Proteomics-based discovery of a novel, structurally unique, and developmentally regulated plasminogen receptor, Plg-RKT, a major regulator of cell surface plasminogen activation

Short Title: Plg-RKT, a novel plasminogen receptor

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**Abstract**

Activation of plasminogen, the zymogen of the primary thrombolytic enzyme, plasmin, is markedly promoted when plasminogen is bound to cell surfaces, arming cells with the broad spectrum proteolytic activity of plasmin. In addition to its role in thrombolysis, cell surface plasmin facilitates a wide array of physiological and pathological processes. Carboxypeptidase B-sensitive plasminogen binding sites promote plasminogen activation on eukaryotic cells. However, no integral membrane plasminogen receptors exposing carboxyl terminal basic residues on cell surfaces have been identified. Here we utilize the exquisite sensitivity of multidimensional protein identification technology and an inducible progenitor cell line to identify a novel differentiation-induced integral membrane plasminogen receptor that exposes a C-terminal lysine on the cell surface, Plg-RKT (C9orf46 homolog). Plg-RKT was highly co-localized on the cell surface with the urokinase receptor, uPAR. Our data suggest that Plg-RKT also interacts directly with tissue plasminogen activator. Furthermore, Plg-RKT markedly promoted cell surface plasminogen activation. Database searching revealed that Plg-RKT mRNA is broadly expressed by migratory cell types, including leukocytes, breast cancer, leukemic and neuronal cells. This structurally unique plasminogen receptor, represents a novel control point for regulating cell surface proteolysis.
**Introduction**

Localization of plasminogen on cell surfaces is a crucial control point for positive regulation of cell surface plasmin proteolytic activity that facilitates both physiological and pathological processes \(^1,^2\), including macrophage recruitment during the inflammatory response \(^3-^6\), tissue remodeling \(^7\), wound healing \(^8,^9\), tumor cell invasion and metastasis \(^10-^12\), skeletal myogenesis \(^13\), neuroendocrine prohormone processing \(^14,^15\), and neurite outgrowth \(^16,^17\). Cell surface plasminogen binding sites promote plasminogen activation by reducing the Km (11-60-fold) for plasminogen activation \(^18-^24\). Active plasmin also associates with the cell surface, where its activity is protected from inhibitors \(^25,^26\).

Plasminogen binding sites are very broadly distributed on both eukaryotic and prokaryotic cells \(^27\). Of the many eukaryotic cells examined to date, only erythrocytes do not bind plasminogen \(^28\). The interactions of plasminogen with eukaryotic cells are mediated by lysine binding sites within the disulfide-bonded kringle domains of plasminogen \(^18,^29\). Therefore, plasminogen binding to eukaryotic cells is blocked in the presence of lysine and lysine analogs, including \(\varepsilon\)-aminocaproic acid (EACA) \(^27\). Because most cell types have a very high capacity for plasminogen, no single molecule can account for the entire plasminogen binding capacity of a given cell type \(^27\). However, a subset of plasminogen binding proteins exposing C-terminal basic residues on cell surfaces are predominantly responsible for the ability of eukaryotic cells to enhance plasminogen activation because carboxypeptidase B (CpB) treatment abrogates cell-surface-dependent plasminogen activation \(^24\). Correspondingly, plasminogen-dependent macrophage recruitment *in vivo* is mediated by CpB-sensitive plasminogen receptors, and
plasminogen binding to recruited macrophages is increased, compared to peripheral blood monocytes \(^6,30\). Therefore, we probed the monocyte proteome as a source of an inducible integral membrane plasminogen receptor(s) exposing a C-terminal basic residue on the cell surface.

Several plasminogen binding proteins with established intracellular functions that are synthesized with C-terminal lysines associate with the monocytoid cell surface [e.g. \(\alpha\)-enolase \(^{29,31}\), TIP49a \(^{32}\), histone H2B, and p11 \(^{33}\)]. Other functional plasminogen binding proteins that are not synthesized with C-terminal basic residues are present on monocytoid cells, including annexin II \(^{34}\), amphoterin \(^{35}\), tissue factor \(^{36}\) and \(\alpha_{\text{II}}\beta_2\) \(^{37}\). However, no integral membrane plasminogen binding proteins that are synthesized with C-terminal basic residues have been identified to date. The existence of a receptor with such a structure would constitute a novel mechanism for stimulating plasminogen activation because its induction would endow cells with the ability to bind plasminogen and promote plasminogen activation, without requiring release and re-binding of intracellular proteins or proteolytic cleavage of a membrane protein to reveal C-terminal basic residues. Therefore, we used the exquisite sensitivity of multidimensional protein identification technology (MudPIT), to search for an integral membrane plasminogen receptor(s), exposing a C-terminal basic residue on the cell surface and upregulated during differentiation.
Materials and Methods

Proteins

Glu-plasminogen was purified from fresh human blood as described \(^{38,39}\). Lys-plasminogen was from Enzyme Research Laboratories (South Bend, IN). Single chain t-PA was from Calbiochem (San Diego, CA). Polyclonal antibodies were raised in rabbits and monoclonal antibodies were raised in rats and mice against the synthetic peptide, CEQSKLFSDK, thiol coupled to keyhole limpet hemocyanin. Antibodies were selected for direct binding to immobilized CEQSKLFSDK coupled to BSA and for the ability to inhibit specific plasminogen binding to CEQSKLFSDK. Anti-α-enolase mAb 9-C12 \(^{31}\) and polyclonal anti-plasminogen \(^{40}\) were prepared in our laboratory. Anti-uPAR (product #3936) was from American Diagnostica, (Stamford CT). FITC conjugated CD marker antibodies and the relevant FITC-conjugated isotype control antibodies were from Pharmagen (San Diego CA). The mouse macrophage differentiation antibody rat anti-mouse F4/80 FITC conjugate was from Serotech (Raleigh, NC). Goat anti-mouse FITC conjugated polyclonal antibody and goat anti-rabbit FITC conjugated polyclonal antibody, were from Calbiochem. Alexa 488- F(ab’)\(_2\) of goat anti-rabbit IgG and Alexa 568- F(ab’)\(_2\) fragment of goat anti-mouse IgG were from Invitrogen (San Diego, CA).

