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PLASMINOGEN RECEPTORS: THE *SINE QUA NON* OF CELL SURFACE PLASMINOGEN ACTIVATION

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1. ABSTRACT

Localization of plasminogen and plasminogen activators on cell surfaces promotes plasminogen activation and serves to arm cells with the broad spectrum proteolytic activity of plasmin. Cell surface proteolysis by plasmin is an essential feature of physiological and pathological processes requiring extracellular matrix degradation for cell migration (1,2) including macrophage recruitment during the inflammatory response (3), tissue remodeling (4), wound healing (5,6), tumor cell invasion and metastasis (7) and skeletal myogenesis (8). Cell associated plasmin on platelets and endothelial cells is optimally localized for promotion of clot lysis. In more recently recognized functions that are likely to be independent of matrix degradation, cell surface-bound plasmin participates in prohormone processing (9,10) as well as stimulation of intracellular signaling (11-14).

This issue of *Frontiers in Bioscience* on ***Plasminogen Receptors*** encompasses chapters focusing on the kinetics of cell surface plasminogen activation (15) and the regulation of plasminogen receptor activity (16) as well as the contribution of plasminogen receptors to the physiological and pathophysiological processes of myogenesis, muscle regeneration and cancer (17-19). The molecular identity of plasminogen receptors is cell-type specific, with distinct molecular entities providing plasminogen receptor function on different cells. This issue includes chapters on the well studied plasminogen receptor functions of α-enolase, cytokeratin 8, annexin II, S100A10 and annexin A2 heterotetramer (17,19-21). In this introductory chapter, we emphasize challenges and future directions in the field.

2. INTRODUCTION

A key control point by which plasmin activity is positively regulated is by the localization of Glu-plasminogen (the native circulating form of the plasminogen molecule) on specific binding sites on cell surfaces. Glu-plasminogen is activated to plasmin by the plasminogen activators, urokinase (u-PA) and tissue plasminogen activator (t-PA), much more efficiently when bound to cells than when in solution, due to a reduction in Km (11-60-fold) for the plasminogen activation reaction (22-31). Although binding of plasminogen activators to cells is clearly important, when plasminogen binding is prevented, stimulation of plasminogen activation on the cell surface is lost (29). Conversely, cell surface plasminogen activation is still enhanced when cell-surface plasminogen is activated by forms of plasminogen activators that do not interact with cells [e.g. low molecular weight urokinase (32)]. Hence, plasminogen receptors are the *sine qua non* of cell surface plasminogen activation.

The reduction in Km for plasminogen activation on the cell surface is predominantly due to enhanced plasmin cleavage of cell-associated Glu-plasminogen to the more readily activated Lys-plasminogen on the cell surface (32) (Figure 1). Active plasmin remains associated with the cell surface, where the activity of plasmin is relatively protected from inhibitors (33). Furthermore, the enzymatic activity of plasmin is enhanced in the environment of the cell surface (34).

Plasminogen receptors are very broadly distributed on both prokaryotic and eukaryotic cells. For example, plasminogen receptors are present on

