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Contribution of the 37-kDa laminin receptor precursor in the anti-metastatic PSP94-derived peptide PCK3145 cell surface binding ☆

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Abstract

Purpose: PCK3145 is an anti-metastatic synthetic peptide with promising therapeutic efficacy against hormone-refractory prostate cancer. The characterization of the PCK3145 peptide cell surface binding/internalization mechanisms and of the receptors involved remained to be explored.

Results: [^{14}C]PCK3145 cell surface binding assays showed rapid and transient kinetic profile, that was inhibited by RGD peptides, laminin, hyaluronan, and type-I collagen. RGD peptides were however unable to inhibit PCK3145 intracellular uptake. Far-Western ligand binding studies enabled the identification of the 37-kDa laminin receptor precursor (37LRP) as a potential ligand for PCK3145. Overexpression of the recombinant 37LRP indeed led to an increase in PCK3145 binding but unexpectedly not to its uptake.

Conclusions: Our data support the implication of laminin receptors in cell surface binding and in transducing PCK3145 anti-metastatic effects, and provide a rational for targeting cancers that express high levels of such laminin receptors.

Keywords: Laminin receptor precursor; Prostate cancer; Metastasis; MMP-9; EGCg

Prostate cancer is frequently associated with bone metastases, which are in fact the main cause of morbidity and mortality for this tumor. It is also the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in American males [1]. Androgen ablation as initial therapy for advanced prostate cancer provides high response rates but does not cure disease, as nearly all men with metastases will eventually progress to hormone-refractory prostate cancer (HRPC) [2]. Currently no effective treatments exist for patients where hormone treatment has failed, and the management of HRPC is solely palliative. It thus becomes crucial to develop new strategies to circumvent the progression of prostate cancer from localized growth to the invasion of surrounding tissues, and the development of distant bone and visceral organ metastasis. Prostate secretory protein 94 (PSP94), also known as prostatic inhibin or β-microseminoprotein [3], is a naturally occurring protein synthesized primarily in the prostate and found in large quantities in the seminal fluid [4]. Although the complete physiological role of PSP94 is not completely known, it is believed to be involved as a growth inhibitor and a promoter of cell death

☆ *Abbreviations:* ECM, extracellular matrix; EGCg, epigallocatechin-3-gallate; HRPC, hormone-refractory prostate cancer; 37LRP, 37-kDa laminin receptor precursor; 67LR, 67-kDa laminin receptor; MMP-9, matrix metalloproteinase-9.

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in the natural control of excessive and/or abnormal proliferation of epithelial cells both in normal prostate tissue as well as in malignant prostate tissues.

Recently, promising roles of PSP94 and, more particularly of one of its derived peptides PCK3145, were highlighted for its potential therapeutic efficacy against prostate cancer and its associated complications in animal models and in human clinical trials [5–7]. Interestingly, the main biological activity of PCK3145 observed in clinical trials was the significant reduction in the level of matrix metalloproteinase (MMP)-9 in patients with an increased level of this enzyme before treatment [7]. Since a direct role for MMP-9 has been specifically associated with prostate cancer metastasis [8–11], we believe that this marker may accurately be used not only to monitor the efficacy of the treatment using PCK3145 but also to select patients for whom PCK3145 treatment may be beneficial.

Selective interaction of the delivered PCK3145 into the target tissue in vivo is expected to enhance drug efficacy. We have recently provided preliminary in vitro evidence for the molecular mechanism of action of PCK3145 with implications as an anti-metastatic [12] and anti-angiogenic [13] agent. Moreover, we have also shown that laminin interfered with PCK3145 intracellular signaling [14], and that the expression of HuR, an intracellular RNA-binding protein that can stabilize MMP-9 mRNA, was decreased by PCK3145 [14]. The characterization of the PCK3145 cell surface receptors as well as of the peptide's binding/internalization mechanisms involved remained to be explored as this will further unravel the molecular kinetics regulating both the cell surface and intracellular effects of PCK3145. We thus investigated the specificity and cell surface receptor binding affinity of PCK3145 in light of its bioactivity against MMP-9 secretion. The cell surface binding and internalization processes through specific extracellular matrix (ECM) proteins competition experiments confirmed the implication of laminin receptor activity in PCK3145 cell surface recognition. Our results also highlighted the possibility that alternate cell surface receptors may be involved in the internalization mechanisms of PCK3145. Our current observations not only support the implication of laminin receptors in transducing part of the PCK3145 anti-metastatic effects, but also provide a rational for targeting cancers that express high levels of such laminin receptors.

Materials and methods

Materials. Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). FUGENE-6 transfection reagent was from Roche Diagnostics, Canada (Laval, QC). Cell culture media were obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The polyclonal antibody against the 67-kDa laminin receptor (67LR) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), the monoclonal antibody against galectin-3 was from Chemicon (Temecula, CA), and the mouse β-actin monoclonal antibody was from Sigma. The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). Fluorescein isothiocyanate (FITC)-N-terminal-labeled PCK3145 was synthesized by EZBiolab (Westfield, IN). Type-I collagen was extracted from rat tail tendon [12]. The GRGDSP peptide was purchased from Bachem (King of Prussia, CA). All other reagents were from Sigma–Aldrich, Canada.