Cells

For differentiation experiments, monocytes were further isolated from the mononuclear cell population by plating onto tissue culture dishes (Corning, Corning,
New York), prior to the addition of human recombinant macrophage colony stimulating factor (M-CSF) (Calbiochem).

Hoxa9-ER4 cells were cultured as described and were differentiated either with murine M-CSF (Calbiochem) or with conditioned media produced by LADMAC cells (ATCC, Manassas, VA), as a source of M-CSF.

Quantitative Flow Cytometry

Quantitative flow cytometric equilibrium binding of fluorescein isothiocyanate (FITC)-plasminogen to monocytoid cells was analyzed using beads impregnated with FITC as described. Briefly, the output from the flow cytometer was standardized into mean equivalent standard fluorescence units (MESF) using beads impregnated with different MESF units of FITC. The fluorescence intensity change of the EACA-inhibitable FITC-plasminogen conformational change (Q = ΔIf/ΔIi; where Ii and If are the initial and final fluorescence intensities of FITC-plasminogen respectively) was approximated using a F500 fluorometric plate reader (Bio-Rad Model 680, Bio-Rad, Hercules, CA) equipped with a FITC filter set (Extinction 490 nm: Emission 520 nm). Binding parameters were determined using the program, NLREG, version 4.1.

Laser Scanning Confocal Microscopy

Confocal images were captured using a Zeiss 710 Laser Confocal Scanning Microscope (LCSM), with a 63x objective (1.4na), running the latest Zen 2009 Zeiss software suite (Carl Zeiss Inc., Thornwood, NY). All images were then imported and
further analyzed for quantitative colocalization using two independent software packages: LSM examiner (Zeiss Inc., Thornwood, NY) and Image J (NIH imaging; http://rsb.info.nih.gov/ij). Colocalization between fluorescently labeled aggregates of Plg-R\textsubscript{KT} with either uPAR or plasminogen was quantified by obtaining the threshold range of real over background signal and then using the average real threshold range to calculate the correlation coefficients (M values) of at least 40 cells in two separate experiments. To define the number and size of each labeled aggregate, images were imported into Image Pro Plus (Media Cybernetics Inc., Bethesda MD) where each cell was outlined and a similar threshold range (as described above) was used to define a real signal within each cell. Once this range was defined the software then automatically extracts parameters such as area, perimeter total number, and average fluorescence intensity of the fluorescently labeled aggregates per cell.

**Plasminogen Receptor Isolation**

Progenitor and M-CSF-differentiated Hoxa9-ER4 cells (5 X 10\textsuperscript{5}) were separately biotinylated, using EZ-Link Amine-PEO\textsubscript{3}-Biotin (Pierce). The cells were then subjected to dead cell removal on annexin V-coated magnetic microspheres (Miltenyi) that resulted in a 99\% enrichment of viable cells (as determined in fluorescence activated cell sorting (FACS) analysis with propidium iodide and annexin V). Membrane fractions were prepared from the viable cells by dounce homogenization in the presence of Complete Protease Inhibitor Cocktail (Roche, Palo Alto, CA) in Invitrosol (Invitrogen), followed by centrifugation steps as used in our laboratory\textsuperscript{32,43} and 3 mg were applied to a 1 ml
plasminogen-Sepharose affinity column as described. The column was washed in phosphate buffered saline (PBS) containing 1 X Invitrosol until no protein was detected at 280 nm followed by elution with the washing buffer containing 0.2 M EACA. The eluant from the plasminogen-Sepharose column was incubated with 50μl of immobilized avidin for 30 minutes at 4°C. Proteins bound to the immobilized avidin were resuspended in 5μl of Invitrosol and heated at 60°C for 5 minutes. Then, 45μl 80% acetonitrile were added and the samples were digested with trypsin at 37°C for 18 h. After 24 h, the solvent was evaporated in a speedvac, and peptides were dissolved in 50μl of buffer A (95% H2O, 5% acetonitrile, and 0.1% formic acid).

Multidimensional Chromatography and Tandem Mass Spectrometry

The protein digest was subjected to MudPIT [reviewed in 44]. Peptide mixtures were resolved by strong cation exchange liquid chromatography followed by reversed phase liquid chromatography. Eluting peptides were electrosprayed into an LTQ ion trap mass spectrometer equipped with a nano-LC electrospary ionization source (ThermoFinnigan, San Jose, CA). Full MS spectra were recorded over a 400–1600 m/z range, followed by three tandem mass (MS/MS) events sequentially generated in a data-dependent manner on the first, second, and third most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan).
Database Search and Interpretation of MS/MS Datasets

Tandem mass spectra were extracted from raw files, and a binary classifier \(^{49}\), previously trained on a manually validated data set, was used to remove low quality MS/MS spectra. Remaining spectra were searched against a *Mus Musculus* protein database containing 50,370 protein sequences downloaded as FASTA-formatted sequences from EBI-IPI (database version 3.23, released on November 02, 2006), and 124 common contaminant proteins, for a total of 66,743 target database sequences \(^{50}\). To calculate confidence levels and false positive rates, we used a decoy database containing the reverse sequences of the 66,743 proteins appended to the target database, and the SEQUEST algorithm \(^{51}\) to find the best matching sequences from the combined database.

SEQUEST searches were done on an Intel Xeon 80-processor cluster running under the Linux operating system. The peptide mass search tolerance was set to 3 Da. No differential modifications were considered. No enzymatic cleavage conditions were imposed on the database search, so the search space included all candidate peptides whose theoretical mass fell within the 3 Da mass tolerance window, despite their tryptic status.

