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S100A10, ANNEXIN A2, AND ANNEXIN A2 HETEROTETRAMER AS CANDIDATE PLASMINOGEN RECEPTORS

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1. ABSTRACT

The defining characteristic of a tumor cell is its ability to escape the constraints imposed by neighboring cells, invade the surrounding tissue and metastasize to distant sites. This invasive property of tumor cells is dependent on activation of proteinases at the cell surface. The serine proteinase plasmin is one of the key proteinases that participate in the pericellular proteolysis associated with the invasive program of tumor cells. The assembly of plasminogen and tissue plasminogen activator at the endothelial cell surface or on the fibrin clot provides a focal point for plasmin generation and therefore plays an important role in maintaining blood fluidity and promoting fibrinolysis. S100A10, a member of the S100 family of Ca^{2+}-binding proteins, is a dimeric protein composed of two 11 kDa subunits. Typically, S100A10 is found in most cells bound to its annexin A2 ligand as the heterotetrameric $(S100A10)_2(annexin\ A2)_2$ complex, AIIt. In addition to an intracellular distribution, S100A10 is present on the extracellular surface of many cells. The carboxyl-terminal lysines of S100A10 bind tPA and plasminogen resulting in the stimulation of tPA-dependent plasmin production. Carboxypeptidases cleave the carboxyl-terminal lysines of S100A10, resulting in a loss of binding and activity. Plasmin binds to S100A10 at a distinct site and the formation of the S100A10-plasmin complex stimulates plasmin autoproteolysis thereby providing a highly localized transient pulse of plasmin activity at the cell surface. The binding of tPA and plasmin to S100A10 also protects against inhibition by physiological inhibitors, PAI-1 and $alpha_2$-antiplasmin, respectively. S100A10 also colocalizes plasminogen with the uPA-uPAR complex thereby localizing and stimulating uPA-dependent plasmin formation to the surface of cancer cells. The loss of S100A10 from the extracellular surface of cancer cells results in a significant loss in plasmin generation. In addition, S100A10 knock-down cells demonstrate a dramatic loss in extracellular matrix degradation and invasiveness as well as reduced metastasis. Annexin A2 plays an important role in plasminogen regulation by controlling the levels of extracellular S100A10 and by acting as a plasmin reductase. The mechanism by which annexin A2 regulates the extracellular levels of S100A10 is unknown. This review highlights the important part that S100A10 plays in plasmin regulation and the role this protein plays in cancer cell invasiveness and metastasis.

2. INTRODUCTION

An impressive body of scientific data spanning the last several decades has supported the concept of plasminogen activation as a critical factor in both fibrinolysis and the pericellular proteolysis associated with the invasive program of tumor cells (recently reviewed in 1-4). The inactive zymogen, plasminogen, is converted to the active, broad-spectrum serine proteinase, plasmin, by the plasminogen activators; tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). These specific serine proteases cleave the Arg^{561}-Val^{562} site of plasminogen, and the resulting conformation change and formation of the substrate-binding site completes the conversion of plasminogen to plasmin. Since plasminogen is abundant in the vasculature and in most body fluids, it serves as an almost limitless supply of substrate for this reaction. To avoid inappropriate proteolysis, the plasminogen activators and plasmin are regulated by plasminogen activator inhibitors (PAI-1 and PAI-2) and

alpha$_2$-antiplasmin, respectively.

The tPA is typically produced by endothelial cells (5). Carboxyl-terminal lysine residues of endothelial cell surface receptors provide a common binding site for both tPA and plasminogen thereby colocalizing the enzyme and substrate to the cell surface. Once bound to the cell surface, tPA and plasmin are protected from inactivation by their inhibitors. In addition to binding to the endothelial cell surface, plasminogen and tPA colocalize on the fibrin clot. Fibrin is cleaved by the plasmin bound to its surface resulting in the generation of new carboxyl-terminal lysines and additional binding sites for plasminogen and tPA on the fibrin surface (6-9). In this regard fibrin plays the role of a template for binding and colocalization of these proteins and also serves to greatly stimulate plasminogen activation.

In addition to playing a key role in fibrinolysis, plasmin also plays an important role in the pericellular proteolysis associated with tumor cell invasiveness and metastasis. Plasminogen activation by cancer cells is usually initiated by the release of uPA which is tightly bound by its cell surface receptor, uPAR. Plasminogen binds to a heterogeneous group of plasminogen receptors at the cell surface. However, only the receptors that bind plasminogen via carboxyl-terminal lysines appear to participate in plasminogen activation. Mechanistically, the interactions of the carboxyl-terminal residue of the plasminogen receptor with the lysine-binding kringle domains of plasminogen convert plasminogen into a less rigid and more activatable conformation. In addition, a subset of the plasminogen receptors also colocalizes plasminogen to the uPA-uPAR complex. Both induction of this conformational change in plasminogen and its colocalization with the uPA-uPAR complex dramatically stimulate the conversion of plasminogen to plasmin. The plasmin produced by this reaction remains bound to the receptor where it is protected from inactivation by its physiological inhibitor, alpha$_2$-antiplasmin. The cell surface plasmin catalyzes the degradation of proteins of the basement membrane and extracellular matrix such as laminin and fibronectin (10, 11). Plasmin also plays a role in the release of growth factors from the extracellular matrix (12). The most intensively studied examples of this are the activation of latent transforming growth factor-beta and the mobilization of matrix-sequestered fibroblast growth factor-2 (13, 14). The proteolytic processing of plasmin(ogen) also plays a critical role in down-regulation of angiogenesis (15, 16). In addition, plasmin activates several matrix-degrading tumor-cell- and tumor-associated cell-derived metalloproteinases (MMPs), such as the pro-collagenases and pro-gelatinases, thus providing the cell with the necessary enzymatic machinery to breakdown and invade normal surrounding tissue structures (17, 18) (reviewed in 2, 11, 19, 20). Therefore, the generation of plasmin at the cell surface is a key event in the destruction of basement membrane and extracellular matrix that is necessary for the invasion of tumor cells through physiological barriers (20, 21). Identification of the plasminogen receptor(s) is of obvious importance to our understanding of cellular plasmin regulation.

3. CELLULAR PLASMINOGEN REGULATION

3.1. Structure and function of plasminogen

Human plasminogen is synthesized as an 810 amino acid polypeptide chain, the amino-terminus of which consists of an 19-amino acid leader peptide. Upon secretion, the leader peptide is removed, resulting in the mature protein of 791 amino acids. Plasminogen exists as two major isoenzymes; plasminogen-1 has two carbohydrate chains linked to Asn^{289} and Thr^{346}, while plasminogen-2 has one carbohydrate chain linked to Thr^{346} (22-24). The carbohydrate chains appear to influence the binding properties of the proteins, as plasminogen-2 can bind to cellular receptors with 10-fold higher affinity than plasminogen-1 (25-28).

Mature plasminogen consists of several functional domains including the 77-amino acid PAN (plasminogen, apple, nematode) domain, the kringle domains (K) and the serine protease domain. The PAN domain plays an important role in regulating plasminogen by means of its intramolecular interaction with the kringle domains. Specifically, Lys^{50} of the PAN domain is thought to interact with the kringle domains, most likely K4 or K5 resulting in a compact, closed conformation which is relatively resistant to activation (29). A further proteolytic modification, catalyzed by plasmin, involves the removal of the PAN module thereby generating Lys^{77}-plasminogen which adopts an extended conformation and is activated 10-fold more efficiently than the native Glu-plasminogen.