Cell culture and cDNA transfection method. Previous studies from our laboratory have established the antimetastatic effects of PCK3145 using HT-1080 fibrosarcoma cells in vitro model [12,14]. The HT-1080 cell line was purchased from American Type Culture Collection, maintained in Dulbecco's Minimum Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, and 100 U/ml penicillin, 100 μg/ml streptomycin, and were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. The human 37-kDa laminin receptor precursor (37LRP) full-length cDNA was from OriGene Technologies (Rockville, MD). HT-1080 cells were transiently transfected with cDNA using the non-liposomal formulation FUGENE-6 transfection reagent. Transfection efficiency was confirmed by Western blotting. All experiments involving these cells were performed 36 h following transfection. Mock transfections of HT-1080 cultures with pcDNA (3.1+) expression vector alone were used as controls.

Immunoblotting procedures. Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% non-fat dried milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/2500 dilution for 67LR) in TBST containing 5% non-fat dried milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

PCK3145 cell surface binding and uptake assays. Characterization studies on PCK3145 properties to bind to the cell surface and to subsequently be internalized were initiated. This is basically achieved by either performing the experiments at 4 °C, a condition known to minimize internalization/uptake mechanisms, or at 37 °C, a condition that favors both cell surface binding and internalization. The synthesis of ^{14}C-radiolabeled PCK3145 (H-EW-[U-^{14}C]Q-TDNC(Acm)ETC(Acm)TC(Acm)YET-OH) was achieved and this allowed us to develop assays with very low background. Assays were performed in 6-well plates with 80% confluent HT-1080 fibrosarcoma cells. Prior to the assay, cells were washed with HBSS buffer and stabilized at either 4 or 37 °C for approximately 15 min. Reactions were initiated by adding 500 μl of Ringer/Hepes containing 0.5% ovalbumin and 0.5–1.0 μCi [^{14}C]PCK3145. Reactions were stopped with rapid aspiration of the media, and cells were washed rapidly with 1 ml Ringer/Hepes containing 0.5% ovalbumin. Radioactivity was then counted in the whole cells after the addition of 500 μl NaOH 1 N.

Flow cytometry analysis and fluorescein isothiocyanate-labeled PCK3145 binding assay. Fluorescein isothiocyanate (FITC) is currently the most commonly used fluorescent dye for FACS analysis and was conjugated to the N-terminus of PCK3145. This enables us to follow cell binding of FITC-PCK3145 through the shift in fluorescence associated with cells that bind to it. Eighty percent to 90% confluent HT-1080 cells were dislodged after brief trypsinization, washed extensively, resuspended in 10% FBS/DMEM at a concentration of 10^6 cells/ml, washed once with 0.1% PBS/0.1% BSA, and then incubated with 10 μg/ml FITC-PCK3145 for 1 h on ice (4 °C). After washing with PBS/BSA, the cells were suspended in 1 ml PBS/BSA, and analyzed on a FACS Calibur flow cytometer with the CellQuestPro software (BD Biosciences, Mississauga, ON). Results are expressed as the ratio of relative geometric mean values from the PCK3145-treated cells to their untreated controls and are representative of three independent experiments.

PCK3145 biotinylation and Far-Western analyses. PCK3145 was biotin-labeled according to Pierce and purified by FPLC-chromatography using Akta-explorer with a resource RPC-30 ml column (Amersham

Bioscience, QC). The biotin-labeled peptide was eluted with a gradient from 20% CH_3CN to 80% CH_3CN (+0.05% TFA) at a flow rate of 4 ml/min. Products were monitored at different wavelengths (205, 229, and 254 nm) with a UV-900 cell-10. Confluent HT-1080 cell lysates (~50 μg/well) were migrated using 10% SDS-gels and then electrotransferred onto PVDF membranes. Membranes were then incubated with 10 μg/ml of unlabeled PCK3145 or 10 μg/ml biotin-labeled PCK3145 for 1 h, followed by incubation with HRP-streptavidin for another hour and ECL detection.

Statistical data analysis. Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and was used to compare the PCK3145 effect to vehicle-treated cells. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in each figure.

