Conversion of Glu-Plasminogen to Lys-Plasminogen Is Necessary for Optimal Stimulation of Plasminogen Activation on the Endothelial Cell Surface*

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Yun Gong, Sun-OK Kim‡, Jordi Felez§, Davida K. Grella, Francis J. Castellino¶, and Lindsey A. Miles|
From The Scripps Research Institute, La Jolla, California 92037, ‡The University of Notre Dame, Notre Dame 46556, Indiana, and the §Institut De Recerca Oncologica, 08907 Barcelona, Spain

When Glu-plasminogen is bound to cells, plasmin (Pm) formation by plasminogen (Pg) activators is markedly enhanced compared to the reaction in solution. It is not known whether the direct activation of Glu-Pg by Pg activators is promoted on the cell surface or whether plasminolytic conversion of Glu-Pg to the more readily activated Lys-Pg is necessary for enhanced Pm formation on the cell surface. To distinguish between these potential mechanisms, we tested whether Pm formation on the cell surface could be stimulated in the absence of conversion of Glu-Pg to Lys-Pg. Rates of activation of Glu-Pg, Lys-Pg, and a mutant Glu-Pg, [D646E]Glu-Pg, by either tissue Pg activator (t-PA) or urokinase (u-PA) were compared when these Pg forms were either bound to human umbilical vein endothelial cells (HUVEC) or in solution. ([D646E]Glu-Pg can be cleaved at the Arg561–Val562 bond by Pg activators but does not possess Pm activity subsequent to this cleavage because of the mutation of Asp646 of the serine protease catalytic triad.) Glu-Pg activation by t-PA was enhanced on HUVEC compared with the solution phase by 13-fold. In contrast, much less enhancement of Pg activation was observed with [D646E]Glu-Pg (~2-fold). Although the extent of activation of Lys-Pg on cells was similar to that of Glu-Pg, the cells afforded minimal enhancement of Lys-Pg activation compared with the solution phase (1.3-fold). Similar results were obtained when u-PA was used as activator. When Glu-Pg was bound to the cell in the presence of either t-PA or u-PA, conversion to Lys-Pg was observed, but conversion of [D646E]Glu-Pg to [D646E]Lys-Pg was not detected, consistent with the conversion of Glu-Pg to Lys-Pg being necessary for optimal enhancement of Pg activation on cell surfaces. Furthermore, we found that conversion of [D646E]Glu-Pg to [D646E]Lys-Pg by exogenous Pm was markedly enhanced (~20-fold) on the HUVEC surface, suggesting that the stimulation of the conversion of Glu-Pg to Lys-Pg is a key mechanism by which cells enhance Pg activation.

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‡ Present Address: Han Nong Central Research Center, Dongbu Advanced Research Inst., 103-2 Moonji-dong, Daeduck Science Town, Taejon 305-380 Korea.
§ To whom correspondence should be addressed: The Scripps Research Inst., CVN-26 10550 North Torrey Pines Rd, La Jolla, CA 92037. Tel.: 858-784-7105; Fax: 858-784-7374; E-mail: lmiles@scripps.edu.
¶ The abbreviations used are: Glu-Pg, the native form of plasminogen with N-terminal Glu; [D646E]Glu-Pg, a recombinant human plasminogen with Asp646 mutated to Glu; EACA, epsilon aminocaproic acid; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; hmm u-PA, high molecular weight urokinase; HUVEC, human umbilical vein endothelial cells; [D646E]Lys-Pg, [D646E]Glu-Pg cleaved by plasmin; hmw u-PA, low molecular weight urokinase; Lys-Pg, a proteolytic derivative of Glu-Pg with N-terminal Met66, Lys77, or Val56; Pm, plasmin; PAGE, polyacrylamide gel electrophoresis; t-PA, tissue plasminogen activator.