The validity of peptide/spectrum matches was assessed in DTASelect2 \(^{52}\) using SEQUEST-defined parameters, the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, and +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in 9 distinct sub-groups. In each one of the sub-groups, the distribution of XCorr and DeltaCN values for direct and decoy database hits was obtained, and the
two subsets were separated by quadratic discriminant analysis. Outlier points in the two distributions (for example, matches with very low Xcorr but very high DeltaCN) were discarded. Full separation of the direct and decoy subsets is not generally possible; therefore, the discriminant score was set such that a false positive rate of 5% was determined based on the number of accepted decoy database peptides. This procedure was independently performed on each data subset, resulting in a false positive rate independent of tryptic status or charge state.

**Plasminogen and t-PA Binding Assays**

Either the peptide, CEQSKLFSDK coupled to BSA, the reverse peptide, KDSFLKSQEC, or BSA, alone, was coated onto wells of microtiter plates at 10 μg/ml. The wells were blocked with 5% BSA. Either Glu-plasminogen or Lys-plasminogen was then incubated with the wells, followed by anti-plasminogen mAb51 raised in our laboratory, and detection with HRP-conjugated goat anti-mouse IgG (Biosource, Camarillo, CA). Binding of single chain t-PA was determined using anti-t-PA mAb (Calbiochem) followed by HRP-conjugated goat anti-mouse IgG (Biosource).

**Plasminogen Activation Assay**

Cells (8 X 10^4/ml) were preincubated with rat anti-Plg-RKT mAb 35B10 or rat IgG2a for 30 min at 37 °C. Glu-plasminogen (2.7 μM) was then added and incubated for an additional 30 min at 37 °C. Then 20 nM single-chain recombinant t-PA was added. Plasmin activity was measured after 3 min by diluting the reaction mixture 1:10 into S-
2251 (DiaPharma Group, Franklin, OH) to a final concentration of 1 mM and monitoring absorbance at 405 nm as described previously\textsuperscript{24,42}.

**Reagents**

Immuno Floure Mounting Medium was from CICN Biomedical, Inc. (Aurora, OH).
Results

Plasminogen Binding Capacity is Enhanced when Monocytes Differentiate

In order to establish a system whereby plasminogen receptors could be induced, we examined the effect of differentiation on plasminogen binding to monocytes. Peripheral blood monocytes were obtained from mononuclear cells isolated from freshly donated human blood as described. In FACS analysis as described, the cells were identified by their distinct forward scatter/side scatter properties compared with the other cells within the mononuclear cell preparations. Plasminogen binding was analyzed by dual-color FACS analysis using FITC-plasminogen and propidium iodide to gate for only viable cells as described. Specific binding was defined as binding that was inhibited by EACA. The viable peripheral blood monocytes bound plasminogen specifically, as defined by the shift in fluorescence in the presence of EACA. Monocytes were further purified from the mononuclear cell preparation by adherence as described in Materials and Methods and then treated with the differentiation inducing cytokine, M-CSF. The fluorescence signal indicating specific FITC-plasminogen binding to the viable M-CSF differentiated cells was 11-fold greater than that observed with the unstimulated monocytes. The CD antigen profiles were consistent with differentiation into macrophages (Fig.1, compare a and b) and the morphology of the cells changed from a rounded shape to a spindle-type morphology (data not shown).

Thus, differentiation of the monocytes toward the macrophage phenotype markedly upregulated plasminogen binding.
Our search for an integral membrane plasminogen receptor(s), exposing a C-terminal basic residue on the cell surface and upregulated during monocyte differentiation required large quantities of cells. Therefore, we tested whether the Hoxa9-ER4 cell line would behave as peripheral blood monocytes with respect to plasminogen binding capacity. The Hoxa9-ER4 cell line is derived from primary murine bone marrow myeloid precursors immortalized with an estrogen regulated conditional oncprotein, HoxA94-ER\textsuperscript{55}. The Hoxa9-ER4 line is factor-dependent (GM-CSF), and differentiates to monocytes when estrogen is removed from the medium, thereby inactivating the Hoxa9-ER protein. The mature monocytes respond to M-CSF\textsuperscript{41}. We compared plasminogen binding to undifferentiated and M-CSF-differentiated Hoxa9-ER4 progenitor cells. No specific binding of plasminogen to the Hoxa9-ER4 progenitor cells was detected (Fig. 1c, left most column). However, as the cells differentiated along the monocytic pathway, specific plasminogen binding to the cells was observed (Fig. 1d, left most column). The CD antigen profiles were consistent with differentiation into the macrophage phenotype (Fig. 1, compare c and d).

Binding isotherms were constructed for the specific interaction of plasminogen with the monocytoid cells using dual-color quantitative flow cytometry (Fig. 2). FACS data from the gated viable cell populations that were treated with different concentrations of FITC-plasminogen, in the presence or absence of EACA, were used to derive the apparent $K_d$ and $B_{\text{max}}$ of the interactions by fitting these data to the single site binding equation $[LR] = ([L]B_{\text{max}})/([L]+K_d)$. (The FACS signal that was inhibitable by EACA was calculated as specific binding.) The plasminogen binding isotherms with M-CSF-treated monocytes and M-CSF treated Hoxa9-ER4 cells were saturable (Fig. 2a, b). The
binding parameters determined for the M-CSF-differentiated Hoxa9-ER4 cells were apparent $K_d = 0.95$ μM and $B_{\text{max}} = 1.9 \times 10^5$ sites/cell. An apparent $K_d$ of 0.57 μM and a $B_{\text{max}}$ of $9.1 \times 10^5$ molecules/cell were calculated for M-CSF-treated monocytes. In contrast, with peripheral blood monocytes, the specific plasminogen binding isotherms did not reach saturation at a plasminogen concentration of 10 μM (Fig. 2a), suggesting that the $K_d$ is > 5 μM and the $B_{\text{max}}$ is > $4 \times 10^5$ molecules/cell. [Based on the data in Figure 2a, at blood and extracellular concentrations of plasminogen (2 μM 56), ~ $1 \times 10^5$ molecules of plasminogen are predicted to be bound to each circulating peripheral blood monocyte whereas ~ $5 \times 10^5$ molecules of plasminogen are predicted to be bound per macrophage.] Thus, differentiation increased both the affinity and capacity of monocytes and progenitor cells for plasminogen.