Results

Kinetics of PCK3145 cell surface binding and internalization processes

Previous evidence using competitive surface binding approaches has led us to conclude that PCK3145 transduced its inhibitory effect through cell surface receptors bearing laminin-binding activities [14]. Whether any direct cell surface binding and subsequent internalization processes were involved was next investigated. We performed cell surface binding as well as uptake assays by incubating HT-1080 fibrosarcoma cells with radiolabeled [^{14}C]PCK3145 as described in the Materials and methods section. We found that PCK3145 rapidly and transiently bound to the surface of HT-1080 cells peaking within the first 30 s, and rapidly returning to basal levels (Fig. 1A, left panel). Interestingly, when uptake experiments were performed, PCK3145 internalization also peaked within the first 30 s but ^{14}C-radiolabeled cell-associated signal remained sustained for up to 5 min (Fig. 1A, right panel). These observations are consistent with the "hit-and-run" hypothesis that we have previously suggested in PCK3145 intracellular cell signaling and that rapidly led to extracellular signal-regulated kinase (ERK) phosphorylation [14]. When radiolabeled-PCK3145 binding and uptake experiments were performed with increasing PCK3145 concentrations (Fig. 1B), PCK3145 cell surface binding was approximately 2 times lower than uptake. Dissociation constants (K_d) were also

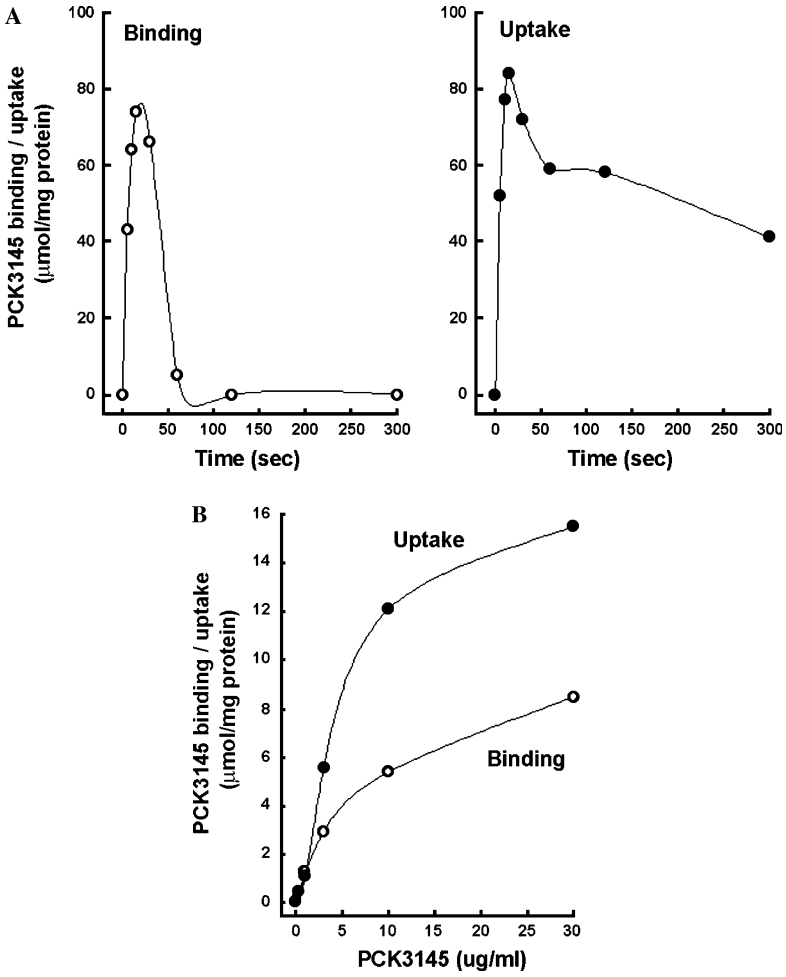

Fig. 1. Differential kinetics in PCK3145 cell surface binding and internalization processes. (A) HT-1080 fibrosarcoma cells were treated with 30 μg/ml [^{14}C]labeled-PCK3145 at 4 °C (binding assay) or 37 °C (uptake assay) as described in Materials and methods. PCK3145 binding/uptake assays were performed for up to 5 min. Data are from one representative experiment. (B) Increasing concentrations of PCK3145 were used to monitor cell surface binding and uptake. Kinetic constants were derived from these data.

computed and were not statistically different for both processes (9.0 ± 1.1 μg/ml vs. 7.7 ± 0.5 μg/ml for binding and uptake, respectively) and one can conclude that the relative affinity for PCK3145, whether it is in binding or internalization processes, is approximately equivalent.

Differential inhibitory effect of RGD peptides and type-I collagen on PCK3145 cell surface binding and internalization processes

We have performed PCK3145 binding and uptake experiments in order to assess whether ECM proteins such as laminin, HA, type-I collagen, or RGD peptides affected cell surface binding processes differently from internalization processes. Results show that besides linear RGD peptides, those three ECM proteins to which PCK3145 inhibited cell adhesion [15] also inhibited PCK3145 internalization process at 37 °C (Fig. 2A, black bars). Interestingly, while RGD peptides, laminin, and HA were still able to inhibit PCK3145 cell surface binding at 4 °C, type-I collagen was ineffective in doing so (Fig. 2A, white bars). An inhibition of PCK3145 cell surface binding was achieved at higher doses of type-I collagen. Relative K_d constants were computed and show a 17-fold magnitude of change between binding (1.5 μg/ml) and uptake (25.4 μg/ml). Because fibronectin, fibrin, elastin, and vitronectin did not initially interfere with cell-to-PCK3145 interaction [12], they were intentionally not further tested. This set of experiments suggests that potential alternate cell surface receptors from either the integrin or non-integrin family may be involved in transducing PCK3145 intracellular effects. Physiologically, this also implies that PCK3145 may have differential effects that would be dictated by the composition of the ECM tumor microenvironment.