When Glu-plasminogen (Glu-Pg), the native circulating form of the zymogen, is bound to cell surfaces, plasmin (Pm) generation by plasminogen (Pg) activators is markedly stimulated compared with the reaction in solution (1–10). This results in arming cell surfaces with the proteolytic activity of Pm. In the case of endothelial cells, Pm becomes localized to sites of thrombus formation and, in the case of leukocytes, the cells become armed with proteolytic activity required for processes in which cells degrade extracellular matrices to migrate. However, a key component of the mechanism of stimulation of Pg activation on the cell surface is not understood. It is not known whether: 1) direct activation of Glu-Pg by Pg activators is promoted on the cell surface or 2) plasminolytic conversion of Glu-Pg to Lys-Pg is necessary to observe enhanced Pm formation on the cell surface. (Pm catalyzes cleavage of Glu-Pg at the carboxyl sides of Lys55, Arg61, Lys77 (11–13) and at additional minor sites (14) to generate new amino termini of Pg, resulting in Pg molecular forms that are collectively termed “Lys-Pg”. Lys-Pg is more readily activated by Pg activators (15–17).) In the first mechanism, Glu-Pg remains uncleaved, yet its direct activation is promoted on the cell surface relative to the solution phase, perhaps through conformational changes induced in the molecule upon its interaction with the cell surface. In the second mechanism, conversion of Glu-Pg by Pm to yield the more readily activated Lys-Pg is necessary, leading to increased Pm production on the cell surface. Furthermore, it is not known whether localization of Glu-Pg on the cell surface enhances its conversion to Lys-Pg by Pm. This question has been addressed also in the current study.

In previous studies, using kinetic assays (1–10) it was not possible to distinguish between the two mechanisms listed above to explain the stimulation of activation of Glu-Pg in the presence of cells. In these earlier studies, cell-associated Glu-Pg was converted to Lys-Pg (~50–60% conversion) on the surfaces of both U937 monocyteoid cells (5) and HUVEC (18) in the absence of added Pg activators or Pm. Thus, it was not possible to distinguish whether the direct activation of Glu-Pg was enhanced on the cell surface compared with the...
reaction in solution. In contrast, in our studies, Glu-Pg remained in its native form, without conversion to Lys-Pg when bound to HUVEC. This enabled us to address the role of conversion of Glu-Pg to Lys-Pg in enhancement of activation on the cell surface. In the current investigation, we employed a Pg recombinant variant, [D646E]Glucose-Pg, to assess the requirement for conversion of Glu-Pg to Lys-Pg on the cell surface for stimulation of Pg activation. This recombinant Pg/Pm variant can be converted to the molecular form of Pm, but does not possess Pm activity because of the absence of the necessary Asp residue in the serine proteolytic catalytic triad (19). Hence, [D646E]Glucose-Pg is not converted to [D646E]Lys-Pg following its cleavage by Pg activators. [D646E]Glucose-Pg was used as the inactive mutant to be as conservative as possible in introducing an amino acid substitution into the active site triad. The rates of activation of Glu-Pg, Lys-Pg, and [D646E]Glucose-Pg by either t-PA or u-PA were compared when these forms were either bound to HUVEC or in solution. These experiments were designed to distinguish between two potential mechanisms by which Pm formation is enhanced on the cell surface compared with the reaction in solution, or 2) plasminolytic cleavage is necessary for stimulation of Glu-Pg activation on the cell surface. Furthermore, we examined whether conversion of [D646E]Glucose-Pg to [D646E]Lys-Pg by exogenous Pm was enhanced on the HUVEC surface.