We examined whether the M-CSF-upregulated plasminogen binding sites were accessible to CpB. The M-CSF differentiated Hoxa9-ER4 cells were incubated with 100 U/ml CpB and washed, prior to determining FITC-plasminogen binding to the viable cells in FACS analysis. CpB treatment decreased specific FITC-plasminogen binding to the viable cells by 78%, consistent with our results with other monocytoid cells 24,29,57.

Isolation of a Regulated Integral Membrane Plasminogen Receptor Exposing a C-Terminal Basic Residue on the Cell Surface

We used MudPIT to probe the membrane proteome of M-CSF-differentiated Hoxa9-ER4 cells for the presence of a regulated integral membrane plasminogen receptor(s), exposing a C-terminal basic residue on the cell surface. Intact M-CSF-differentiated Hoxa9-ER4 cells were biotinylated as described in Materials and Methods.
Initially, using FACS analysis we verified that 1) viable cells were effectively biotinylated using anti-biotin FITC labeled monoclonal antibodies, 2) specific plasminogen binding by the cells was not decreased when cells were treated with the biotinylating reagent and 3) that biotinylated cells exhibited the same reduction in plasminogen binding capacity in response to CpB as non-biotinylated cells, as criteria that the biotinylated plasminogen receptors behaved as native plasminogen receptors. The cell membranes were then isolated, subjected to affinity chromatography on plasminogen-Sepharose, bound to immobilized avidin, digested with trypsin and subjected to MudPIT analysis, as described in Materials and Methods. Using this method, only one protein with a predicted transmembrane sequence and a C-terminal basic residue was identified: the hypothetical protein, C9orf46 homolog (IPI00136293), homologous to the protein predicted to be encoded by human chromosome 9, open reading frame 46. Table 1 lists the peptides corresponding to C9orf46 homolog that were obtained when the membrane fraction from M-CSF-treated Hoxa9-ER4 cells was subjected to MudPIT. Our isolation of peptides corresponding to C9orf46 homolog is, to our knowledge, the first demonstration of the existence of this protein. We have designated the protein, Plg-RKT, to indicate a plasminogen receptor with a C-terminal lysine and having a transmembrane domain (see below).

The C9orf46 homolog/Plg-RKT murine DNA sequence encodes a protein of 147 amino acids with a calculated molecular mass of 17,261 Da (Fig. 3, a, upper line). Notably, a C-terminal lysine is encoded. We blasted the C9orf46 homolog/Plg-RKT sequence against all species using NCBI Blast and obtained unique human, rat, dog and cow predicted orthologs, with high homology (e.g. human versus mouse = 94%
similarity), high identity and no gaps in the sequence (Fig. 3, a and Table 2). Of key importance, a C-terminal lysine was predicted for all of the mammalian orthologs obtained in the blast search (Fig. 1a). In a query of the Ensembl Gene Report, DNA sequences of all 9 other fully sequenced mammalian orthologs (Table 3) and 13 other partially sequenced mammalian orthologs encoded C-terminal lysines.

In addition, the primary sequence of C9orf46/Plg-R\textsubscript{KT} is apparently tightly conserved in humans, with no polymorphisms (cSNPs) within the 6 exons encoded by the gene (on chromosome 9p24.1) yet identified in the NCBI human genome sequence variation database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP).

The C9orf46 homolog/Plg-R\textsubscript{KT} sequence was analyzed in the TMpred site (http://www.ch.embnet.org/software/TMPRED_form.html). The strongly preferred model predicted two transmembrane helices extending from F\textsubscript{53}-L\textsubscript{73} (secondary helix, oriented from outside the cell to inside) and P\textsubscript{78}-Y\textsubscript{99} (primary helix, oriented from inside the cell to outside) (Fig. 3, b). The two transmembrane helices with the same orientation were also predicted using the HMMTOP \textsuperscript{58} site http://www.enzim.hu/hmmtop/. This model takes into account the “positive inside” rule in which positively charged residues in membrane proteins (e.g. residues K\textsubscript{74}R\textsubscript{75}K\textsubscript{76}K\textsubscript{77}) are found predominantly on the cytoplasmic side of the membrane\textsuperscript{59}. Notably, a highly positively charged loop (residues 74-77) between the two predicted transmembrane domains is conserved in the predicted protein sequences of all mammalian orthologs for which either full or partial sequences are available (e.g. Table 3). When analyzed in the Split 4.0 Server (Membrane Protein Secondary Structure Prediction Server) site (http://split.pmfst.hr/split/4/), only the region extending from F\textsubscript{80}-Q\textsubscript{94} was identified as a predicted transmembrane helix. Thus, the
possibility that the N-terminus may be imbedded in the cytoplasm cannot be excluded. Nonetheless, the conservation of the highly charged positive loop (residues 74-77) strongly favors the model in which F53-L73 also is a transmembrane domain (with the N-terminus outside) because the presence of positively charged residues at the C-terminus of apolar domains in proteins favors the insertion of the upstream apolar domains into the cell membrane 60.

Several key aspects of our experimental paradigm support the topological model shown in Fig. 3b. First, recovery of peptides corresponding to C9orf46 homolog relied on accessibility to the biotinylation reagent in the context of intact cells, supporting the exposure of Plg-RKT domains on the cell surface. The biotinylation reagent we used reacts with the carboxyl moieties of the R-groups of either Asp or Glu. Thus, biotinylation of Plg-RKT on intact cells would not occur if the highly basic loop between the transmembrane helices (K74-K77) was exposed on the cell surface. Hence, a model in which the amino and C-termini are both on the cytoplasmic face of the membrane can be excluded.