Identification of the 37-kDa laminin receptor precursor among the potential PCK3145-binding proteins

In light of the above result, we wished to provide initial clues as for the identity of the potential PCK3145 cell surface binding proteins involved. We thus performed a ligand binding (Far-Western) assay onto PVDF membranes using biotin-labeled PCK3145. Autoradiograms showed some non-specific detection of proteins most probably due to the binding of streptavidin to membranes that were not pre-incubated with biotin-labeled PCK3145 (Fig. 3, first lane of left panel). Interestingly, a reproducible staining pattern of approximately 11 candidates was observed in those PVDF membranes that were first incubated with biotin-labeled PCK3145 (Fig. 3, second lane of left panel). Because of the potential role that the 67-kDa laminin receptor (67LR) recently played in MMP secretion [15] and in light of our data that laminin competed with PCK3145 effects [14], when those same PVDF membranes were reprobed with a 67LR antibody, candidate number 9 showed immunoreactive property associated with the 67LR precursor at ~35 kDa (Fig. 3A, right panel) suggesting that the 37-kDa laminin receptor precursor (37LRP) protein of the mature 67LR protein may be one of the PCK3145 binding proteins. Other non-specific (NS) immunoreactive bands were observed at higher molecular weights, and were also observed in data of Fig. 5C. The identity of the remaining PCK3145 binding proteins is under investigation and remains to be elucidated as they can either represent intracellular as well as other cell surface proteins. The potential contribution of cell surface galectin-3, a 31-kDa laminin-binding protein which may cross-react with the 37LRP antibody, was ruled out since when the same PVDF membrane was reprobed, very low

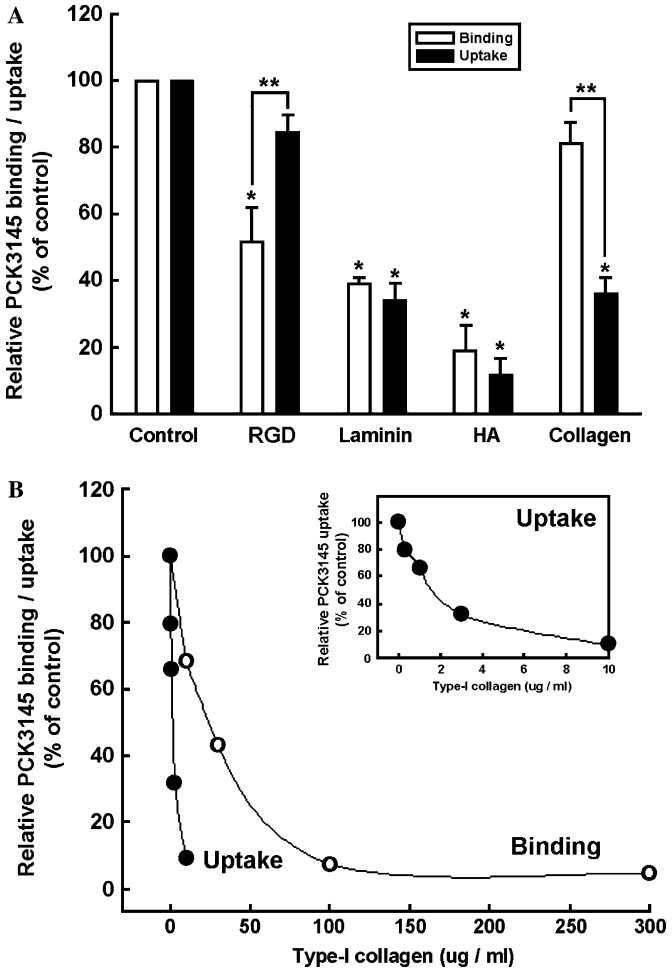

Fig. 2. Differential inhibitory effect of type-I collagen on PCK3145 cell surface binding and internalization processes. (A) Cells were incubated for 15 s with 30 μg/ml PCK3145 in combination or not of 1 μM RGD peptide or 10 μg/ml of laminin, hyaluronic acid (HA), or type-I collagen. Cell surface binding (white bars) or uptake (black bars) were monitored as described in Materials and methods. Data are expressed as the % of untreated controls, and are means of three independent experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*, compared to control conditions) or a double-asterisk (**, uptake vs binding conditions) identifies such significance in cell surface binding and uptake experiments. (B) Relative PCK3145 binding or uptake were monitored in cells that were incubated for 15 s with 30 μg/ml PCK3145 in the presence or not of type-I collagen. Inset represents the representative dose response as monitored for PCK3145 uptake in the presence of low type-I collagen concentrations.