The kringles are polypeptide domains which are found in several blood coagulation and fibrinolytic proteins such as prothrombin (30), factor XII (31), tPA (32), uPA (33) and lipoprotein (a) (34, 35). The kringle domains are highly homologous and consist of about 80 amino acid residues including three disulfide-linked cystine residues, about thirty highly conserved residues and about sixteen invariant residues (36). The crystal structures of several kringles have been solved (37-40). These studies have shown that the kringles contain preformed surface exposed lysine-binding sites. In the case of plasminogen which possesses five kringle domains (K1-K5), an inter-kringle disulfide bond links K2 to K3 (41). UPA contains a single kringle while tPA contains two kringles. However only K2 of tPA contains a lysine-binding site which is similar in binding characteristics to K4 of plasminogen (42). The kringles of plasminogen and tPA appear to be primarily involved in protein-protein interactions, including binding to fibrin(ogen) and cell surfaces as well as to cell-associated actin and to the major physiological inhibitor of plasmin, alpha$_2$-antiplasmin (43-49). It has been suggested that K1 may be the primary site for the binding of plasminogen to the cell surface although possible roles for other kringles cannot be ruled out (50).

Four of the five kringle domains of plasminogen contain binding sites for the amino acid lysine and its analogues such as omega-aminocarboxylic acids. Each of the kringles have different affinity and selectiveness for these

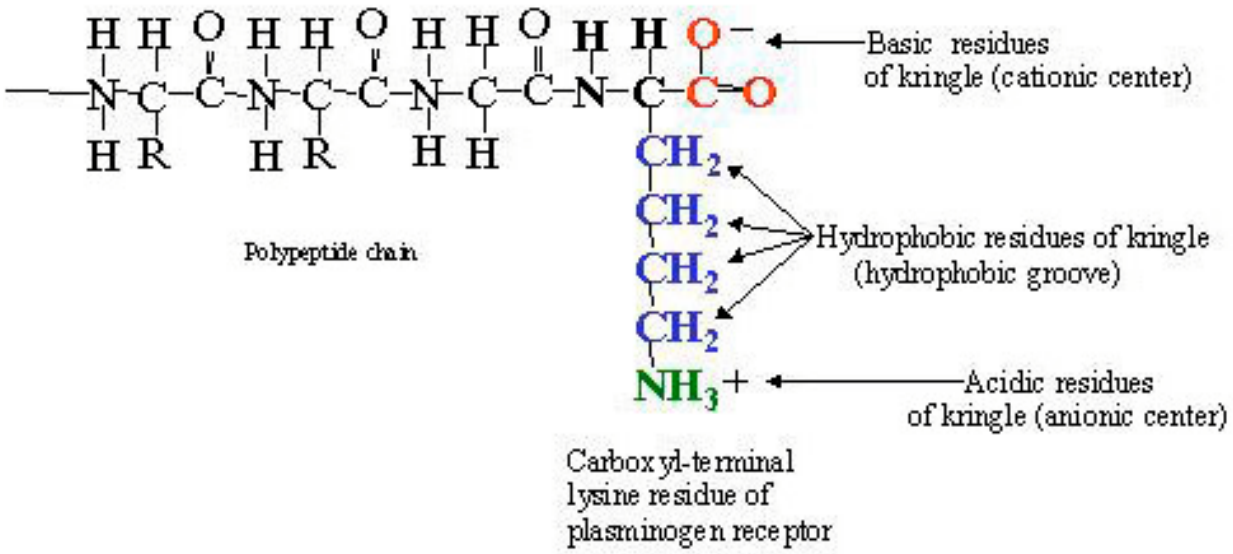

Figure 1. Illustration of the interaction of the carboxyl-terminal lysine residue of the plasminogen receptor with the lysine-binding kringle domain of plasminogen. The binding of plasminogen to either of its physiological targets, fibrin or the cell surface, involves the interaction of the lysine-binding site of the kringle domain with a carboxyl terminal lysine of the plasminogen receptor. The lysine-binding site of the kringle domain has three distinct binding regions, the anionic center, the hydrophobic groove and the cationic center. The anionic center is composed of two acidic residues which interact with the amino group side-chain of lysine. The hydrophobic groove is characteristically formed by several residues, typically tryptophan or tyrosine, and these residues interact with the side-chain methylene backbone of lysine. The cationic center is composed of one or two basic residues, namely a lysine or arginine, and interacts with the free carboxylate group of lysine. Only carboxyl-terminal residues display this free carboxylate group. The carboxyl-terminal lysine of the plasminogen receptor is a preferential ligand for the lysine-binding site of the kringle domains of plasminogen because only a carboxyl-terminal lysine possesses all three binding regions.

ligands. Markus *et al*. initially reported that human plasminogen possessed one high affinity and five low affinity binding sites for omega-aminocarboxylic acid (51). Subsequently, several investigators have established that K1 and K4 have the highest affinity binding sites for lysine while K5 has moderate affinity and K2 has the lowest affinity. K3 lacks a functional binding site for lysine. These studies have formed the framework for an understanding of how lysine interacts with the plasminogen and tPA kringles (37, 41, 52-57) (Figure 1). Three regions of lysine are known to interact with kringles. First, the side-chain amino group of lysine interacts with the anionic center of the kringle, Asp^{54} and Asp^{56} (numbering based on sequence alignments of the kringles). Second, the side-chain methylene backbone of lysine interacts with the hydrophobic groove of the kringle. Implicated in this interaction are Trp^{61} and Tyr/Trp^{71} (K1, K4, K5) as well as other residues such as Phe^{36} (K5), and Phe/Tyr^{63}. Third, the free carboxylate group of lysine interacts with cationic center, Arg^{34} (K1), Lys^{34} (K4) or Arg^{70} (K1, K4). Since only a carboxyl-terminal lysine possesses the free carboxyl group which interacts with cationic center, the carboxyl-terminal lysine is the preferred ligand for the kringles. The moderate affinity of K5 for lysine can be attributed to a mutation in the cationic center ($Arg^{71}Leu$) which prevents the interaction of K5 with the lysine carboxylate group.

The kringles play key roles in regulating the conformation of plasminogen as well as serving to localize the protein to its major binding partner, fibrin(ogen) or to cell surface receptors. The importance of the interaction of plasminogen kringles with fibrin has been well documented. Cleavage and removal of fibrinopeptides A and B from soluble fibrinogen is catalyzed by thrombin, the terminal enzyme of the coagulation cascade. The cleaved fibrinogen, or fibrin monomers, spontaneously polymerize, forming a fibrin network that constitutes the major proteinaceous component of a hemostatic plug (58, 59). Subsequent removal of a fibrin clot occurs by plasmin-dependent proteolysis, a process called fibrinolysis. The binding of plasmin to the fibrin clot is mediated by one or more of the kringle domains which bind to the lysine residues in fibrin (8, 60-62). Activation of plasminogen also is rendered clot specific by virtue of the affinity of tPA for fibrin and also because of the fibrin-dependent stimulation of tPA-catalyzed plasminogen activation (7). Fibrin binds both tPA and plasminogen and thereby functions not only as the fibrinolytic substrate but also as a cofactor in plasminogen activation. Upon fibrin polymerization, tPA is stimulated to activate Glu-plasminogen. The resulting plasmin simultaneously converts fibrin to intermediate fragments and Glu-plasminogen to Lys-plasminogen. The Lys-plasminogen shows enhanced binding to intact fibrin while both Glu-plasminogen and Lys-plasminogen show enhanced binding to the newly formed carboxyl-terminal lysines of partially degraded fibrin. This serves to colocalize both forms of plasminogen, as well as tPA and fibrin and thus further enhance plasminogen activation and fibrin degradation (27, 60, 63, 64). It is currently believed that the conversion of

Glu-plasminogen to Lys-plasminogen during fibrinolysis is responsible for positive feedback that ensures sufficient activation of plasminogen to accomplish fibrinolysis under conditions of potent circulating inhibitors and limiting amounts of activator. Thus, the carboxyl-terminal lysines of fibrin play a key role in regulating plasmin formation and consequently fibrin digestion.