Second, a key prediction from the model in Fig. 3b is the presence of a 48 amino acid C-terminal tail with a C-terminal lysine exposed on the cell surface. We addressed this prediction experimentally. When intact M-CSF-treated Hoxa9-ER4 cells were incubated with CpB prior to subcellular fractionation, purification and MudPIT analysis, no peptides corresponding to C9orf46 homolog were detected. These data are consistent with exposure of the C-terminal lysine of Plg-RKT on the cell surface and, hence, accessibility to CpB 32, so that CpB treatment of intact cells resulted in loss of the ability of Plg-RKT to bind to the plasminogen-Sepharose column.
Upregulation of Plg-RKT in Membranes of M-CSF-treated Cells

To assess whether expression of C9orf46 homolog/Plg-RKT was increased during cellular differentiation, biotinylated membrane fractions from undifferentiated progenitor Hoxa9-ER4 cells were subjected to the purification method described above. In the absence of M-CSF treatment, no peptides corresponding to C9orf46 homolog/Plg-RKT were detected, suggesting that membrane expression of Plg-RKT was markedly upregulated by M-CSF treatment.

To further assess the M-CSF-responsiveness and membrane localization of Plg-RKT we raised a monoclonal antibody in rats against the synthetic peptide, CEQSKLFSDK (corresponding to the nine C-terminal amino acids of murine Plg-RKT with an amino terminal cysteine added for coupling). Membrane and cytoplasmic fractions from progenitor and M-CSF-differentiated Hoxa9-ER4 cells were electrophoresed and western blotted with either anti-Plg-RKT mAb or isotype control. A specific immunoreactive band migrating with an Mr_{app} of ~17,000, consistent with the predicted molecular mass of C9orf46 homolog/Plg-RKT, was detected in membranes from M-CSF-differentiated cells (Fig. 4, a), clearly demonstrating the existence of this novel protein. The protein was not detected in membrane fractions from progenitor Hoxa9-ER4 cells or in cytoplasmic fractions from either progenitor or differentiated cells (Fig. 4, a). These data further demonstrate the responsiveness of the C9orf46 homolog gene to a maturation-inducing signal, and verify the presence of C9orf46 homolog /Plg-RKT in the cell membrane.
Plg-R_KT Partitions to the Detergent Phase

To test the prediction that Plg-R_KT is an integral membrane protein, M-CSF-differentiated Hoxa9-ER4 cell membranes were subjected to phase separation in Triton X-114 as described 61,62. In this technique, integral membrane proteins form mixed micelles with the nonionic detergent and are recovered in the Triton X-114 detergent phase, whereas hydrophilic proteins remain in the aqueous phase. An immunoreactive band migrating with a Mr_{app} of ~17,000 was detected in the detergent phase in western blotting with anti-Plg-R_KT mAb, but was not detected in the aqueous phase (Fig. 4, b). These data further support the prediction that Plg-R_KT is an integral membrane protein.

Plg-R_KT is Dispersed Over the Cell Surface and Colocalizes with uPAR

Confocal microscopy was performed to examine the subcellular expression of Plg-R_KT and to examine whether Plg-R_KT co-localized with uPAR, an additional key component of the cell-surface plasminogen activation system. Plg-R_KT was immunodetected in small aggregates (~0.2 μm^2) dispersed over the cell surface (Fig. 5, panel a, green). uPAR was also immunodetected in small aggregates (~0.2 μm^2) dispersed over the cell surface (Fig. 5a, red), consistent with published data 63. Plg-R_KT colocalized with uPAR as revealed by merged images (Fig. 5.a). The extent of colocalization of Plg-R_KT with uPAR was 73 ± 3 %. These results suggest that Plg-R_KT and uPAR are present in very close proximity on the cell surface in an orientation to promote plasminogen activation.

In order to determine whether the epitope recognized by anti-Plg-R_KT mAb functioned as a plasminogen binding site, the cells were either preincubated with buffer
or plasminogen, fixed and then stained with polyclonal anti-plasminogen or anti-Plg-RKT mAb. As with the polyclonal anti-Plg-RKT, Plg-RKT was immunodetected in small aggregates (0.16+/−0.03 to 0.26+/−0.03 μm²) dispersed over the cell surface (5,b, red). However, following pre-incubation with plasminogen, the ability to immunodetect Plg-RKT was reduced by half (Fig. 5,b,c). These results suggest that plasminogen and the anti-Plg-RKT mAb (directed against the C-terminal peptide) share binding sites on the cell and that plasminogen binds to the C-terminal domain of Plg-RKT on the cell surface.

**Plasminogen Binds Specifically to the Carboxyl Terminal Peptide of Plg-RKT**

To further explore the functional importance of the C-terminal lysine, we tested whether the synthetic peptide, corresponding to the C-terminus of Plg-RKT could bind plasminogen. The peptide, CEQSKLFSDK, was coupled to BSA and then coated onto wells of microtiter plates. Glu-plasminogen was incubated with the wells, followed by our anti-plasminogen mAb ⁵³ and detection with HRP-conjugated goat anti-mouse IgG. Glu-plasminogen bound to the peptide in a concentration dependent manner, achieving half saturation at a concentration of 7.6 nM (Fig. 6, a). The binding was specific because it was blocked in the presence of EACA, consistent with the ability of EACA to inhibit plasminogen binding to differentiated Hoxa9-ER4 cells (Fig. 1,d). Lys-plasminogen also bound specifically to the peptide (Fig. 6, b) and the concentration for half saturation was < 2.7 nM, consistent with the higher affinity of Lys-plasminogen compared to Glu-plasminogen for cell surfaces ⁶⁴,⁶⁵. We investigated the interaction of t-PA with the C-terminal peptide because t-PA and plasminogen share binding sites on monocytoid cells and t-PA binding to monocytoid cells is sensitive to CpB ⁵⁷. Concentration dependent
binding of t-PA to the peptide was observed (Fig. 6,c) and the concentration for 50% saturation was 3.2 nM, consistent with the relative affinities obtained when comparing Glu-plasminogen and t-PA binding to cell surfaces \(^6\). We noted that the concentration for 50% saturation of plasminogen binding to immobilized CEQSKLFSDK-coupled to BSA were much greater than the Kd value we had determined for plasminogen binding to cells. These differences in apparent affinity when plasminogen binds to the immobilized peptide compared to the cell surface may be due to our use of BSA-conjugated peptide to coat the plate. If multiple peptides were conjugated to the BSA that may provide a higher affinity surface than the cell surface receptor, since plasminogen has multiple kringle domains that may interact in a cooperative lysine-dependent manner with multiple Plg-R\(_{KT}\) peptides on a single BSA molecule. In order to resolve this issue, we tested the ability of the soluble C-terminal peptide to inhibit Glu-plasminogen binding under solution phase equilibrium conditions. The soluble peptide competed for Glu-plasminogen binding in a dose-dependent manner with an IC\(_{50}\) of 2 \(\mu\)M (Fig. 6, d), similar to the Kd values we had determined for Glu-plasminogen binding to both human M-CSF-treated monocytes and M-CSF treated Hoxa9-ER4 cells, above. In addition, a mutated peptide with the C-terminal lysine substituted with alanine did not compete for plasminogen binding at concentrations up to 1 mM (Fig. 6, d), further supporting the role of the C-terminal lysine in the interaction of Plg-R\(_{KT}\) with plasminogen.