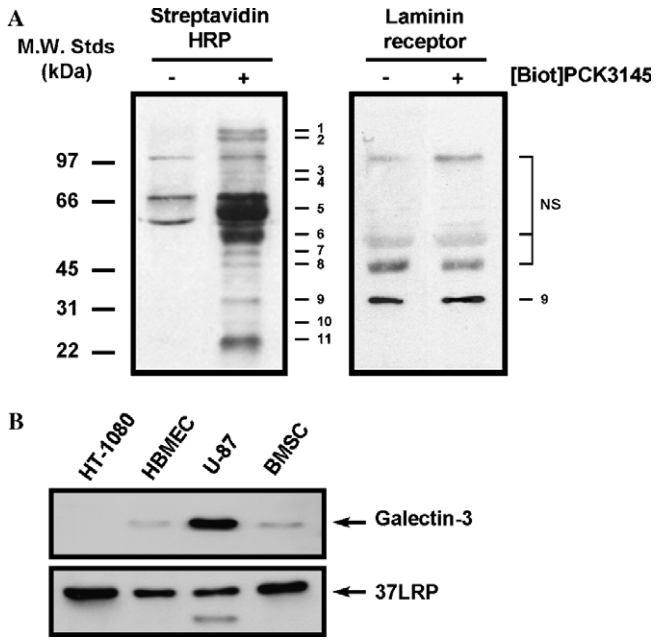

Fig. 3. Identification of the 37-kDa laminin receptor precursor among the potential PCK3145-binding proteins. PCK3145 was biotin-labeled and purified as described in Materials and methods. (A) One hundred micrograms of proteins was loaded in each well of a 10% SDS–PAGE from lysates isolated from confluent HT-1080 cells. Migration was followed by PVDF membranes electroblotting and incubation with HRP-streptavidin or biotin-labeled PCK3145 as described in Materials and methods. The left panel was incubated (+) or not (−) with biotin-labeled PCK3145 and bands revealed using streptavidin-HRP coupled to ECL detection. Right panel represents the same PVDF membrane blotted with an anti-67 kDa laminin receptor (67LR) antibody. Numbers (1–11) next to the left panel represent the potential proteins that interact with PCK3145. Band number 6 represents the 37LRP immunoreactive band that is similar to the same band identified in the left panel. NS, non-specific immunoreactive band. (B) Lysates (30 μg/well) from HT-1080 cells, U-87 glioma cells, human brain endothelial microvascular cells (HBMEC), and bone marrow stromal cells (BMSC) were electrophoresed, transferred to PVDF membranes, and immunodetection performed to assess the expression of the 37LRP and of the galectin-3 proteins.

to undetectable immunoreactivity was observed in HT-1080 cell lysates in comparison to positive galectin-3 immunoreactivity for the other cell lysates tested (Fig. 3B).

Differential regulation of PCK3145 cell surface binding and internalization by the 37-kDa laminin receptor precursor

We have further investigated the impact of the 37LRP expression on PCK3145 cell surface binding and internalization processes by transiently transfecting HT-1080 cells with a cDNA plasmid encoding the 37LRP protein (Fig. 4A). Interestingly, when PCK3145 cell surface binding was assessed in 37LRP-transfected cells, we noticed a significant ~35% increase when compared to untransfected mock cells (Fig. 4B, black bars). This result confirms and supports that obtained in high confluent cells where the increased expression of the 37LRP correlates with an increase in PCK3145 cell surface binding (see next Fig. 5). Intriguingly, when these same 37LRP-transfected cells were used to assay PCK3145 internalization, there

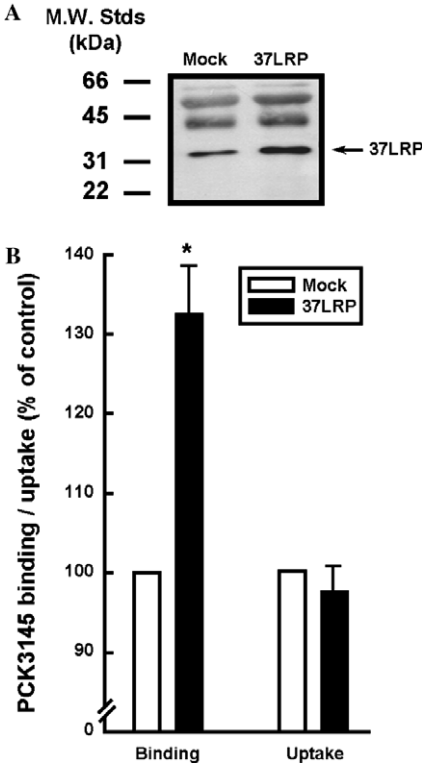

Fig. 4. The expression of the 37-kDa laminin receptor precursor regulates PCK3145 cell surface binding but not internalization. (A) HT-1080 cells were transiently transfected with a cDNA encoding the 37LRP. Cell lysates were isolated and immunoblotting was performed as described in Materials and methods. (B) Mock (white bars) and 37LRP (black bars) HT-1080 cells were trypsinized and PCK3145 cell surface binding and uptake monitored as described in the legend of Fig. 2B. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance between Mock and 37LRP-transfected cells in cell surface binding and uptake experiments.