**EXPERIMENTAL PROCEDURES**

**Proteins—**Glu-Pg was purified from fresh human blood collected into 3 mM benzamidine, 3 mM EDTA, 100 units/ml Trasylol (Pentex Miles, Inc., Kankakee, IL), and 100 μg/ml soybean trypsin inhibitor (Sigma). The plasma was subjected to affinity chromatography on lysine-Sepharose (20) in phosphate-buffered saline (0.01 M sodium phosphate pH 7.3, 0.15 M NaCl) with 1 mM benzamidine, 0.02% NaN₃, and 3 mM EDTA, followed by molecular exclusion chromatography on Biogel A 1.5 M (Bio-Rad, Hercules, CA). The Pg concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 16.8. Lys-Pg was from the National Institute for Biological Standards and Control (Holly Hill, Hampstead, London). The Lys-Pm control was prepared by incubating Lys-Pg with 10 mM high molecular weight (hmw) u-PA. (Calbiochem, San Diego, CA) for 30 min at 37 °C. Pm was from Amersham Pharmacia Biotech/Chromogenix (Uppsala, Sweden). [D646E]Glucose-Pg was generated by primer-directed mutagenesis of single-stranded p119/HpG (21) as previously described (22) and expressed in baculovirus-infected lepidopteran cells, followed by purification on lysine-Sepharose as described (20). The characteristics of this mutant have been described (19). Pg forms were radiolabeled using a modified chloramine T procedure as described (1). t-PA was from Genentech (South San Francisco, CA). Low molecular weight (lwm) u-PA was from Calbiochem (San Diego, CA).

**Cells, Cell Culture, and Ligand Binding Analyses—**HUVECs were purchased from Clonetics/BioWhittaker (Walkersville, MD) and cells of passage four and below were used in these experiments. HUVEC, grown previously described from our laboratory (23). Briefly, HUVEC, grown to confluence in 131 medium containing 2% fetal calf serum, 12 μg/ml bovine brain extract, 50 ng/ml amphotericin B, 50 μg/ml gentamicin, 1 μg/ml hydrotassium, 1 ng/ml human epidermal growth factor.

The binding of radiolabeled Pg forms to HUVEC was performed as previously described from our laboratory (23). Briefly, HUVEC, grown to confluence in 24-well culture dishes (6–8 × 10⁵ cells/cm²), were washed three times with HBSS. The radiolabeled Pg forms were incubated with the cells in a final total volume of 200 μl at a final concentration of 25 nm. Reactions were terminated by aspirating the fluid from the wells and rapidly washing the cultures twice with HBSS-BSA. The cell-bound radioactivity was extracted with 100 μl of reduced sample buffer (31.2 mM TrisHCI, pH 7.2, 2% SDS, 10% sucrose, 0.002% bromphenol blue, 15 mM diethiothreitol, 10 mM EDTA, 10 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor (Sigma), 0.02% Na azide, 5 units/ml Trasylol (Miles, Inc., Kankakee, IL)). Greater than 90% of the cell-associated ligand was eluted by this procedure as assessed by comparing counts bound to the cells prior to elution, with counts eluted. Samples were subjected to 7% SDS-PAGE under reducing conditions that distinguish two-chain Pm from the single chain Pg and were exposed to Biomax MR film. The autoradiograms were scanned on an Alpha Imager™ 2000.

**RESULTS**

Role of the Glu-Pg to Lys-Pg Conversion in Pg activation on the cell surface. A concentration of 25 nm of either [125I]-Glu-Pg (A), [125I]-[D646E]Glucose-Pg (B), or [125I]-Lys-Pg (C) was incubated with buffer (HBSS-0.4% BSA) or HUVEC (5 × 10⁴) in the presence or absence of 20 μM t-PA for the indicated times at 37 °C. Cell-bound ligand was obtained as described under “Experimental Procedures” and subjected to electrophoresis on 7% SDS-PAGE under reducing conditions.