**Plg-R\(_{KT}\) Regulates Cell Surface Plasminogen Activation**

We found that plasminogen activation was stimulated 12.7-fold in the presence of M-CSF treated Hox9-ER4 cells, compared to the reaction in the absence of cells and that
cell-dependent plasminogen activation was stimulated 4.4-fold on differentiated cells, compared to undifferentiated cells (Fig. 7). In order to verify the role of Plg-RKT in plasminogen activation, we tested the effect of a monoclonal antibody (anti-Plg-RKT mAb 35B10) raised in rats against the synthetic peptide, CEQSKLFSDK. The IgG fraction reacted with the C-terminal peptide of murine Plg-RKT, blocked plasminogen binding to CEQSKLFSDK (Fig. 6,e), and specifically recognized a protein with a Mr_app of ~17,000 in western blotting of M-CSF treated Hoxa9-ER4 cells (Fig. 4). In FACS analysis of M-CSF treated Hox9-ER4 cells, anti-Plg-RKT mAb 35B10 reacted specifically with the cell surface giving a mean fluorescence intensity value of 93.2 compared to the mean fluorescence intensity value for the rat IgG2a isotype control of 32.8. Anti-Plg-RKT mAb 35B10 substantially suppressed cell-dependent plasminogen activation by 46% and suppressed cell differentiation-dependent plasminogen activation by 58% (Fig.7). In controls, plasminogen activation in the absence of cells or on undifferentiated cells was not affected by anti-Plg-RKT mAb.
Discussion

Localization of plasminogen on cell surfaces is a crucial control point for positive regulation of cell surface plasmin proteolytic activity that facilitates both physiological and pathological processes. Plasminogen binding sites, with C-terminal basic residues, are predominantly responsible for the ability of eukaryotic cells to bind plasminogen and promote plasminogen activation. In the current study, we identified a novel protein, Plg-R\text{KT}, the first integral membrane plasminogen receptor, synthesized with a C-terminal lysine.

Our results support a mechanism in which Plg-R\text{KT} binds plasminogen via an interaction requiring the C-terminal lysine of Plg-R\text{KT} that is exposed on the cell surface. When intact, differentiated cells were pre-treated with CpB, prior to membrane fractionation, Plg-R\text{KT} was not detectable in the MudPIT analysis, indicating that the C-terminal lysine of Plg-R\text{KT} was accessible to CpB on the cell surface and, thus, lost the ability to bind plasminogen-Sepharose following CpB treatment of intact cells. Secondly, Glu-plasminogen bound specifically to a synthetic peptide corresponding to the C-terminus of Plg-R\text{KT} and the binding interaction was inhibited by the lysine analog, EACA that inhibits the interaction of plasminogen with the cell surface. Lys-plasminogen also bound specifically to the peptide consistent with the ability of Lys-plasminogen to also bind to the cell surface. Thirdly, plasminogen binding to a mutated peptide with the C terminal lysine substituted with alanine, was not detected, further supporting the key role of the C-terminal lysine in the interaction with plasminogen. Of key importance, anti-Plg-R\text{KT} antibodies directed against the C-terminal peptide of Plg-R\text{KT}, detected Plg-R\text{KT} dispersed over the cell surface in small aggregates. A similar immunolocalization
pattern of small aggregates dispersed over the cell surface has been published for other cell surface plasminogen binding proteins, α-enolase, histone 2B, p11 and annexin 2. Of key importance, following pre-incubation of the cells with plasminogen, the ability to immunodetect Plg-RKT was reduced by half (Fig. 5b, c). These results suggest that plasminogen and the anti-Plg-RKT mAb share binding sites on the cell and that plasminogen binds to the C-terminal domain of Plg-RKT on the cell surface. Notably, a C-terminal lysine is encoded for all 26 mammalian orthlogs of Plg-RKT for which the C-terminal sequence is available, consistent with a fundamental role of Plg-RKT to bind plasminogen in mammals.

T-PA also bound specifically to the C-terminal peptide of Plg-RKT, consistent with sharing of binding sites with plasminogen and with the CpB-sensitivity of the interaction of t-PA with cells. Despite potentially sharing a binding site on Plg-RKT, the relative concentrations of t-PA and plasminogen in the circulation should permit simultaneous binding of both ligands to the cell surface, and each t-PA molecule should be bound proximally to several plasminogen molecules. We also found that that Plg-RKT was spatially highly colocalized with the uPAR receptor. These results suggest a mechanism for co-localization of plasminogen with plasminogen activators to promote cell surface plasminogen activation. Of key importance, treatment of the cells with anti-Plg-RKT mAb markedly reduced the ability of M-CSF treated Hoxa9-ER4 cells to stimulate plasminogen activation.

Our experimental data support the prediction that Plg-RKT is a multipass transmembrane protein, exposing a C-terminal lysine on the cell surface. Our results summarized above supported the cell surface exposure of the C-terminus. Regarding
transmembrane topology, Plg-R\textsubscript{KT} was immunochemically detected in the membrane fraction, but not the cytoplasmic fraction of differentiated cells. And, in phase partitioning experiments, Plg-R\textsubscript{KT} partitioned to the detergent phase.