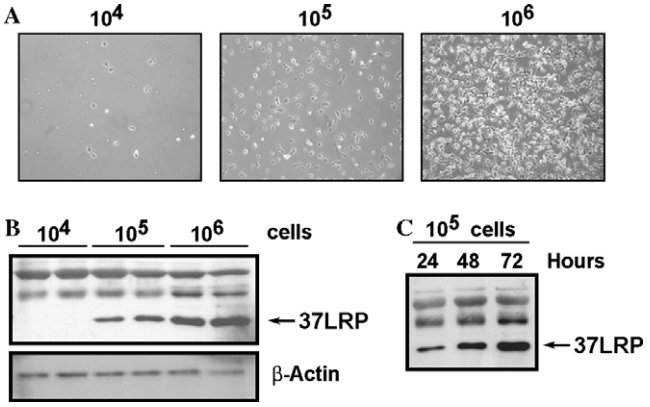

Fig. 5. Cell confluence regulates the 37-kDa laminin receptor precursor expression and PCK3145 cell surface binding. (A) HT-1080 cells were seeded in individual wells of a 6-well plate at increasing confluence (10^4–10^6 cells/well) and pictures taken 24 h later. (B) Cell lysates were isolated in duplicate from each cell confluence culture condition. SDS–PAGE and immunoblotting with the anti-67LR were performed at 24 h. (C) HT-1080 cells were seeded at 10^5 cells/well in a 6-well culture plate and lysates were isolated at 24, 48, and 72 h.

was no evidence for increase in PCK3145 uptake (Fig. 4B). Collectively, these data suggest that the 37LRP is involved in PCK3145 cell surface binding but that

alternate cell surface receptors must be engaged in the PCK3145 internalization process.

Cell confluence regulates the 37-kDa laminin receptor precursor expression and PCK3145 cell surface binding

In the setting of our experimental conditions to test the different effects of PCK3145, we noticed that the 37LRP was expressed at very low to undetectable levels in low (10^4 cells/well) density, while its expression drastically increased at high (10^6 cells/well) confluent cells cultured for 24 h (Fig. 5A and B). This potential cell–cell contact effect was also observed when cells were harvested at different times of incubation (from 24 to 78 h) (Fig. 5C). This unexpected observation is of crucial importance as it may first explain and optimize the reproducibility of PCK3145 cellular effect, and basically provides an interesting "model" to study the impact of the 37LRP in binding PCK3145 in low 37LRP-expressing cells versus high 37LRP-expressing cells.

The expression of the 37-kDa laminin receptor precursor potentiates EGCg inhibition of cell surface PCK3145 binding

Although the mature 67LR is thought to be the receptor for the green tea-derived catechin EGCg [16], not much is known about the 37LRP that would enable the generation of a mature 67LR. Interestingly, EGCg is also known for its ability to inhibit MMP-9 secretion [17,18] an end-point effect that is similar to that of PCK3145 but that does not necessarily involve the same mechanism of action. We sought to investigate the involvement of that potential common 37LRP/67LR in the ability of the cells to bind biotin-labeled PCK3145 by flow cytometry. Low and high confluent cells were harvested and cells were incubated with or without biotin-labeled PCK3145 and in the presence or not of 100 μM EGCg at 4 °C for 1 h. Cells were then analyzed for the shift in fluorescence following a last incubation with FITC-labeled avidin by flow cytometry (profiles not shown). We show that PCK3145 binds more significantly (~25%) to the cell surface of high confluent HT-1080 cells (Fig. 6A, black bars control). Interestingly, we also show that EGCg only effectively competed with PCK3145 cell surface binding and completely abolished it in high confluent cells while it only inhibited PCK3145 cell surface binding by approximately 7% in low confluent cells (Fig. 6A, black bars EGCg). When the inhibitory effect of PCK3145 on MMP-9 secretion was assessed using these two cell populations, we observe that PCK3145 was ineffective in low confluent cells while it significantly inhibited MMP-9 secretion in high confluent cells as assessed by gelatin zymography (Fig. 6B) and quantified by scanning densitometry (Fig. 6C). This observation correlates and supports the fact that the higher expression of the 37LRP at the cell surface may better transduce the effect of PCK3145. This highlights the potential contribution of 37LRP/67LR as one of the cell surface laminin receptors that may transduce the effects of PCK3145. Collectively, we provide more evidence for the involvement of the 37LRP as