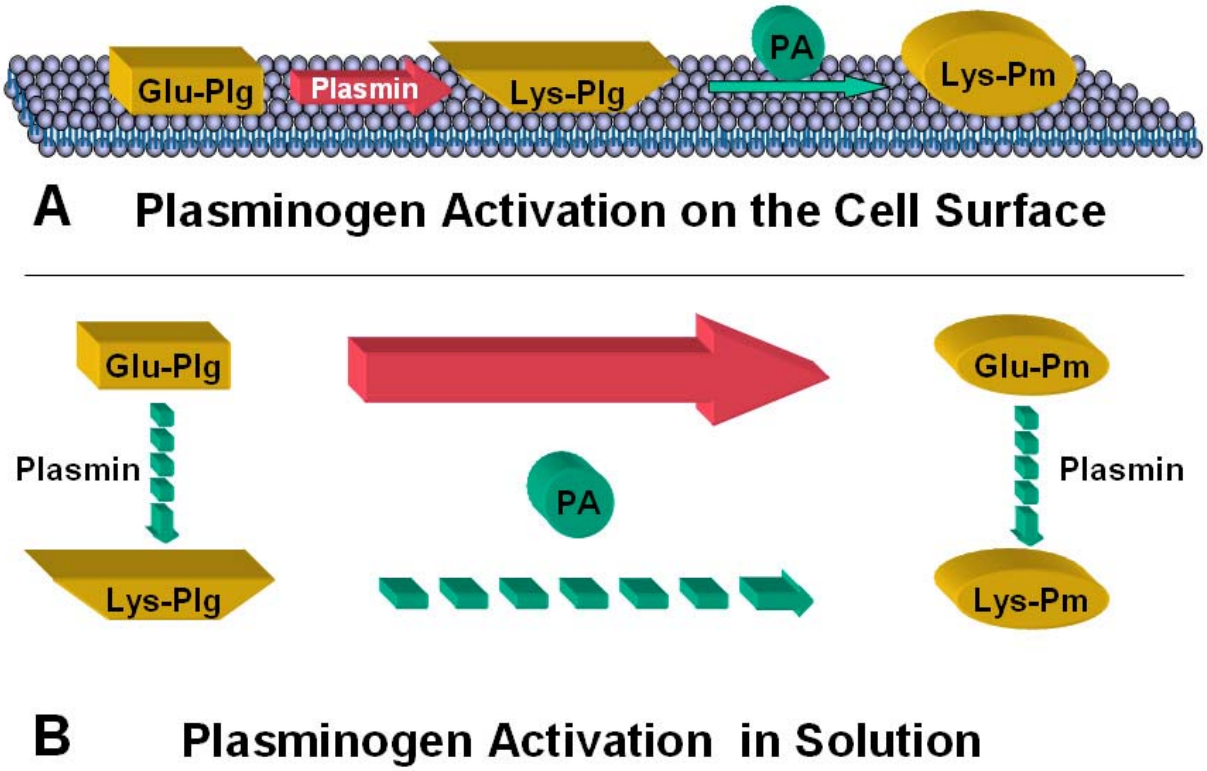

Figure 1. Mechanisms of Glu-plasminogen (Glu-Pg) activation on cell surfaces compared to the reaction in solution. On the cell surface (A), the conversion of Glu-Plg to Lys-Plg is enhanced so that Lys-Plg is the predominant substrate for plasminogen activators. In contrast, in the solution phases (B), the major substrate for plasminogen activators is Glu-Plg. Therefore, on cells, the conversion to the more readily activated Lys-Plg is necessary for optimal stimulation of plasminogen activation. PA, plasminogen activator, PM, plasmin.

monocytes(35), monocytoid cells (36), macrophages (37), endothelial cells (24,38,39), fibroblasts (40), platelets (22,41), adrenal medullary cells (9,10) and carcinoma cells (42,43). This broad distribution of plasminogen receptors among cell types with distinct functions suggests a broad functional importance of these receptors. Among the many cell types examined for plasminogen binding capacity, only red cells (22) and a murine monocyte progenitor cell line (44) are negative for plasminogen binding ability.

The Kd for the interaction of Glu-plasminogen with cells is ~ 1μM (45). Although this is a relatively low affinity receptor-ligand interaction, more than 50% occupancy of plasminogen receptors is predicted in plasma and interstitial fluid, based on the circulating plasminogen concentration of 2.2.μM (46) and this prediction has been borne out experimentally (47,48).

The capacity of cells for plasminogen is very high, ranging from 37,000 sites/platelet (22) to $>10^7$ sites on endothelial cells (38). Therefore, no single molecule is expected to account for the entire complement of plasminogen binding sites on a cell. Plasminogen can interact with both protein (49) and non-protein [e.g. gangliosides (50) cell surface components.

3. STRUCTURE OF PLASMINOGEN RECEPTORS

3.1. Role of carboxyl terminal lysines in the interaction of plasminogen with cells

The interactions of plasminogen with all eukaryotic cells are blocked by lysine and lysine analogs, e.g. ϵ-aminocaproic acid (EACA) (22,49) as well as peptides with carboxyl terminal lysines (49). This implys a requirement for unoccupied lysine binding sites within the plasminogen kringle structures in the interaction and isolated kringle domains of plasminogen can bind directly to cells (47). Furthermore, the participation of cell surface proteins with carboxyl terminal lysines as plasminogen receptors is also suggested by these studies.

Evidence for the participation of cell surface proteins with carboxyl terminal lysines in plasminogen binding was provided in studies in which monocytoid cells treated with carboxypeptidase B (CpB) lost ~60% of their plasminogen binding capacity (29). However, the cell surface-dependent enhancement in plasminogen activation was reduced by >95% (29). This suggests that the class of receptors with carboxyl terminal lysine residues accessible to CpB is predominantly responsible for the cell surface-dependent enhancement in plasminogen activation.