3.2. Activation of plasminogen

The conversion of the zymogen, plasminogen to plasmin involves the proteolytic cleavage of Arg^{561}-Val^{562} by the urokinase plasminogen activator (uPA) or the tissue-type plasminogen activator (tPA) (65, 66). This results in the generation of two-chain Glu-plasmin which consists of a heavy chain of 561 residues disulfide linked by two disulfide bonds to the light chain of 230 residues. The light chain contains the serine protease domain including the catalytic triad of His^{603}, Asp^{646}, and Ser^{741}. The proteolytic cleavage of plasminogen by the plasminogen activator creates a new amino-terminal Val^{562} residue. The free amino group of the Val^{562} residue stabilizes the structure of plasmin by interacting via a salt bridge with Asp^{740}. This interaction leads to the stabilization of the oxyanion hole and substrate binding pocket of the active site of plasmin, resulting in the formation of fully active plasmin. The plasminogen activators are also converted into more active forms through the action of plasmin (63, 67, 68). The tPA is synthesized by vascular endothelial cells as a single chain 72 kDa polypeptide (sctPA) and is converted to the more active two-chain molecule (tctPA) in the presence of plasmin. The plasmin-dependent conversion of sctPA to a tctPA results in a 15-fold increase in the activity of tctPA in the absence of fibrin, but in the presence of fibrin both forms of tPA have similar activity (69). This renders tctPA particularly efficient on a fibrin-free extracellular surface (70). Similarly, uPA is converted from a relatively inactive scuPA to an active tcuPA when exposed to plasmin. Upon conversion to the two-chain form, uPA loses its ability to bind to fibrin, which suggests that the two-chain form of uPA, like two-chain tPA, may be preferentially destined for plasminogen activation on the vascular endothelial extracellular surface (71).

4. OBSERVATIONS FROM MODEL SYSTEMS THAT DEFINE THE ROLE OF PLASMINOGEN RECEPTORS IN PLASMINOGEN REGULATION

4.1. Binding of plasminogen to cell surface proteins

Glu-plasminogen binds rapidly and reversibly to cell surface receptors with moderate affinity (K_d of 0.3-2 micromolar) and high capacity (up to 10^7 sites per cell) (47, 72-75). Plasminogen binding sites on cells are heterogeneous and both proteins and non-proteins such as gangliosides (76) and glycosaminoglycans (77, 78) can serve as plasminogen binding sites (reviewed in 79). It was originally reported that plasminogen binding to cells was inhibited by lysine analogues such as epsilon-aminocaproic acid (EACA) (49) or peptides containing carboxyl terminal lysines (80). A series of studies has established that only plasminogen receptors with carboxyl-terminal lysine residues that are exposed and susceptible to cleavage by basic carboxypeptidases participate in cell surface plasminogen activation although they account for only a fraction of the cell's total plasminogen binding capacity (80-84) (reviewed in 85). This heterogeneity among plasminogen receptors with respect to binding of plasminogen and enhancement of plasminogen activation was initially reported in U937 monocytoid cells. For example, treatment of U937 monocytoid cells with carboxypeptidase B reduced plasminogen binding by 60%, but reduced the enhancement of plasminogen activation by 95%, suggesting that the subset of receptors with carboxyl-terminal lysine residues are responsible for the stimulation of cell-associated plasminogen activation (82).

Plasminogen and plasmin share common receptors at the cell surface although plasmin is bound with much higher affinity (Kd of 60 nM) (86, 87). It has also been demonstrated that tPA and plasminogen share the same cellular binding sites. It was originally demonstrated with nine different cell types that tPA could inhibit the binding of plasminogen to cells and that both plasminogen and tPA binding sites were lost upon treatment of cells with carboxypeptidase B (88). These authors also showed that under several conditions of cell treatment, tPA and plasminogen receptor expression were modulated in parallel. Based on these studies, it has been assumed that the carboxyl terminal lysine of the plasminogen receptor is capable of binding either tPA or plasminogen.

4.2. Regulation of cellular plasminogen binding sites

Plasminogen-binding to cells has been shown to be up-regulated by a protease-dependent pathway which appears to influence the availability of plasminogen-binding proteins with carboxyl-terminal lysines. Trypsin-like proteases, such as plasmin or trypsin, can cleave certain cell surface proteins resulting in the exposure of new carboxyl-terminal lysines and increased cell surface plasminogen binding (82, 83, 89, 90). For example, freshly isolated monocytes show a substantial up-regulation (about 30-fold) of their plasminogen receptors when cultured overnight. Since this change was attenuated by protease inhibitors, it was concluded that the proteolysis of cell surface proteins and generation of new carboxyl-terminal lysines was partially responsible for the enhanced plasminogen binding. Proteolytic remodeling of the cell surface resulting in the exposure of preexisting receptors with carboxyl-terminal lysines was also partially responsible for enhanced plasminogen binding to the cell surface. In contrast, other enzymes may decrease cell surface plasminogen binding by removal of carboxyl-terminal lysines from the plasminogen receptors. The basic carboxypeptidases are a family of enzymes that regulate this interaction by cleaving carboxyl-terminal lysine or arginine residues from the plasminogen-binding sites on fibrin clots (91, 92) or cell surface proteins (82, 93). Cellular plasminogen binding, which is mediated by both proteins and nonproteins such as gangliosides, is only partially sensitive to carboxypeptidase treatment. In contrast, the plasminogen receptors that play a role in acceleration of cell-associated plasmin are extremely sensitive to carboxypeptidase treatment and therefore contain lysine at the carboxyl-terminus (82, 94). The plasma carboxypeptidases TAFIa and CpN eliminate

plasminogen binding sites and serve to dampen fibrinolysis and cell-associated proteolysis. CpB is primarily a digestive enzyme produced by the pancreas (95), while CpN is constitutively active in plasma and has been implicated in peptide hormone processing (96), protection against anaphylatoxins (97), and binding of plasminogen to cells (93). TAFI has been primarily implicated in fibrinolysis (91-93, 98, 99) but also appears to play a role in regulating cellular plasminogen binding (93).

A non-protease dependent pathway for modulation of plasminogen receptors may be initiated by growth factors, chemokines or cytokines that alter the cell membrane and/or cytoskeleton architectures to expose plasminogen binding sites. Although this pathway has not been well characterized it has been shown that 1,25-dihydroxyvitamin D, interferon-gamma, and uPA can increase cell surface plasminogen binding (47, 100). Since this up-regulation of plasminogen binding is attenuated by carboxypeptidase treatment it is likely that newly exposed carboxyl-terminal lysines mediate this increased binding.