Our data also indicate that Plg-R\textsubscript{KT} is upregulated during differentiation. In the MudPIT analysis Plg-R\textsubscript{KT} peptides were detected in M-CSF-differentiated cells, but not in progenitor cells. Similarly, in western blotting Plg-R\textsubscript{KT} was detected in membranes of differentiated cells, but not in the progenitor cells.

The C9orf46 homolog/Plg-R\textsubscript{KT} transcript is broadly expressed in normal human and mouse tissues [as determined in high-throughput gene expression profiling in which RNA samples from human and murine tissues were hybridized to high-density gene expression arrays \textsuperscript{67,68}], including spleen, thymus, lymph node, lung, intestine, bone marrow, adrenal, pituitary, and other endocrine tissues, vascular tissue, kidney, liver, stomach, bladder, and neuronal tissue (hippocampus, hypothalamus, cerebellum, cerebral cortex, olfactory bulb and dorsal root ganglion). We examined Plg-R\textsubscript{KT} protein expression in murine tissue and detected Plg-R\textsubscript{KT} in lymph node, bone marrow and intestine, tissues rich in leukocytes but not in adipose tissue, as a negative control (Fig. S1 in Supplemental Data). Differences in mRNA expression data and western blotting results may be explained by high expression by specific cells within tissues. In western blotting, we also have detected Plg-R\textsubscript{KT} in rat hippocampal neurons, bovine adrenal tissue and in rat pheochromocytoma PC12 cells (Parmer, et al., unpublished observations).

We searched for C9orf46 homolog/Plg-R\textsubscript{KT} mRNA microarray expression data at http://www.ebi.ac.uk/gxa/ (Table 4). C9orf46 homolog/Plg-R\textsubscript{KT} mRNA is present in monocytes, leukocytes, NK cells, T cells, myeloid, dendritic, and plasmacytoid cells,
breast cancer, acute lymphoblastic leukemia and Molt-4 acute lymphoblastic leukemia cells. These data are consistent with previous reports documenting expression of plasminogen binding sites on peripheral blood leukocytes \(^5\), breast cancer cells \(^1\), and other tissues [reviewed in \(^2\)]. In addition, results obtained by searching the ArrayExpress Warehouse (http://www.ebi.ac.uk/gxa/) indicated that the C9orf46 homolog gene is also regulated in other tissues by lipopolysaccharide, canrenoate, H\(_2\)O\(_2\) and dexamethasone (Table 5). The broad distribution and regulation in tissues that express plasminogen binding sites, suggest that C9orf46 homolog/Plg-R\(_{KT}\) provides plasminogen receptor function that may serve to modulate plasmin proteolytic functions in these tissues, as well. In genome-scale quantitative image analysis, overexpression of more than 86 cDNAs, including C9orf46 homolog, conferred dramatic increases in cell proliferation, while knockdown of C9orf46 homolog mRNA resulted in apoptosis \(^7\). In microarray studies, C9orf46 homolog mRNA expression has a high power to predict cervical lymph node metastasis in oral squamous cell carcinoma \(^7\). We have previously shown that cell-bound plasminogen protects monocytoid cells from TNF\(\alpha\)-induced apoptosis \(^7\). Future studies should elucidate the relationship of these roles of C9orf46 homolog/ Plg-R\(_{KT}\) to its plasminogen receptor function.

In summary, regulation of the cell surface expression of plasminogen receptors is a mechanism by which cells modulate the proteolytic activity of their environment. We have identified a novel plasminogen receptor with unique characteristics: Plg-R\(_{KT}\) is an integral membrane protein that exposes a C-terminal lysine and serves to markedly promote plasminogen activation on the cell surface.
Acknowledgments

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Authorship

Reference List


Table 1. Peptides obtained corresponding to C9orf46 homolog

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SEQUEST-defined parameters (Xcorr, DeltCN, and Conf%) are shown for each peptide. (core: cross-correlation score; DeltCN: normalized difference in cross-correlation scores; Conf%: confidence level of the peptide; ObsM+H+: observed peptide mass; CalcM+H+: theoretical peptide mass). Observed peptide mass, theoretical peptide mass, and charges of the peptide identified (3+ or 2+) are also shown to demonstrate accurate peptide identification.
Table 2. Plg-R\textsubscript{KT} sequence identities and homologies

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B. % Interspecific amino acid sequence homologies

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Table 3 - Alignment of known mammalian orthologs of C9orf46 homolog/Plg-RKT

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C9orf46 homolog/Plg-RKT microarray mRNA expression data are from [http://www.ebi.ac.uk/gxa/](http://www.ebi.ac.uk/gxa/).
Table 5. Regulation of C9orf46 homolog/Plg-R$_{KT}$ mRNA in Tissues

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Data were obtained from ArrayExpress Warehouse (http://www.ebi.ac.uk/gxa/).
* ↓ = downregulation.
**Figure Legends**

**Figure 1.** Specific plasminogen binding is enhanced by M-CSF treatment of human peripheral blood monocytes and Hoxa9-ER4 cells. Human peripheral blood monocytes were either untreated (a) or treated with 0.44 nM M-CSF for 8 days (b) and Hoxa9-ER4 cells were either untreated (c) or treated with 20% LADMAC conditioned media (a source of M-CSF) for two days (d). The cells were analyzed by dual color fluorescence activated cell sorting (FACS) analysis for specific plasminogen binding and CD antigen expression as described 10. Viable (propidium iodide negative and annexin V negative) cells were gated from the non-viable cells. Histogram plots of FITC-plasminogen (left columns) or specific anti-CD antibody binding to viable cells are shown. Gray tracings = either FITC-plasminogen or specific anti-CD antibody. Black tracings = either FITC-plasminogen + 0.2 M EACA or isotype controls.