3.2. Identity of plasminogen receptors

The molecular identity of plasminogen receptors is cell-type specific. A subset of cell-surface plasminogen binding proteins that are synthesized with carboxyl-terminal lysines have been demonstrated to promote plasminogen activation. These include α-enolase on monocytes (49,51), neurons (52), carcinoma cells (53), lymphoid cells (54), myoblasts (8) and pathogenic streptococci (55) and TATA-binding protein-interacting protein (TIP49a) on monocytoid cells (55), cytokeratin 8 on the surfaces of hepatocellular and breast carcinoma cells (17,43) p11 on endothelial cells (56) and glyceraldehyde-3-phosphate dehydrogenase on the surface of bacteria (57).

A second subset of plasminogen binding proteins includes proteins that promote plasminogen activation, but are not synthesized with carboxyl terminal lysine, but expose carboxyl terminal lysines on the cell surface. These include annexin II on endothelial cells (58) and actin on endothelial cells (59), carcinoma cells (60), catecholaminergic cells (61), PC-3 and HT1080 cells (62). Therefore, post-translational processing to generate carboxyl terminal lysines in order to stimulate plasminogen activation is likely to be required for members of this subset. *The elucidation of these mechanisms is a crucial line of investigation in the field.*

A third subset of plasminogen receptors promotes plasminogen activation, are not synthesized with carboxyl terminal lysines and the exposure of carboxyl terminal lysines by these proteins has not been investigated. This subset of plasminogen receptors includes αIIbβ3 on platelets (41) and rheumatoid arthritis synovial fibroblasts (41,63,63), activated $\alpha_M\beta_2$ on PMA-stimulated neutrophils (64), amphoterin (65) and GP330 (66,67). Thus, either mechanisms exist for generation of carboxyl terminal lysines on these proteins, or these plasminogen receptors may represent a small percentage of eukaryotic plasminogen receptors [e.g.<5% on monocytes (29)] that stimulate plasminogen activation without exposing a carboxyl terminal lysine. This mechanism may be analogous to the predominant prokaryotic plasminogen receptor on group A streptococcal surface protein, PAM, that interacts with plasminogen via an internal lysine (68,69).

A fourth subset of plasminogen receptors binds plasminogen, but does not promote plasminogen activation. Tissue factor is a member of this class because although tissue factor exhibits plasminogen binding function on the cell surface (70), this interaction does not result in enhancement of plasminogen activation, consistent with the lack of an encoded carboxyl terminal lysine. Non-protein gangliosides are also included within this class of plasminogen receptors (50). *The functional consequences of plasminogen binding to this class of receptors remain to be elucidated. Modulation of intracellular signaling pathways is a potential role of this class of binding interactions.*

A fifth subset of plasminogen receptors interacts with immobilized plasminogen but with different characteristics than the interaction of soluble Glu-plasminogen with cells. Integrin $\alpha_5\beta_1$ interacts with plastic-immobilized Glu-plasminogen (but not plasmin or Lys-plasminogen) via a lysine-binding site-independent mechanism (71). Thus, this integrin-dependent interaction with immobilized plasminogen is clearly distinct from the interactions of soluble circulating plasminogen with cells (that require the lysine binding sites in plasminogen and intact with Glu- and Lys-plasminogen as well as plasmin). *The physiologic role of this interaction remains to be elucidated.*

The majority of cell surface plasminogen binding proteins appear to be "moonlighting" proteins, members of a growing list of proteins that are recognized as identical gene products exhibiting multiple functions at distinct cellular and extracellular sites through "gene sharing" (72,73). For example, α-enolase is a lens crystallin in several vertebrates (74), binds to F-actin and tubulin (75), binds to single-stranded DNA (76) and has been localized to the centrosome (77,78). Recently, a 35 kDa protein (MBP-1) that acts as a transcriptional regulator of c-myc has been identified as a carboxyl terminal fragment of α-enolase and is an alternative translation product of the α-enolase gene (79).