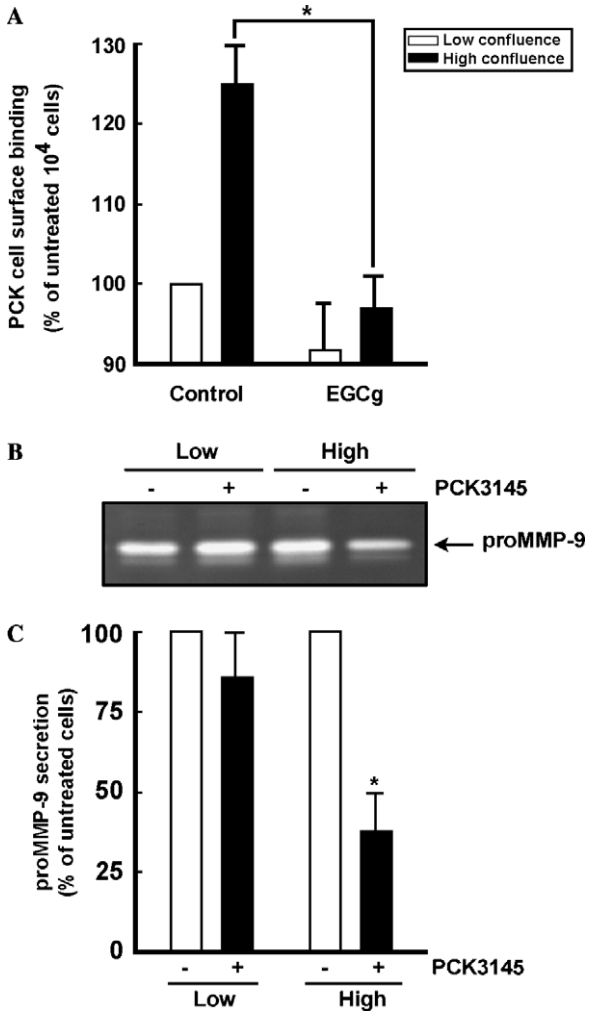

Fig. 6. The expression of the 37-kDa laminin receptor protein potentiates EGCg inhibition of cell surface PCK3145 binding. (A) Low confluent HT-1080 cells (10^4 cells/well, white bars) were trypsinized and incubated with biotin-labeled PCK3145 at 4 °C as described in Materials and methods. PCK3145 cell surface binding was monitored by flow cytometry in cells that were incubated or not with 100 μM EGCg and binding compared to that of high confluent cells (10^6 cells/well, black bars). Statistical significance (*) for the effect of EGCg on PCK3145 cell surface binding between high and low confluent cells is indicated. (B) proMMP-9 secretion from low and high confluent cells was monitored by gelatin zymography in serum-starved cells that were incubated or not with 300 μg/ml PCK3145 for 48 h. (C) The extent of proMMP-9 gelatinolytic activity was quantified by scanning densitometry from three different zymograms and expressed as the percent of untreated cells in low and high confluent cells. Statistical significance is denoted by the asterisk (*).

one of the PCK3145 cell surface binding proteins as EGCg is being able to efficiently compete with PCK3145 for cell surface binding. Since the 37LRP expression is induced in high confluent cells, we may safely infer that the 37LRP may indeed transduce, at least in part, the effects of PCK3145.

Discussion

Mechanisms by which circulating PCK3145 crosses or interacts with the plasma membrane are still unknown. The kinetics and specificity of interaction of PCK3145 have

thus been investigated in order to characterize the potential actors regulating cell surface binding and internalization processes that ultimately lead to decreased expression and secretion of MMP-9. The major observations made in our study highlight the implication of cell surface laminin receptor activity, and more particularly that of the 37LRP. The novel mechanism of PCK3145 laminin receptor-mediated regulation of MMP-9 expression provides foundation of our hypothesis that PCK3145 acts through receptor-mediated signaling and that targeting and inhibiting the 37LRP-mediated signaling may explain the anti-metastatic property of PCK3145. Interestingly, increased expression of the 37LRP/67LR has been reported in a variety of human carcinomas (colon, breast, stomach, liver, and ovary) and directly correlates with a higher proliferation rate of malignant cells and tendency to metastasize [19]. In addition, the 67LR is detectable in anaplastic large cell lymphomas and in small subsets of high-grade B-cell non-Hodgkin's or Hodgkin's lymphomas [20], and found to mediate acute myeloid leukemia cell adhesion to laminin and to be frequently associated with monocytic differentiation [21]. In light of these documented expression of the 67LR, it is thus tempting to suggest that cancers, or specific stage of cancer development such as in monocytic-oriented acute myeloid leukemia, could potentially be efficiently targeted by PCK3145.