**Fig. 1. Role of the Glu-Pg to Lys-Pg Conversion in Pg activation by t-PA on the Cell Surface**—To monitor the activation of [125I]-Glu-Pg, [125I]-[D 646E]Glucose-Pg and [125I]-Lys-Pg by t-PA, SDS-PAGE was employed because kinetic assays could not be performed on [D646E]Glucose-Pg because its Pm form is proteolytically inactive. We found that in the absence of added t-PA, >95% of the added [125I]-Glu-Pg and [125I]-[D646E]Glucose-Pg remained in their Glu-Pg forms, and Pg activation was not detected on these cells (Fig. 1). Activation of the three ligands by 20 μM t-PA was compared on HUVEC with the reaction in the solution phase, in the absence of cells. The cell-bound ligand was recovered and subjected to 7% SDS-PAGE under reducing conditions, which distinguished native Glu-Pg from Lys-Pg and distinguish the heavy chains of Glu- and Lys-Pm (Fig. 1). Greater than 90% of the cell-bound ligand was recovered by the elution procedure. The percent Pm formation was calculated by dividing the sum of the densities of the Glu-Pm heavy chain and Lys-Pm heavy chain bands by the sum of the densities of the Glu-Pg and Lys-Pg bands and the Glu-Pm and Lys-Pm heavy chain bands. (The light chain of Pm does not incorporate [125I] in proportion to the heavy chain and was not used in the calculation of 100%
Role of the Glu-Pg to Lys-Pg Conversion in Pg Activation by u-PA on the Cell Surface—We also examined cleavage of the Pg forms by another Pg activator, hmw u-PA. Cleavage of Glu-Pg to Pg by hmw u-PA (10 nM) was stimulated 4-fold at 10 min, when Glu-Pg was bound to the HUVEC surface compared with the reaction in solution (Fig. 3, panels A and B). The predominant form of the Pg heavy chain was Lys-Pg (Fig. 3, panel A). In addition, Lys-Pg accounted for 43% of the uncleaved Pg on the cell surface. In contrast, cleavage of cell-associated [D646E]Pg was still markedly less than cleavage of cell-associated 125I-Glu-Pg and cleavage of cell-associated [D646E]Pg was not enhanced compared with the reaction in solution (Fig. 3). The formation of [D646E]Lys-Pg was not detected on the cell surface. The percentage cleavage of Lys-Pg on the cell surface was similar to that of Glu-Pg, but cleavage of Lys-Pg in solution was markedly greater than that of Glu-Pg. At 10 min, 71% of the Lys-Pg in solution was cleaved to Pm whereas only 17% of the Glu-Pg in solution was cleaved to Pm). Hence, cleavage of Lys-Pg was enhanced only 1.1-fold.

The foregoing data showed that cell-associated [D646E]Glu-Pg was less readily cleaved than either cell-associated 125I-Glu-Pg or 125I-Lys-Pg, which is consistent with less direct cleavage of Glu-Pg forms compared with Lys-Pg. However, the case of [D646E]Glu-Pg was also distinct from that of the other ligands because Pm was not produced following cleavage by the Pg activators. Pm cleaves single-chain t-PA to Pm and Pm also cleaves hmw u-PA to Pm (10 nM) was stimulated 4-fold at 10 min, when Glu-Pg was bound to the HUVEC surface compared with the reaction in solution (Fig. 3, panels A and B). The predominant form of the Pg heavy chain was Lys-Pg (Fig. 3, panel A). In addition, Lys-Pg accounted for 43% of the uncleaved Pg on the cell surface. In contrast, cleavage of cell-associated [D646E]Pg was still markedly less than cleavage of cell-associated 125I-Glu-Pg and cleavage of cell-associated [D646E]Pg was not enhanced compared with the reaction in solution (Fig. 3). The formation of [D646E]Lys-Pg was not detected on the cell surface. The percentage cleavage of Lys-Pg on the cell surface was similar to that of Glu-Pg, but cleavage of Lys-Pg in solution was markedly greater than that of Glu-Pg. At 10 min, 71% of the Lys-Pg in solution was cleaved to Pm whereas only 17% of the Glu-Pg in solution was cleaved to Pm). Hence, cleavage of Lys-Pg was enhanced only 1.1-fold.

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In this study we provide the first demonstration that activation of cell-bound Glu-Pg is markedly enhanced only when its conversion to Lys-Pg on the cell surface is permitted. This result allowed us to distinguish between two potential mechanisms for stimulation of Pm formation when Glu-Pg is bound to the cell surface (1–10). In the first mechanism, the direct activation of Glu-Pg is promoted on the cell surface. In the second mechanism, initial conversion of Glu-Pg to Lys-Pg is necessary, so that formation of Pm is enhanced because the more readily activated Lys-Pg becomes the predominant substrate for Pm activators. These two potential mechanisms for this key step in cell surface Pg activation have not been distinguished in previous studies.