4.3. Activation of plasminogen at the cell surface

Cancer cells constitutively secrete the plasminogen activators, tissue plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA). The uPA is considered to be the predominant plasminogen activator secreted by cancer cells. Elevations in uPA and uPAR have been demonstrated in a variety of tumors including those of breast, bladder, colon, lung, ovary, and prostate (4, 101, 102). Early studies showed uPA and uPAR overexpression to be associated with areas of with invasive growth and also correlated with degradation of surrounding normal tissue (103, 104). The overexpression of uPA by neoplastic cells at the invading front of cancerous tissue leads to a cascade of proteolytic activities critical to local tumor growth and metastasis (105). UPA expression has been shown to be necessary for cancer cell invasion and metastasis as measured in experimental model systems and there is a strong association between uPA expression and the invasive-metastatic phenotype. In fact, enhanced expression of uPA is a significant prognostic marker for various human tumors (106). It was therefore concluded that high uPA/uPAR levels play a role in the proteolysis of the extracellular matrix that occurs at the invasive front of tumors. Subsequent studies have shown that although cancer cells can overexpress uPAR, often the stromal cells secrete uPA which is then utilized by the cancer cells (107). The expression of uPA can be regulated by *ras*, and other tyrosine kinase-encoding oncogenes (108-112). The observation that overexpression or constitutive activation of *ras* genes induces uPA and uPAR expression and uPA enzymatic activity in a variety of cell lines may afford an explanation of why cancer cells overexpress these proteins, since Ras overexpression is a common feature of many types of cancer (113). Growth factors such as basic fibroblast growth factor and phorbol ester stimulation also can activate uPA secretion. Their effect is mediated by the uPA enhancer, located upstream of the transcriptional start site. This element contains an Ets-2 site juxtaposed to an octameric AP-1_A site at the 5' end and an eptameric AP-1_B site at the 3' end. The region between the AP-1 sites is defined as cooperativity mediator (COM) and is necessary for the combined action of the AP-1-binding transcription factors. This inducible uPA expression is dependent on MEKK1 activation by these factors (114). Interestingly, the expression of uPAR is also transcriptionally regulated. Growth factors, cytokines and hormones can activate expression of this gene (115-117). It is also interesting to note that in addition to the enhanced transcription of uPA and uPAR, the plasminogen activator inhibitor, PAI-1 is commonly overexpressed by cancer cells and is associated with a poor prognosis in cancer (118, 119). However, it is likely that the enhanced PAI-1 levels may be related to the role of this protein in binding to vitronectin and hence blocking integrin-mediated cell adhesion and migration rather than as an inhibitor of uPA (120, 121).

The uPA secreted by cancer cells is in its single-chain proenzyme form, scuPA. The scuPA is rapidly converted to its active two-chain form by cell-bound plasmin (122). The tcuPA produced by the action of plasmin on scuPA then converts plasminogen to plasmin thus participating in an exponential double-reciprocal zymogen activation cycle. While plasminogen may bind to all cell-surface proteins with carboxyl-terminal lysine residues, only the binding of plasminogen to proteins with carboxyl-terminal residues which are colocalized to cell-surface receptors for plasminogen activators such as uPA is important for plasminogen activation. The critical importance of the accessibility of plasminogen receptors to the plasminogen activators explains why although the number of plasminogen receptors is 4-5 orders of magnitude higher than that of uPAR, bound plasminogen is rate-limiting for cell-surface-mediated activation of plasminogen by uPA (122, 123).

5. THE PLASMINOGEN RECEPTORS

The binding of plasminogen to its receptors not only converts plasminogen into a less rigid and more activatable conformation but also serves to localize plasminogen to the cell surface in close proximity to its activator, uPA. The proximity of the enzyme (uPA-uPAR complex) and substrate complex (plasminogen-receptor complex) results in an enhancement in plasminogen activation. In addition, the retention of plasmin, once formed, to the cell surface restricts the proteolytic activity of plasmin to the immediate vicinity of the cell. The retention of plasmin to the cell surface by its receptor is most likely important for allowing the cell to harness the proteolytic activity of the enzyme. This serves to direct plasmin towards particular substrates such as extracellular matrix proteins like fibronectin or to the cleavage and activation of other proteolytic enzymes such as the matrix metalloproteinases. In addition, a major consequence of the retention of plasmin at the cell surface is the protection of plasmin from inactivation by its major serum inhibitor, $alpha_2$-antiplasmin. The presence of this inhibitor in the serum serves to limit plasmin-dependent proteolysis to the immediate vicinity of the cell (reviewed in 11).

Plasminogen receptors have been detected on virtually all cell types tested, and their occupancy has also

been demonstrated under a number of biological settings. Characteristic features of plasminogen receptors include their relatively low affinity (0.3-2 micromolar) and their high density (10^5-10^7 receptors/cell) on many cells. There are many distinct receptors on the cell surface that bind plasminogen with comparable affinity (47, 124). Candidate plasminogen receptors possessing carboxyl-terminal lysines include S100A10 (125, 126), cytokeratin-8 (127-130), TIP49a (131), histidine-proline-rich glycoprotein (HPRG) (132-135), the integrins alphaMbeta2 and alpha5beta1 (136, 137) as well as alpha-enolase (75, 80, 138) (recently reviewed in 3, 79). Plasminogen binds to these receptors via the interaction of its kringles with the carboxyl-terminal lysines of the receptor. Interestingly, most of these plasminogen receptors have well characterized intracellular functions. Plasminogen or plasmin also binds to proteins that do not have a pre-existing carboxyl-terminal lysine residue. Included in this group are actin (84, 139, 140), glycoprotein IIIb/IIa (141), amphoterin (142, 143), annexin A2 (144, 145), and gp330 (146, 147). In addition to cell surface proteins, uPA and plasminogen can also bind to cell surface gangliosides (76, 88). Lysine analogues also prevent binding to gangliosides which implicate the kringle domains of plasminogen in the binding event. Whether or nor gangliosides are plasminogen regulatory proteins has not been established since enhanced plasminogen activation upon binding to gangliosides has not been demonstrated.

The first protein identified as a plasminogen receptor that possessed a carboxyl-terminal lysine, was present on the cell surface of several leukocyte cell types and was capable of binding plasminogen and stimulating plasminogen activation was alpha-enolase (148). The binding of plasminogen to alpha-enolase was inhibited by lysine analogues and removal of the carboxyl-terminal lysine residue by carboxypeptidase B significantly reduced its plasminogen binding capacity, suggesting that binding required a carboxyl-terminal lysine residue (149). Alpha-enolase has been shown to account for about 10% of the total plasminogen binding capacity of U937 cells. The mechanism by which this cytosolic glycolytic enzyme is transported to the cell surface is unclear. It is likely that alpha-enolase, which lacks a secretory signal sequence, is transported to the cell surface by a leaderless secretion mechanism. Recently, it was proposed that alpha-enolase localizes and enhances plasmin generation on the cell surface and via this function acts as an important plasminogen regulatory protein in skeletal myogenesis (150).

Other plasminogen receptor proteins have been shown to play important physiological role. HPRG is a relatively abundant protein in plasma (concentration of 1.5 micromolar) that binds plasminogen with micromolar affinity (Kd approximately 1 micromolar, in solution). It has been estimated that about 50% of plasminogen circulates bound to HPRG. Immobilized HPRG increases the efficiency of plasminogen activation by tPA or uPA about 30-fold and 2-fold, respectively (151, 152). Cytokeratin-8 has been identified on the external surfaces of epithelial cells and it has been proposed that this protein is the major plasminogen-binding protein of breast cancer cells (128, 130). An antibody to cytokeratin-8 was shown to decrease cellular plasminogen binding by 80% and decrease the rate of tPA-dependent plasminogen activation by about 80%, therefore suggesting an important role for this protein in tPA-dependent plasminogen regulation.

It has also been reported that actin may play a role as a plasminogen receptor despite the fact that this protein does not possess a carboxyl-terminal lysine. In experiments using anti-actin antibodies, it was observed that binding of plasminogen to endothelial cells was inhibited by 45%, while tPA binding was inhibited by 46%. This suggested that actin could act as a cellular binding site for tPA and plasminogen (140, 153, 154). Other studies have shown that actin may colocalize with uPA on the cell surface (139). Monomeric or polymerized actin has been shown to bind to tPA (Kd of 0.5 micromolar). Actin also stimulates plasmin generation from plasminogen and this reaction was blocked by EACA. The stimulation of plasmin generation by actin was enhanced by plasmin-dependent degradation of actin and plasmin-treated actin bound tPA and plasminogen simultaneously. Denaturation of actin also enhanced its stimulation of plasmin formation (45, 46).