In contrast to the 37LRP role that we highlight in PCK3145 cell surface binding, our current observations however preclude any involvement of the 37LRP in PCK3145 uptake processes. Indeed, although a role for the 67LR was recently ascribed in the internalization of proteins or peptides such as CNF-1 [22], the overexpression of the 37LRP recombinant protein did not lead, under our experimental conditions, to increased PCK3145 uptake/internalization whereas cell surface binding was increased in 37LRP-transfected cells. This suggests that either the mature form of the 67LR must be generated or that alternate yet uncharacterized cell surface processes mediate PCK3145 internalization. Whether this occurs via receptor-mediated endocytosis upon PCK3145 binding to a specific cell surface receptor or by a clatherin- or caveolin-(in)dependent mechanisms remains to be investigated. In fact, our explanation can, at this point, only be speculative, since the relationship between 37LRP and 67LR is unknown so far [23]. One possible explanation would be that the LRP/LR complex would serve in efficient receptor-mediated endocytosis processes such as of the extracellular prion protein [24]. Keeping in mind that laminin plays a central role in cell growth, differentiation, and migration and that any interference with these functions, such as by PCK3145, may be deleterious confirm the potential therapeutic actions of PCK3145 in interacting with 37LRP. The presence of a functional 37LRP/67LR complex may thus be required in order to further promote PCK3145 internalization. Several lines of evidence from the current study suggest that PCK3145 physical interaction occurs potentially with proteins isolated from cell lysates and which comprise intracellular as well as plasma membrane bound proteins as demonstrated by the multiple candidates found to interact with PCK3145 in our ligand binding assay. Among the 11 candidates that we have identified, we believe that we have now clearly characterized the implication of the 37LRP. Other proteins of low (less than 30 kDa) or high (more than 75 kDa) molecular weight have also been identified. Their exact identity however remains to be confirmed.

Among the ECM proteins known to potentially bind and interact with laminin receptors, RGD peptides and type-I collagen were found to differentially compete with PCK3145 internalization process and cell surface binding (Fig. 2). The fact that RGD peptides inhibited PCK3145 cell surface binding but not internalization processes suggests that those two processes occur through different cell surface-mediated mechanisms. These mechanisms may however integrate the cooperative functions of both integrin and non-integrin cell surface receptors which remain to be characterized. In fact, $\alpha 3\beta 1$ integrin was recently shown to regulate MMP-9 mRNA stability in immortalized keratinocytes [25], and provides a similar mechanism of integrin-mediated regulation of MMP gene expression as that involving PCK3145. Recently, emerging features in the regulation of MMP-9 gene expression for the development of novel molecular targets and therapeutic strategies aim at targeting the integrin-mediated cell–cell or cell–ECM signaling pathways [26].

MMP-9 is a matrix metalloproteinase involved in prostate cancer progression and its expression can be regulated at several levels [27]. Although most published studies have focused on transcriptional control of MMP-9, there is increasing evidence that its expression can also be regulated at the steps of mRNA stability, translation, and protein secretion. The ability to modulate MMP-9 expression at multiple steps through distinct signaling pathways may be particularly important during malignant conversion and metastasis, when tumor cells need to induce or maintain MMP-9 levels in response to changing environmental cues. While PCK3145 did not affect MMP-9 enzymatic activity per se, it however significantly reduced HuR, a MMP-9 mRNA stabilizing factor, and its gene expression which, consequently, led to decreased extracellular MMP-9 secreted levels [14]. The fact that the expression of HuR, an intracellular protein, was targeted by PCK3145 keeps open the possibility that, besides intracellular transduction mechanisms regulating HuR expression, some crucial internalization mechanisms may also regulate part of PCK3145 mechanism of action. As HuR also binds to the AU-rich elements of RNAs encoding genes for cytokines, growth factors, tumor suppressor genes, proto-oncogene, and cell cycle regulators, one can envision that downregulation of HuR by PCK3145 may also inhibit cell proliferation or induce apoptosis. These alternate cellular processes are currently under investigation.

In fact, our data suggest that intracellular signaling of PCK3145, that leads to the inhibition of MMP-9

extracellular levels, is mediated by the laminin receptor-like activity such as that transduced by the 37LRP. This highlights the potential contribution of 37LRP as one of the cell surface laminin receptors that may transduce the effects of PCK3145. Noteworthy, an alternative interpretation of these results may also rely on the fact that the increase in 37LRP protein expression in high confluent cells represents approximately 5–6 times that expression in low confluent cells. In contrast, an increase of only 25% is observed in PCK3145 cell surface binding. This suggests that a potential cell surface 37LRP sub-population may not be as effective in binding PCK3145 or that these may not be in the right conformation. This is further re-enforced by the unexpected observation that PCK3145 shares, to some extent, homology with different structural chains of laminin (not shown) and suggests that it may indeed also share the potential to interact with the same cell surface receptors. Moreover, EGCg, a green tea catechin that similarly to PCK3145 inhibits MMP-9 secretion, is also a 67LR ligand [16] and antagonized cell binding to PCK3145. This confirms that such cell surface receptors regulate PCK3145 effects. Collectively, our data support the implication of cell surface laminin receptors in binding and transducing PCK3145 effects that lead to MMP-9 diminished secretion and provide a rational for targeting cancers that express high levels of such laminin receptors.

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