki, S., Nakase, I., Suzuki, T., Youjun, Z., and Sugiura, Y. (2002) Translocation of branched-chain arginine peptides through cell membranes: flexibility in the spatial disposition of positive charges in membrane-permeable peptides, *Biochemistry 41*, 7925−7930.
8. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13003−13008.
9. Rothbard, J. B., Kreider, E., VanDeusen, C. L., Wright, L., Wylie, B. L., and Wender, P. A. (2002) Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake, *J. Med. Chem. 45*, 3612−3618.
10. Umezawa, N., Gelman, M. A., Haigis, M. C., Raines, R. T., and Gellman, S. H. (2002) Translocation of a beta-peptide across cell membranes, *J. Am. Chem. Soc. 124*, 368−369.
11. Futaki, S. (2005) Membrane-permeable arginine-rich peptides and the translocation mechanisms, *Adv. Drug Delivery Rev. 57*, 547−558.
12. Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, *J. Biol. Chem. 278*, 585−590.
13. Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J. C., Jones, A. T., Sugiura, Y., and Futaki, S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement, *Mol. Ther. 10*, 1011−1022.

14. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, *Nat. Med. 10*, 310−315.
15. Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis, *J. Controlled Release 102*, 247−253.
16. Swanson, J. A., and Watts, C. (1995) Macropinocytosis. *Trends Cell Biol. 5*, 424−428.
17. Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S., and Greber, U. F. (2002) Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake, *J. Cell Biol. 158*, 1119−1131.
18. Meier, O., and Greber, U. F. (2003) Adenovirus endocytosis, *J. Gene Med. 5*, 451−462.
19. Conner, S. D., and Schmid, S. L. (2003) Regulated portals of entry into the cell, *Nature 422*, 37−44.
20. Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K., and Sugiura, Y. (2002) Possible existence of common internalization mechanisms among arginine-rich peptides, *J. Biol. Chem. 277*, 2437−2443.
21. Console, S., Marty, C., García-Echeverría, C., Schwendener, R., and Ballmer-Hofer, K. (2003) Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans, *J. Biol. Chem. 278*, 35109−35114.
22. Ziegler, A., and Seelig, J. (2004) Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters, *Biophys. J. 86*, 254−263.
23. Fuchs, S. M., and Raines, R. T. (2004) Pathway for polyarginine entry into mammalian cells, *Biochemistry 43*, 2438−2444.
24. Richard, J. P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., and Chernomordik, L. V. (2005) Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors, *J. Biol. Chem. 280*, 15300−15306.
25. Chang, H. C., Samaniego, F., Nair, B. C., Buonaguro, L., and Ensoli, B. (1997) HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region, *AIDS 11*, 1421−1431.
26. Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001) Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans, *J. Biol. Chem. 276*, 3254−3261.
27. Rusnati, M., Coltrini, D., Oreste, P., Zoppetti, G., Albini, A., Noonan, D., d'Adda di Fagagna, F., Giacca, M., and Presta, M. (1997) Interaction of HIV-1 Tat protein with heparin. Role of the backbone structure, sulfation, and size, *J. Biol. Chem. 272*, 11313−11320.
28. Liu, Y., Jones, M., Hingtgen, C. M., Bu, G., Laribee, N., Tanzi, R. E., Moir, R. D., Nath, A., and He, J. J. (2000) Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands, *Nat. Med. 6*, 1380−1387.
29. Sanderson, R. D., Yang, Y., Suva, L. J., and Kelly, T. (2004) Heparan sulfate proteoglycans and heparanase—partners in osteolytic tumor growth and metastasis, *Matrix Biol. 23*, 341−352.
30. Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanasami, U. (2002) Roles of heparan-sulphate glycosaminoglycans in cancer, *Nat. Rev. Cancer 2*, 521−528.
31. Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P. K., and Shinkai, H. (2001) A unique sequence of the laminin alpha 3 G domain binds to heparin and promotes cell adhesion through syndecan-2 and -4, *J. Biol. Chem. 276*, 28779−28788.
32. Katoh, H., and Negishi, M. (2003) RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo, *Nature 424*, 461−464.
33. Schlunck, G., Damke, H., Kiosses, W. B., Rusk, N., Symons, M. H., Waterman-Storer, C. M., Schmid, S. L., and Schwartz, M. A. (2004) Modulation of Rac localization and function by dynamin, *Mol. Biol. Cell 15*, 256−267.
34. Marcoux, N., and Vuori, K. (2003) EGF receptor mediates adhesion-dependent activation of the Rac GTPase: a role for phosphatidylinositol 3-kinase and Vav2, *Oncogene 22*, 6100−6106.
35. Noda, Y., Takeya, R., Ohno, S., Naito, S., Ito, T., and Sumimoto, H. (2001) Human homologues of the Caenorhabditis elegans cell polarity protein PAR6 as an adaptor that links the small GTPases Rac and Cdc42 to atypical protein kinase C, *Genes Cells 6*, 107−119.
36. Burbelo, P. D., Drechsel, D., and Hall, A. (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases, *J. Biol. Chem. 270*, 29071−29074.
37. Ueno, M., Yamada, S., Zako, M., Bernfield, M., and Sugahara, K. (2001) Structural characterization of heparan sulfate and chondroitin sulfate of syndecan-1 purified from normal murine mammary gland epithelial cells. Common phosphorylation of xylose and differential sulfation of galactose in the protein linkage region tetrasaccharide sequence, *J. Biol. Chem. 276*, 29134−29140.
38. Pellegrini, L. (2001) Role of heparan sulfate in fibroblast growth factor signalling: a structural view, *Curr. Opin. Struct. Biol. 11*, 629−634.
39. Mitola, S., Sozzani, S., Luini, W., Primo, L., Borsatti, A., Weich, H., and Bussolino, F. (1997) Tat-human immunodeficiency virus-1 induces human monocyte chemotaxis by activation of vascular endothelial growth factor receptor-1, *Blood 90*, 1365−1372.
40. Rubio Demirovic, A., Canadi, J., Weiglhofer, W., Scheidegger, P., Jaussi, R., and Kurt, B. H. (2003) HIV TAT basic peptide is not a high-affinity ligand for VEGF receptor 2, *Biol. Chem. 384*, 1435−1441.
41. Wu, R. F., Gu, Y., Xu, Y. C., Mitola, S., Bussolino, F., and Terada, L. S. (2004) Human immunodeficiency virus type 1 Tat regulates endothelial cell actin cytoskeletal dynamics through PAK1 activation and oxidant production, *J. Virol. 78*, 779−789.
42. McQuade, K. J., and Rapraeger, A. C. (2003) Syndecan-1 transmembrane and extracellular domains have unique and distinct roles in cell spreading, *J. Biol. Chem. 278*, 46607−46615.

BI0612824