Several experimental approaches have been utilized to identify the relative contributions of plasminogen receptors to total cellular plasminogen binding. Ligand blots of U937 cell membranes with labeled plasminogen revealed that membrane-associated alpha-enolase, actin and annexin A2 showed minimal changes in plasminogen binding following carboxypeptidase B treatment of intact cells, suggesting that although these proteins may play a role in cellular plasminogen binding, it is unlikely that they play a role in cellular plasminogen activation. In other studies, ligand blots of monocyte membranes revealed that plasminogen binding to alpha-enolase was reduced 71% by treatment of intact cells with carboxypeptidase B while binding to annexin A2 was reduced only by 14%. Thus, it was likely that enolase but not annexin A2 might play an important role as a plasminogen regulatory protein in this cell line. An additional study utilized immunofluorescence microscopy to examine the distribution of plasminogen receptors with uPA. These investigators found that plasminogen partially colocalized with actin on the surface of the breast cancer cells, while other candidate plasminogen receptors were either not expressed (alpha-enolase, cytokeratin-8) or did not significantly colocalize with plasminogen (annexin A2) (139, 155). Actin also colocalized with uPA suggesting the formation of a plasminogen-actin-uPA ternary complex on the cell surface. It is important to point out that with the exception of annexin A2 and S100A10, knockdown studies have not been performed for any of the plasminogen receptors. In many cases such as actin and enolase plasminogen receptors have other critical cellular functions so it is not possible to perform such studies. It is therefore difficult to elucidate the contribution of these plasminogen receptors to cellular plasminogen binding or to establish if they play a significant role in plasminogen activation.

It is important to differentiate between a plasminogen receptor and a plasminogen regulatory protein. As discussed, a variety of protein and non-protein

molecules have been identified as plasminogen-binding molecules. However, only a few of these molecules have been suggested to actually be involved in cellular plasminogen activation. Furthermore, although some plasminogen receptors such as alpha-enolase and actin are known to stimulate plasmin formation *in vitro*, the plasminogen-binding sites of these proteins may not be accessible *in vivo* (84). As discussed by others (156), in order for a plasminogen-binding protein to be considered a *bone fide* plasminogen regulatory protein several critical criteria must be met. First, the binding of plasminogen to the candidate receptor must fulfill several prerequisites. It must be demonstrated that the candidate protein binds plasminogen with low micromolar affinity. Binding of plasminogen must be dependent on a carboxyl-terminal lysine and blocked by carboxypeptidase B treatment. The binding of plasminogen to the plasminogen regulatory protein must convert plasminogen into the open, activation-susceptible conformation thereby resulting in enhanced plasminogen activator-dependent conversion of plasminogen to plasmin. Plasminogen regulatory proteins that bind plasminogen activator or plasmin must protect these enzymes from inactivation by their inhibitors. Second, it must be demonstrated that the candidate protein either directly binds a plasminogen activator or colocalizes with a plasminogen activator on the cell surface. Third, loss of the candidate protein from the cell surface, by selective elution, blocking export or by knock-down must result in a loss in cellular plasmin generation. Under certain circumstances blocking antibodies are a reasonable option. However, it is critically important that the binding site on the protein and mechanism of action of the blocking antibodies is characterized. As discussed in Section 8 and shown in Figure 2, S100A10 is one of the only plasminogen regulatory proteins to satisfy all aspects of these criteria.

6. ANNEXIN A2, S100A10, AND THE ANNEXIN A2 HETEROTETRAMER

The annexins are classically defined as proteins that bind to anionic phospholipids in a Ca^{2+}-dependent manner (for a recent review, see (157)). To date, at least twelve annexins have been described in higher vertebrates and at least one member of the family has been found in all mammalian tissues and cell types except erythrocytes. The annexins appear to be widely distributed throughout nature and have been reported in a diverse number of organisms including *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, *Giardia,* several fungi, and all plants examined thus far. The only organisms studied that do not encode any of the annexins are viruses, prokaryotes, and yeast. In most biological contexts studied, the annexins are highly abundant proteins, reaching levels as high as 0.5-2% of the total cellular proteins (158). All annexins share a core domain of four similar repeats, each approximately seventy residues long with each repeat composed of five alpha-helixes. Typically, the repeats contain several type II Ca^{2+}-binding sites bearing the Gly-x-Gly-Thr-[38 residues]-Asp/Glu motiff. Based on their Ca^{2+}-dependent interaction with membranes, numerous putative roles have been described for the annexins including exocytosis, endocytosis, formation of ion channels, cell-cell and cell-matrix interactions, and inhibitors of phospholipase A_2 activity. Additionally, some members of the annexin family appear to be involved in aspects of cellular differentiation, proliferation and mitogenesis.

Annexin A2 was originally identified as a 36 kDa protein that was phosphorylated upon transformation of cells with the Rous sarcoma virus (159) (recently reviewed in160). The amino-terminal domain (ATD) contains the amino-terminal thirty amino acid residues, and incorporates two phosphorylation sites at Tyr-23 and Ser-25. In addition to the phosphorylation sites, the ATD also contains the site for interaction with the S100A10 dimer. The remaining carboxyl core domain (CCD), encompassing residues 31-338, consists of multiple ligand-binding sites including binding sites for F-actin (161), fibrin (162) RNA (163), and heparin (164) (reviewed in 157, 158, 160). AIIt is an oxidatively labile protein whose level of activity is regulated by the redox status of its sulfhydryl groups. Oxidative stress of cells results in the glutathionylation of annexin A2. The glutathionylation of AIIt, *in vitro*, results in the modification of Cys^{8} and Cys^{132} of the annexin A2 subunit and results in the inhibition of phospholipid and F-actin binding activity of AIIt. Deglutathionylation of AIIt by glutaredoxin, however, restores activity (165).

The crystal structure of an amino-terminally truncated form of annexin A2 has been reported (166). The protein is planar and curved with opposing convex and concave sides. The convex side faces the biological membrane and contains the Ca^{2+}- and phospholipid-binding sites. The concave side faces the cytosol and contains both the amino- and carboxyl-termini. The amino-terminal region contains the binding site for the S100A10 subunit.

The expression of annexin A2 is induced in various transformed cells, including v-*src*-, v-H-*ras*-, v-*mos* or SV40-transformed cells (167). Furthermore, the *ANXA2* gene is growth-regulated and its expression is stimulated by growth factors such as insulin, FGF and EGF (168). Up-regulated annexin A2 has also been reported in human hepatocellular carcinoma (169), pancreatic adenocarcinoma (170), high-grade glioma (171), gastric carcinoma (172) and in acute promyelocytic leukemia (173). In contrast, loss of annexin A2 by gene silencing has been reported in prostate cancer specimens (174-177).

Annexin A2 exists as three major species - a monomer, a heterodimer or a heterotetramer (AIIt) (158). The heterodimer is composed of a single subunit of annexin A2 bound to a subunit of 3-phosphoglycerate kinase (178). The heterotetramer, on the other hand, comprises two subunits of annexin A2 linked together by a dimer of S100A10 (also referred to as p11), a member of the S100 family of Ca^{2+}-binding proteins (179-182). The binding of S100A10 to annexin A2 is essentially irreversible. The relative amounts of heterotetrameric versus monomeric annexin A2 are variable depending on the cell or tissue examined, and range from 100% heterotetrameric annexin A2 in intestinal epithelium, to about 50% monomeric annexin A2 monomer in cultured fibroblasts (180, 183).