The DNA sequences encoding many plasminogen receptors do not predict classical signal sequences (e.g. α-enolase, actin, annexin II, cytokeratin 8, p11 or TIP49a). Thus, other mechanisms for release of these plasminogen binding proteins and their subsequent membrane localization or direct translocation of these proteins to the cell membrane must exist (80). *Identification of these mechanisms is a crucial area within the field.*

3.3. Identification of proteins exposing carboxyl terminal lysines on the cell surface using proteomics approaches

Because more than one protein can provide plasminogen binding function on a given cell, proteomics approaches are ideal for analysis of cell surface plasminogen receptors. We developed a proteomics-based approach to exclusively identify proteins exposing carboxyl terminal lysines on the cell surface. This approach was designed to distinguish plasminogen-binding proteins with available carboxyl terminal lysines on cell surfaces from cell surface proteins with carboxyl terminal lysines that are masked or in other inaccessible orientations on the cell surface, proteins exposing carboxyl terminal lysines on the cytoplasmic face of the plasma membrane or cytoplasmic contaminants of membrane preparations (Figure 2, Panel A). To exclusively identify plasminogen-binding proteins that expose carboxyl terminal lysines in an accessible orientation on the cell surface, intact cells were treated with CpB to remove exposed carboxyl terminal lysines (Figure 2, Panel B). Cell membranes were subsequently prepared and subjected to two-dimensional polyacrylamide gel electrophoresis followed by ligand blotting with ^{125}I-plasminogen. Comparison of the plasminogen ligand blots of membranes of untreated intact cells with membranes of CpB treated intact cells revealed the plasminogen-binding

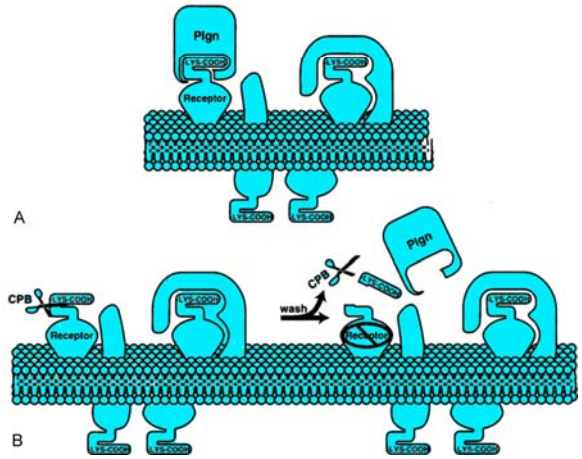

Figure 2. Plasminogen binding to carboxyl terminal lysines on the cell surface. Panel A) The binding of plasminogen to cell surface proteins occurs via receptors exposing carboxyl terminal lysines to the extracellular environment. Cell surface proteins with carboxyl terminal lysines that are masked or in other inaccessible orientation on the cell surface, or membrane-associated proteins with carboxyl terminal lysines that are located on the inner face of the membrane, cannot serve as plasminogen receptors. Panel B) CpB treatment of intact cells removes carboxyl terminal lysines from plasminogen receptors, and plasminogen binding to the cell surface is reduced. Reprinted with permission from (81).

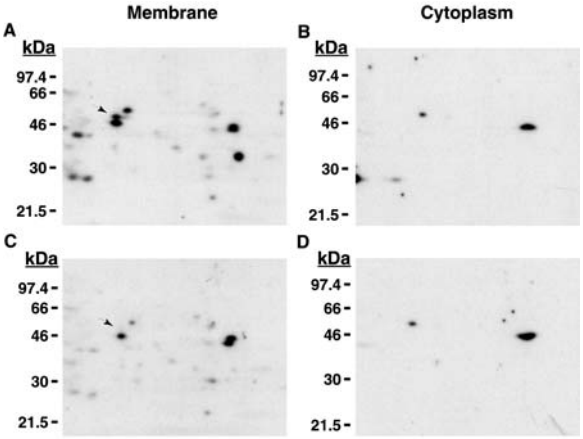

Figure 3. 2-D gel analysis of plasminogen-binding proteins that expose carboxyl-terminal lysines on the cell surface. Intact U937 cells were treated either in the absence (A and B) or presence (C and D) of 100 units/ml CpB for 30 min at 37° C prior to fractionation into membrane and cytosolic fractions. 2-D gel electrophoresis was performed on 100 µg of membrane proeins (A and C) or 10 µg of the cytoplasmic proteins (B and D), followed by ligand blotting with 50 nM ^{125}I-plasminogen. First dimension isoelectric focusing pH 6-8, is along the horizontal axis of the gel, and second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis is along the vertical axis. The arrow indicates the location of the 54-kDa plasminogen binding protein. Reprinted with permission from (82).

proteins exposing carboxyl terminal lysines on the cell surface.