**Figure 2.** Equilibrium binding of plasminogen is observed to viable human peripheral blood monocytes and viable Hoxa9-ER4 cells. Human peripheral blood monocytes (a) were either untreated (open circles) or treated with M-CSF (closed circles) as in Fig.1 and Hoxa9-ER4 cells (b) were treated with M-CSF as in Fig.1 and subjected to quantitative flow cytometry with increasing concentrations of FITC-plasminogen as described in Materials and Methods.

**Figure 3.** Alignment of predicted amino acid sequences of mouse, human, rat, dog and cow orthologs of Plg-RKT (a) and the structural model of Plg-RKT (b) show high interspecies sequence homology. Green indicates amino acids within the predicted
primary transmembrane helix. Orange indicates amino acids within the predicted secondary transmembrane helix. Red indicates basic amino acids.

**Figure 4.** **Plg-RKT behaves as a regulated integral membrane protein.** a. Membrane fractions or cytoplasmic fractions from either undifferentiated or M-CSF-treated Hoxa9-ER4 cells (30 μg/lane) were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels under reducing conditions and western blotted with either anti-Plg-RKT mAb, anti-α-enolase mAb as a loading control, or isotype control IgG. b. M-CSF-treated Hoxa9-ER4 cell membranes were solubilized in 3% Triton X-114. After heating at 37°C and separation of the phases by centrifugation, an aliquot of both phases was electrophoresed and western blotted with anti-Plg-RKT mAb 35B10. In controls for the method, when the cell lysates were spiked with BSA and subjected to phase partitioning, BSA was detected in the aqueous, but not the detergent phase (data not shown).

**Figure 5.** **Plg-RKT is dispersed over the cell surface and colocalizes with uPAR.** M-CSF-differentiated Hoxa9-ER4 cells were grown on coverslips and incubated with a combination of polyclonal rabbit anti-Plg-RKT IgG (20 μg/ml) and mouse monoclonal anti-uPAR (20 μg/ml) (a). Cells were washed, fixed in 1% formaldehyde and then stained with a combination of Alexa 488- F(ab’)2 of goat anti-rabbit IgG and Alexa 568-F(ab’)2 fragment of goat anti-mouse IgG for 60 min at 20 °C in PBS containing 0.001% Triton X-100. Controls are samples incubated without first antibody. In panel b, cells were either preincubated with PBS (- plasminogen) or 2 μM plasminogen (+ plasminogen) for 10 min at 4°C. Then, the cells were fixed in 1% formaldehyde, washed and then stained.
with polyclonal anti-Plg IgG or mouse anti-Plg-RKT mAb and stained with a combination of Alexa 488- F(ab’)2 of goat anti-rabbit IgG and Alexa 568- F(ab’)2 fragment of goat anti-mouse IgG. Cells were washed and mounted in Immunofloure Mounting Medium. Images were captured using a Zeiss Confocal Laser Scanning Microscope, then imported into LSM Examiner and Image J for further processing as described in Materials and Methods. The data in panel c were quantified and the number and size of each labeled aggregate were determined as described in Materials and Methods. The results reflect counts (panel c) and colocalization correlation coefficients (M1) values (last column in panels a and b) from over 40 cells in 2 independent experiments. *p < 0.001.

**Figure 6. Plasminogen binds to the C-terminal peptide of Plg-RKT.** The peptide, CEQSKLFSKDK, corresponding to the amino terminus of Plg-RKT was coupled to BSA and coated onto wells of microtiter plates. Either Glu-plasminogen (a) or Lys-plasminogen (b) or t-PA (c) was then incubated with the wells, followed by anti-plasminogen mAb 53 (a,b) or anti-t-PA polyclonal mAb (c) and detection with HRP-conjugated goat anti-mouse IgG (closed circles) as described in Materials and Methods. The binding was specific because it was blocked in the presence of 0.2 M EACA (open triangles), consistent with the ability of EACA to inhibit plasminogen binding to differentiated Hoxa9-ER4 cells. In additional controls, nonspecific binding to either BSA (closed triangles), or to the reverse peptide (open circles) was <10% of binding to CEQSKLFSKDK. (At high input concentrations of t-PA nonspecific binding increased, but was <10% of binding to CEQSKLFSKDK at the concentration required for 50% saturation (3.2 nM). In controls for the detection method, O.D.490 values obtained using
an isotype control antibody or in the absence of added plasminogen or t-PA were <5% of the values for plasminogen or t-PA binding to immobilized CEQSKLFSDK. (Panels d and e: Biotinylated-Glu-plasminogen (25 nM) was incubated with immobilized CEQSKLFSDK in the presence of increasing concentrations of (d) the C-terminal peptide, CEQSKLFSDK (closed circles) or a mutated C-terminal peptide with K147 substituted with alanine, CEQSKLFSDA (open circles) or (e) anti-Plg-RKT mAb 35B10 (closed circles) or isotype control (open circles). Biotinylated Glu-plasminogen binding was detected with HRP-streptavidin and was 96% inhibited in the presence of 0.2 M EACA (not shown). Data are as mean ± SEM, n=3, for each determination.

Figure 7. Plg-RKT regulates cell surface plasminogen activation. Plasminogen activation was determined after 12 min as described in Materials and Methods in either the presence or absence of either undifferentiated Hoxa9-ER4 progenitor cells or M-CSF-differentiated Hoxa9-ER4 cells and in the presence of either rat anti-Plg-RKT mAb 35B10 (filled bars) or isotype control rat IgG2a (open bars). ***p < 0.001, compared to the corresponding isotype control.
Figure 1
Figure 2

(a) Graph showing the relationship between bound molecules/cell x 10^5 and plasminogen concentration (μM). The solid line represents the experimental data, while the dashed line is a theoretical curve.

(b) Enlarged view of the lower part of Figure 2(a) to illustrate the data points more clearly.

Legend:
- Solid circles: Experimental data points
- Open circles: Theoretical model predictions

Y-axis: Bound molecules/cell x 10^5
X-axis: Plasminogen concentration (μM)
Proteomics-based discovery of a novel, structurally unique, and developmentally regulated plasminogen receptor, Plg-R_KT, a major regulator of cell surface plasminogen activation