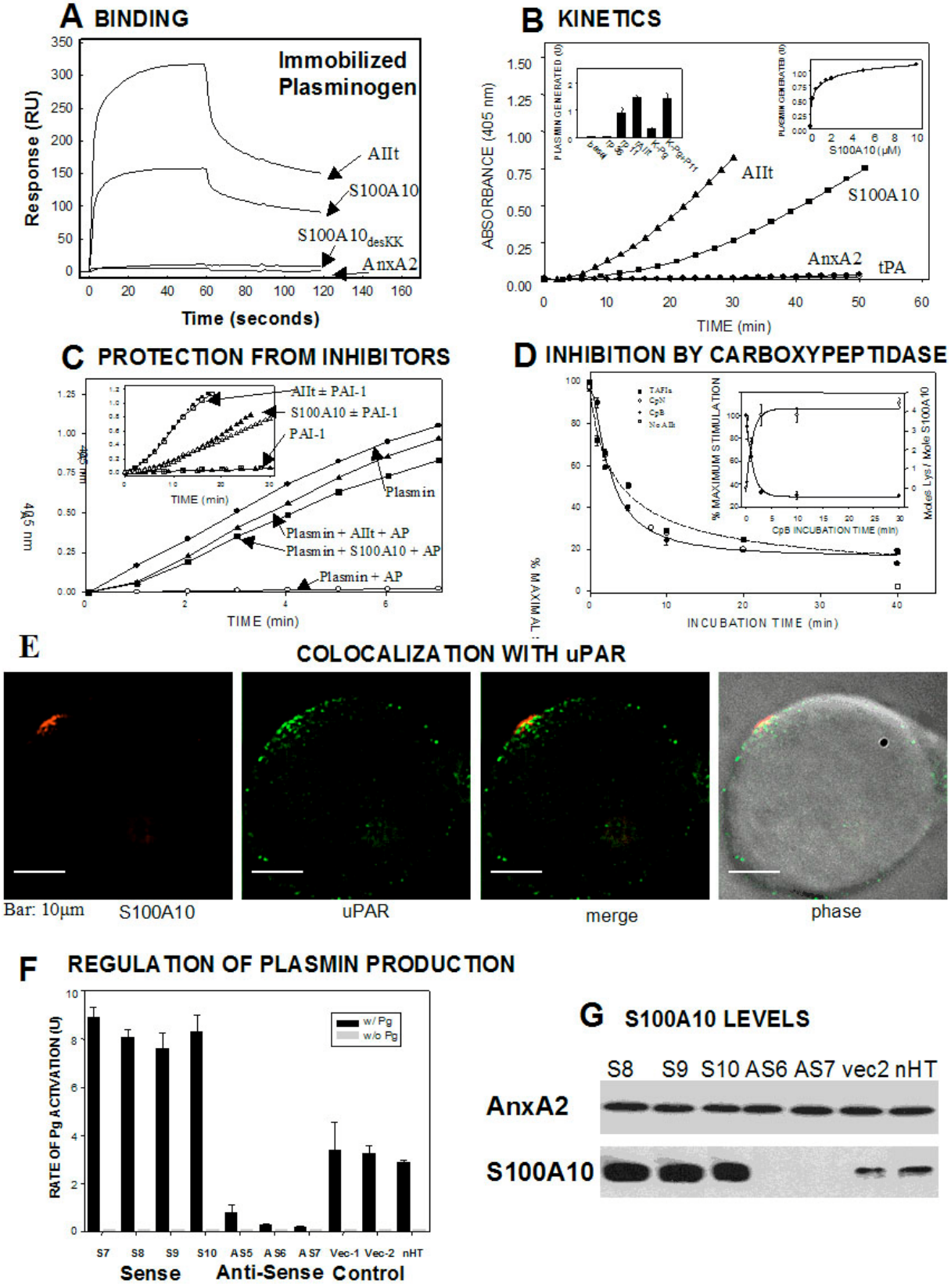

Figure 2. S100A10 is a plasminogen regulatory protein. A. Interaction of plasminogen with annexin A2-S100A10 heterotetramer or its subunits. Surface plasmon resonance studies of the binding of AIIt, S100A10, S100A10$_{des\text{-}KK}$, and annexin A2 to an

immobilized plasminogen surface. About 575 RU of plasminogen was immobilized on the biosensor chip and 500 nM AIIt, S100A10, $S100A10_{desKK}$ or annexin A2 were applied at a flow rate of 30 μL/minute. Removal of the carboxyl-terminal lysines of S100A10 ($S100A10_{desKK}$) attenuates binding, therefore establishing the carboxyl-terminal lysines as the plasminogen binding site of S100A10. Tissue or recombinant annexin A2 did not bind to plasminogen. Similarly, annexin A2 immobilized on a phospholipid surface did not bind plasminogen or tPA. B. Stimulation of t-PA-dependent plasminogen activation by annexin A2, S100A10, and AIIt. T-PA (5.6 nM) was incubated at 25 °C in buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $CaCl_2$) in the absence (*basal*) or presence of purified recombinant annexin A2 (2 micromolar), 2 micromolar recombinant S100A10, 2 micromolar recombinant AIIt and Spectrozyme #251 substrate (104 micromolar). The reaction was initiated by the addition of 0.11 micromolar [Glu]-plasminogen and the amidolytic activity of plasmin was monitored. *Left inset*, comparison of the rates of plasmin generation calculated from plots of $A_{405\ nm}$ *versus* t^2 (mean ± S.D.), *basal*, 0.019 ± 0.006 U (n = 9); *rp36* (annexin A2), 0.028 ± 0.005 U (n = 5); *rp11* (S100A10), 0.866 ± 0.20 U (n = 9); *rAIIt*, 1.47 ± 0.24 (n = 4). The stimulation of the rates of t-PA dependent [Lys]-plasminogen activation in the absence (0.306 ± 0.05, n = 3) and presence of 2 micromolar S100A10 (1.4 ± 0.2, n = 3) is also compared. *Right inset*, concentration-dependence of the stimulation of t-PA-dependent [Glu]-plasminogen activation by recombinant S100A10. C. AIIt and S100A10 protect tPA and plasmin from inactivation. Plasmin (105 nM) was incubated at 25 °C in the absence (*filled circles*) or presence (*open circles*) of 210 nM $alpha_2$-antiplasmin. In other experiments, plasmin was preincubated with recombinant S100A10 (*filled triangles*) or bovine lung AIIt (*filled squares*). After 5 min, 210 nM $alpha_2$-antiplasmin was added and the reaction was initiated by the addition of substrate. The preincubation of plasmin with S100A10 or AIIt but not annexin A2 (not shown) protects plasmin from inactivation. *Inset*, Comparison of the rates of t-PA-dependent plasmin production. Recombinant S100A10 (2 micromolar) (*triangles*) or bovine lung AIIt (2 micromolar) (*squares*) were preincubated with tPA for 5 min., followed by incubation in the absence (*filled symbols*) or presence of 58 nM PAI-1. The reaction was initiated by addition of plasminogen and amidolytic substrate. In other experiments, AIIt (*open crossed square*) or S100A10 (*closed crossed triangle*) were preincubated with PAI-1 for 5 min before addition of tPA and the rate of tPA-dependent plasmin production was determined. These experiments (*inset*) showed that the incubation of AIIt or S100A10 with tPA was required to protect tPA from inactivation by PAI-1. D. Inactivation of AIIt or S100A10 by plasma carboxypeptidases. AIIt was treated with 0.1 micromolar thrombin-activated fibrinolysis inhibitor (TAFI) (*filled squares*), 0.5 micromolar carboxypeptidase N (CpN) (*open circles*) or 0.1 micromolar carboxypeptidase B (CpB) (filled circles) and aliquots were removed at the indicated time points and the reaction was quenched with 10 micromolar MGTA. The rates of plasmin formation by CpB-, CpN- or TAFIa- digested AIIt (2 micromolar) was then determined. *Inset*, Loss in stimulation of plasminogen activation corresponds with release of lysine from S100A10**.** 35 micromolar recombinant S100A10 was incubated with 43 nM CpB at 37°C. At the indicated time points the carboxypeptidase was inactivated with MGTA and aliquots were removed and assayed for plasmin production (*closed circles*) or for the amount of free lysine in solution (*open circles*). These experiments show that several carboxypeptidases remove the carboxyl-terminal lysines from S100A10 or S100A10 complexed with annexin A2 (AIIt) and this results in a dramatic loss in activity. E. Colocalization of S100A10, and uPAR on the CCL-222 cell surface. Colorectal cells grown on glass coverslips were fixed with 4% paraformaldehyde and stained for both S100A10 and uPAR using anti-S100A10 monoclonal antibody and anti-uPAR polyclonal antibody. Immunofluorescence photomicrographs detailing the extracellular expression of S100A10 and uPAR were compared using confocal microscopy. F. S100A10 regulates plasminogen activation on the cell surface of HT1080 fibrosarcoma cells. The S100A10 gene was inserted in the pLin plasmid in the sense (*S7, S8, S9, S10*) or antisense (*AS5, AS6, AS7*) orientation. HT1080 fibrosarcoma cells were transfected with pLin-sS100A10, pLin-aS100A10, and pLin (vector control-*Vec-1, Vec-2*) using LIPOFECTAMINE 2000 reagent and stable transfectants were cloned and clonal cell lines propagated. Untransfected cells were also compared (nHT). Confluent cells were incubated in the absence or presence of 1 micromolar [Glu]-plasminogen and cell-generated plasmin activity was measured at 405 nm after addition of the plasmin substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide. G. Western blot of annexin A2 and S100A10 levels of transfected HT1080 cells. Transfected cells were lysed, resolved by SDS PAGE, transferred to nitrocellulose and analyzed for annexin A2 or S100A10. Figures are reprinted with permission.