As an example of the application of this methodology, we examined U937 cell membrane-associated proteins for the presence of carboxyl terminal lysines exposed to the extracellular environment (81,82). Comparison of ligand blots from membrane fractions of intact control cells (Figure 3, Panel A) with ligand blots of membrane fractions from intact cells treated with CpB (Figure 3, Panel B) showed that a prominent plasminogen-binding protein (mass ~54 kDa, pI 6.5) was undetectable after CpB treatment of intact cells (arrow), indicating that it lost its ability to bind plasminogen. Cytoplasmic proteins showed no changes in plasminogen binding following CpB treatment of intact cells (Figure 3, compare panels B and D), suggesting that proteolysis by CpB did not occur in the interior of intact cells.

To purify the 54 kDa protein, we eluted the protein from 2-D gels and digested it with trypsin and, using tandem mass spectrometry, obtained two peptide sequences (82). These peptide sequences were identical to sequences contained within the rat TATA-binding protein-interacting protein (TIP49a), but were not present in the human protein data base at the time of our analysis. BLAST searches of the nonredundant human EST data base yielded an EST whose calculated translation products were an exact match to both peptide sequences in frame with each other and two other EST's whose calculated translation products were an exact match to the other peptide sequence. These EST's were used to search the TIGR Human Gene Index data base for tentative human consensus (THC) sequences (83). The EST's used to assemble the THC sequence were then used to search GenBank for additional EST's with significant sequence identity. Alignment of the EST's revealed that there were significant amounts of sequence not contained within the original THC and that the aligned EST's extended over two THC sequence. All EST's mapped to this alignment, suggesting that only one molecule could account for the peptide sequences generated.

One EST clone was screened for a possible full-length cDNA insert by performing PCR with primers to the cDNA sequence outside of the calculated open reading frame of the full-length consensus cDNA and this EST clone contained a full-length human TIP 49a cDNA that was identical to the human TIP 49a cDNA that was reported by Makino and coworkers (84). The predicted translation products of the human and rat TIP49a cDNA's both had carboxyl terminal lysines (84), consistent with plasminogen binding capacity.

In order to verify that TIP49a corresponded to the 54 kDa plasminogen-binding protein, we re-probed 2-D ^{125}I-plasminogen ligand blots of U937 membrane proteins as Western blots with anti-rat TIP49a antibody. The immunoreactive protein observed with the anti-rat TIP49a antibody directly overlaid the 54 kDa ^{125}I-plasminogen-binding spot observed at pI 6.5. Using recombinant

TIP49a, we verified that plasminogen could bind directly to TIP49a and that this binding enhanced plasminogen activation (82).

4. FUNCTION OF PLASMINOGEN RECEPTORS

Localization of plasmin activity on cells generates a local nidus of protected proteolytic activity that exhibits distinct functions on different cell types. For example, on leukocytes and cancer cells, the association of plasmin with these cell surfaces arms the cells with the proteolytic activity of plasmin required in processes in which cells must degrade extracellular matrices in order to migrate (2,85). On platelets and endothelial cells, plasmin becomes localized to sites of thrombus formation where plasmin proteolytic activity can contribute to fibrin clot lysis (22,24). Association of plasmin with neuroendocrine cells enhances prohormone processing (9,61). On myoblasts, plasmin promotes myogenesis (8). Plasmin bound to bacterial plasmin(ogen) receptors serve to enhance invasive potential of bacteria by increasing penetration of extracellular matrices, basement membranes and tissues (86).

Induction of intracellular signaling pathways by plasminogen independently of the proteolytic activity of plasmin, has not been described in the literature. However, induction of signaling pathways that requires the proteolytic activity of plasmin has been reported in monocytes (11-13), neutrophils (87), and endothelial cells [(88) (14). The domain of plasminogen comprised of K1-4 (angiostatin), as well as K1-3, also inhibit endothelial cell proliferation and migration (89,90), consistent with an intracellular signaling mechanism. However, the activity of angiostatin is not mimicked by the plasminogen molecule.