With both native Glu-Pg and [D646E]Glu-Pg, we detected <5% conversion to either Lys-Pg or [D646E]Lys-Pg following binding to HUVEC in the absence of a Pg activator. This finding allowed us to carry out our study to address the role of the Glu-Pg to Lys-Pg conversion in stimulation of Pg activation on the HUVEC surface. In contrast, in an earlier study ~50–60% of Glu-Pg was converted to Lys-Pg on the HUVEC surface, in the absence of the addition of exogenous Pm (18). These studies also suggested that Pm was not the source of proteolytic activity for conversion of Glu-Pg to Lys-Pg (5, 18)). This difference from the previously published data may be ascribed to differences in the culture conditions of the HUVEC in different laboratories or to differences in the added ligand. Thus, the ability to convert Glu-Pg to Lys-Pg, in the absence of exogenous Pm or Pg activator is not a consistent property of the HUVEC surface.

We found that when Glu-Pg was bound to the cells and activated with either t-PA or hmw u-PA (conditions where Lys-Pg and Lys-Pm formation occurred), the rate of Pm formation was similar to that of cell-bound Lys-Pg. This resulted in stimulation of Glu-Pg activation 4–13-fold compared with the reaction in solution. This first observation would be compatible with either of the two activation mechanisms proposed above. To distinguish between these mechanisms we studied cleavage by t-PA of a mutant Glu-Pg, [D646E]Glu-Pg, that does not produce Pm activity following cleavage by Pg activators, so that a [D646E]Lys-Pg is not produced. Cleavage of [D646E]Glu-Pg on the HUVEC surface was not markedly enhanced compared...
Plasminogen Activation on the Cell Surface

The rate of cleavage of cell-bound Lys-Pg by t-PA was similar to the rate of cleavage of cell-bound Glu-Pg. However, binding to the cells only minimally increased Lys-Pg activation by the Pg activators compared with the reaction in solution. The small amount of stimulation of activation of [D646E]Glu-Pg by Pg activators can occur (as it does in the solution phase) but direct cleavage of [D646E]Glu-Pg does not appear to be stimulated upon binding of the ligand to the cell. Analogously, a small amount of direct activation of native Glu-Pg on the cell surface, should provide a source of Pm for conversion of cell-bound Glu-Pg to Lys-Pg, leading to amplification of Pg activation on these cells.

Cleavage of the variant Pg, [D646E]Glu-Pg, was not markedly stimulated when bound to the HUVEC, although [D646E]Glu-Pg was still susceptible to cleavage by both t-PA and by u-PA when bound to the cell surface. Thus, cleavage of [D646E]Glu-Pg by Pg activators can occur (as it does in the solution phase) but direct cleavage of [D646E]Glu-Pg does not appear to be stimulated upon binding of the ligand to the cell. Analogously, a small amount of direct activation of native Glu-Pg on the cell surface, should provide a source of Pm for conversion of cell-bound Glu-Pg to Lys-Pg, leading to amplification of Pg activation on these cells.

Our results also suggest an additional new profibrinolytic function of localization of plasmin(ogen) on the cell surface.
Conversion of Glu-Pg to Lys-Pg by Pm was enhanced when the ligand was cell-associated compared with being in the solution phase. This may be because of colocalization and concentration of Pm and Pg on the cell surface, enhanced enzymatic activity of cell-bound Pm as described for U937 cells (26) and/or a more accessible conformation of cell-associated Glu-Pg compared with the solution phase.

Taken together, our results suggest that the conversion of Glu-Pg to Lys-Pg by Pm is necessary for maximal enhancement in Glu-Pg activation on cell surfaces relative to the reaction in solution and that the conversion of Glu-Pg to Lys-Pg is enhanced when Glu-Pg is bound to cells. Thus, the enhancement of formation of the more readily activated Lys-Pg allows cells to promote Pg activation on their surfaces, a key step in both thrombolysis and in physiologic and pathophysiologic processes involving cell migration.

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