A multitude of intracellular functions have been suggested for annexin A2, including roles as a mediator of Ca^{2+}-regulated exocytosis (184-187) or endocytosis (188-191) as well as a role in modulating sarcolemmal phospholipid raft organization during smooth muscle cell contraction (192, 193) modulating cell membrane cytoarchitecture (194) and regulation of ion channels (195). Hajjar's group first reported that annexin A2 was present on the endothelial cell surface where it bound tPA, plasminogen and plasmin (144, 145). Subsequent studies have shown that the majority of this extracellular annexin A2 is bound to S100A10 (125). Since annexin A2 does not possess a carboxyl-terminal lysine, it has been postulated that proteolytic processing of annexin A2 could result in the exposure of a carboxyl-terminal lysine. However, this putative processing event has never been demonstrated either *in vitro* or *in vivo*.

S100A10 is a member of the S100 family of proteins which contain 2 EF-hand calcium-binding motifs (reviewed in 196). S100 proteins are present in the cytoplasm and/or nucleus of a wide range of cells, and are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members which are located as a cluster on chromosome 1q21. S100A10 is the only member of the S100 family that has suffered deletions and mutations in its two EF hand domains thus rendering the Ca^{2+}-binding sites nonfunctional. However, these mutations have resulted in the locking of the protein in a permanently active conformation. Thus, the binding of S100A10 to its

primary ligand, annexin A2, is Ca^{2+} independent.

S100A10 exists intracellularly as a cytosolic protein which, upon binding annexin A2, directs the complex to the submembranous region of the cell (197). It has been reported that that S100A10 regulates cytosolic phospholipase A2 (cPLA2) by interacting with the carboxyl-terminal region of cPLA2, resulting in inhibition of cPLA2 activity and arachidonic acid release (198-200). Also, EGF treatment was shown to increase S100A10 bound to cPLA2, leading to the late suppression of arachidonic acid release induced by EGF. Additionally, the S100A10-annexin A2 complex has been suggested to form a complex with and regulate epithelial Ca^{2+} channels (201). Extracellular S100A10 has been shown to be present on the surface of many cells (reviewed in 79). Initially, extracellular S100A10 was shown to be complexed with annexin A2 on the cell surface (125, 202). However, it was recently shown that S100A10 but not annexin A2 was present on the surface of colorectal cells, suggesting that S100A10 could associate with the cell surface without its annexin A2 binding partner (203). As discussed in Section 8 and presented in Figure 2, studies from our laboratory have suggested that S100A10 is a key plasminogen regulatory protein (125, 126, 202-206). As discussed in Section 8, S100A10 possesses a carboxyl-terminal lysine and binds plasminogen via a lysine-dependent mechanism. Interestingly, S100A10 may also regulate other cell surface proteases such as cathepsin B (207, 208).

7. IS ANNEXIN A2 A PLASMINOGEN RECEPTOR?

In order for a possible role of annexin A2 in plasminogen regulation to be established, it must be determined whether annexin A2 is present at the cell surface as a monomer or complexed with S100A10 as the heterotetramer (AIIt). Although the weight of evidence suggests that the majority of annexin A2 is present as AIIt, it is not possible to rule out the presence of the annexin A2 monomer at the cell surface. Therefore, we will consider the possible role of either the annexin A2 monomer or the annexin A2 subunit of AIIt in plasminogen regulation.

Hajjar's group originally demonstrated that annexin A2 monomer bound tPA, plasminogen and plasmin and stimulated the tPA-dependent-activation of plasminogen (144, 145, 209). These studies utilized annexin A2 that was purified from placenta by elution from a SDS polyacrylamide gel. The binding studies conducted with this annexin A2 involved the drying of the protein on polystyrene plates. The kinetic studies were conducted with purified components, however a one hour preincubation of annexin A2 with plasminogen was required before initiation of the reaction. Since the binding and activation of plasminogen were lysine-dependent and annexin A2 does not possess a carboxyl-terminal lysine, the authors speculated that their annexin A2 was *proteolytically processed* thus exposing a new carboxyl-terminal lysine. This *processed* annexin A2 has never been isolated or characterized nor has it been shown to exist either *in vitro* or *in vivo* (see Figure 3A). Unfortunately, the authors did not address the possibility that their annexin A2 preparation or the polystyrene-adsorbed annexin A2 used in their binding studies was not *processed* but actually denatured. This is an important point because many denatured proteins stimulate plasmin formation nonspecifically (151, 152).

We have directly addressed the issue of the role of annexin A2 in plasminogen regulation (reviewed in 79, 160, 210). For these studies we used native annexin that was isolated from bovine lung or human recombinant protein isolated from bacteria (211) or yeast (204). We observed that annexin A2 stimulated tPA-dependent plasminogen activation only about two-fold and that in fact annexin A1 was a more potent stimulator (212). These results were consistent with our observation that AIIt but not annexin A2 caused a quenching of the fluorescence of plasminogen that was FITC-labeled at its active site. This meant that AIIt, but not annexin A2 promoted an open activatable conformation of plasminogen. We have also used surface plasmon resonance to examine the interaction between annexin A2 and plasminogen in real time. We observed that annexin A2 either directly amine-coupled to the biosensor or tightly bound to a phospholipid surface on the chip did not bind plasminogen or tPA. Furthermore, when amine-coupled plasminogen was used as a ligand it did not bind to annexin A2. We did however, observe that phospholipid-associated annexin A2 did bind to plasmin (Kd of 0.78 micromolar). The binding of plasmin to annexin A2 was consistent with our hypothesis that annexin A2 was a plasmin reductase which functioned to convert plasmin into angiostatin (15, 16). These results also suggested that the minor stimulation of tPA-dependent plasminogen activation by annexin A2 could be due to its binding and therefore sequestration of plasmin, the product of the plasminogen activation reaction.