Strong support for key functions of cell-associated plasminogen *in vivo* is provided by studies with plasminogen deficient (Plg-/-) mice. A direct involvement of cell surface plasminogen in monocyte recruitment during the inflammatory response is supported by results of several studies: Monocyte recruitment is decreased in Plg (-/-) mice compared to Plg (+/+) mice following thioglycollate injection to induce a peritoneal inflammatory reaction (3,91). In addition, in a lung injury model, lung macrophage recruitment is decreased in Plg -/- mice in response to bleomycin treatment (92). Most tellingly, monocyte recruitment in response to injection of Plg +/- mice with thioglycollate is enhanced in mice either heterozygous or homozygous for carboxypeptidase B (CpB) deficiency (92). The role of CpB in cell migration may be its removal of carboxyl-terminal lysines, that function as plasminogen binding sites on the cells (29). These data are consistent with the interpretation that plasminogen bound to the monocyte/macrophage surface plays a key role in the inflammatory response.

The function of cell surface plasminogen receptors *in vivo* is also demonstrated in a murine model in which co-expression of transgenes encoding both u-PA and u-PAR results in epidermal thickening, extensive loss of hair follicles and blistering. The pathology is only observed upon co-expression of both transgenes and requires active u-PA. When the doubly transgenic mice are crossed into a Plg-/- background the phenotype is lost, suggesting that plasminogen activation on the cell surface is required for the pathological phenotype (93).

There are less data available on the relationship between specific plasminogen receptors and phenotypes. A role of α-enolase is supported in studies showing that Plg-/- mice show a severe regeneration defect after cardiotoxin-induced muscle injury *in vivo* (8). Because specific anti-α-enolase antibodies inhibit plasmin generation on the surfaces of myoblast cells *in vitro* (8), these results suggest that cell surface plasmin, bound to α-enolase is required for muscle regeneration. Annexin II knockout mice have been produced recently and these mice show a defect in endothelial cell-dependent plasmin generation (20), although further studies are needed to determine whether this effect is due to a direct reduction in plasminogen binding to endothelial cells and/or reduction in levels of p11 (21) .

5. INCREASED PLASMINOGEN RECEPTOR EXPRESSION ON APOPTOTIC CELLS

A key observation in the understanding of plasminogen receptor biology was that early apoptotic and non-viable/necrotic monocytoid cells exhibit markedly enhanced plasminogen binding ability, compared to viable cells (94,95). Plasminogen binding is also increased on non-viable epithelial cells (96), necrotic breast cancer cells (7,97) and damaged amniotic epithelial cells (98,99).

The function of plasminogen receptors on apoptotic cells is a key area for investigation. Recent studies support a role for plasminogen in promotion of anoikis on adherent cells (100,101) (102). *The function of plasminogen receptors on cells in suspension and not subject to anoikis is also a key area for investigation.*

5.1. Technical Considerations Regarding Plasminogen Binding to Apoptotic Cells

As pointed out by O'Mullane and Baker (94), in order to measure kinetics of plasminogen binding and activation on viable, vs either apoptotic or necrotic cells, these subpopulations should be analyzed separately. An effective method to accomplish this goal is the use of fluorescence activated cell sorting (FACS) analysis. In this method, the binding of fluorescein isothiocyanate-labeled plasminogen to cells is measured simultaneously with analysis of the viability state of the cells, using markers of early apoptosis and late apoptosis/necrosis. The viable, early apoptotic and late apoptotic cell populations can be gated out and plasminogen binding measured on each subpopulation, separately (94). Analogously, plasminogen activation can be determined on these subpopulations by measuring the binding of fluorescein isothiocyanate-labeled aprotinin to the subpopulations (94).

6. PERSPECTIVES

Novel functions of plasminogen receptors under physiological and pathological conditions are increasingly

being recognized. Studies of plasminogen knockout mice have provided key insights into functions of plasminogen receptors in general. Application of knockout technology to the analysis of specific plasminogen receptors is a promising means to understand roles of specific plasminogen receptors. This technology is in its infancy with respect to analysis of specific plasminogen receptors, with analysis of only annexin II knockouts having been performed to date (20). This technology should also be effective in determining the functional consequences of plasminogen binding to specific receptors that do not stimulate plasminogen activation as well as the consequences of plasminogen binding to apoptotic cells.

Answers to basic mechanistic questions are also crucial for understanding mechanisms by which specific plasminogen receptors function *in vivo* and *in vitro.* These include determining the enzymes responsible for generation of carboxyl terminal lysines on plasminogen receptors that are not synthesized with carboxyl terminal lysines and determining how plasminogen receptors that lack classical signal sequences arrive at the cell surface. These answers obtained should provide key understanding of pathophysiological processes including inflammation, wound healing, tumor cell invasion, metastasis, myogenesis and prohormone processing.

7. ACKNOWLEDGMENTS

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