Perhaps the most compelling evidence for a role of annexin A2 in plasminogen regulation is the observation that annexin A2 knockdown or knockout blocks cellular plasmin generation (213). Homozygous annexin A2-null mice display deposition of fibrin in the microvasculature and incomplete clearance of injury-induced arterial thrombi. However, interpretation of these studies is complicated by the observations that changes in annexin A2 levels affect S100A10 levels. For example, it has been reported that annexin A2 regulates the expression of S100A10 protein by a post-translational mechanism (174, 214). Our laboratory has also shown that transfection of 293 cells, which lack both annexin A2 and S100A10, with annexin A2, results in the appearance of S100A10 (204). Recently, Benaud *et al.* (194), down-regulated annexin A2 of MDCK cells by transfection with annexin A2-specific small interfering RNA (siRNA). They observed that the annexin A2-specific siRNA caused a significant down-regulation of both annexin A2 and S100A10. In contrast, our laboratory observed that down-regulation of S100A10 by antisense to S100A10 or S100A10-siRNA blocks S100A10 but not annexin A2 protein expression (202, 203). Therefore, it is apparent from these studies that annexin A2 regulates S100A10 levels and that studies utilizing knock-down of annexin A2 cannot be used to support a role for annexin A2 in plasminogen regulation. Data showing the regulation of S100A10 levels by annexin A2 is presented in Figure 3B, C.

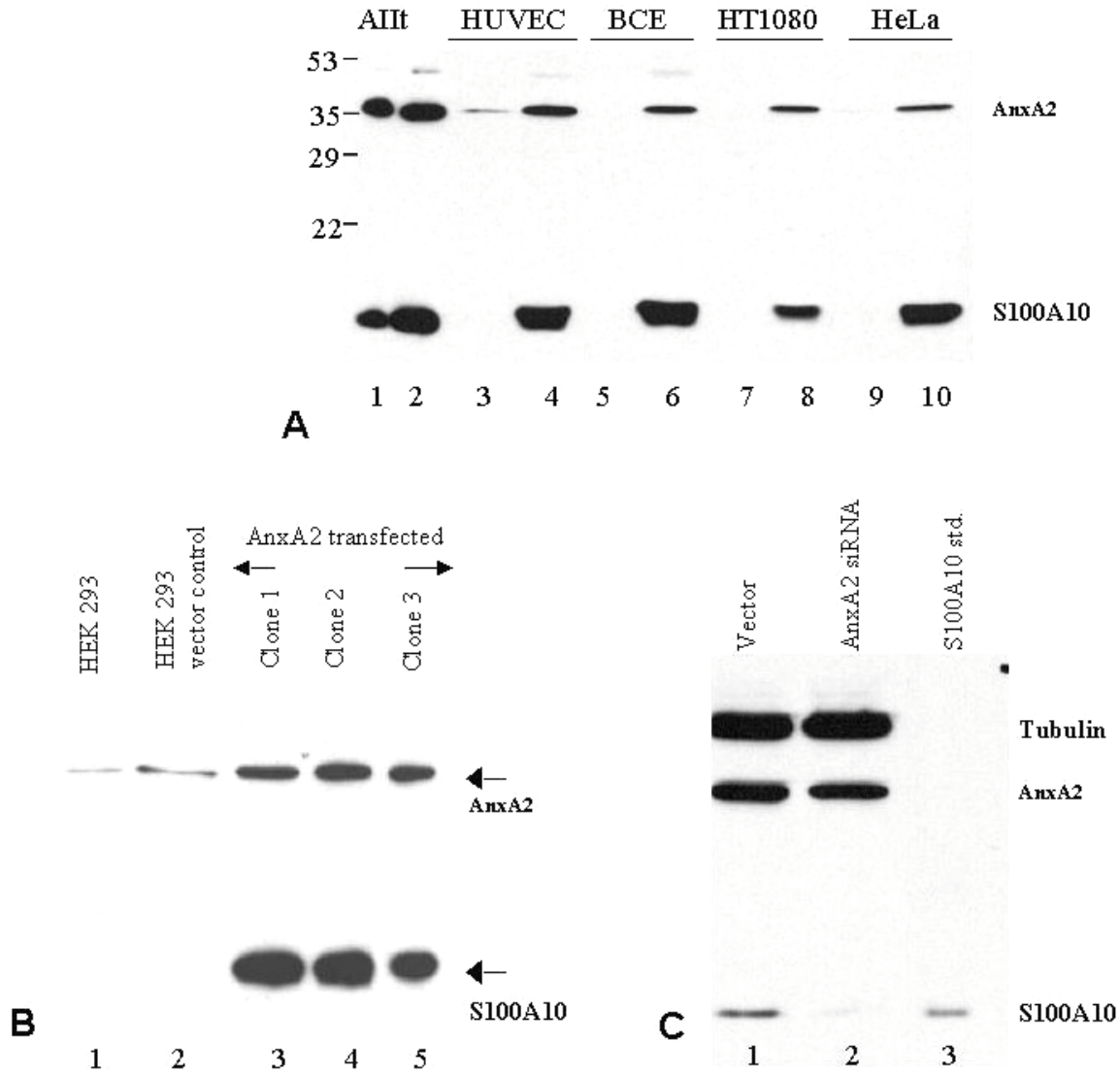

Figure 3. Association of S100A10 and annexin A2. A. Detection of annexin A2 and S100A10 on the cell surface of cultured cells. Cultured cell monolayers were surface biotinylated by incubation with PBS containing 0.5 mg/ml sulfo-NHS-biotin at room temperature for 30 min. The monolayers were washed five times with PBS, then incubated with Versene (0.5 mM EDTA in PBS) at 37 °C for 15 min. The supernatant (Versene extract) was incubated with either control-agarose beads or streptavidin-agarose beads, and the bound proteins were subjected to Western blot analysis using antibody against annexin A2 or S100A10. Control-agarose bound (*lane 3*) or streptavidin-agarose bound (*lane 4*) annexin A2 and S100A10 from primary HUVECs (human umbilical vein endothelial cells); Control-agarose bound (*lane 5*) or streptavidin-agarose bound (*lane 6*) annexin A2 and S100A10 from primary BCE (bovine capillary endothelial) cells; Control-agarose bound (*lane 7*) or streptavidin-agarose bound (*lane 8*) annexin A2 and S100A10 from HT 1080 (human fibrosarcoma) cell line; Control-agarose bound (*lane 7*) or streptavidin-agarose bound (*lane 10*) annexin A2 and S100A10 from HeLa (human cervical cancer) cell line; AIIt standard (*lane 1, 2*) is also shown. This experiment demonstrates that when cells are cultured under normal conditions, their cell surface annexin A2 and S100A10 are not proteolyzed (*processed*). B. Coexpression of annexin A2 and S100A10 in annexin A2-transfected HEK 293 cells. HEK 293 cells were transfected with pLin-annexin A2 (containing full length annexin A2) and pLin vector control with LIPOFECTAMINE 2000 reagent, and stable transfectants were cloned and propagated. Expression of annexin A2 and S100A10 was examined by Western blotting. *lane 1*, untransfected; *lane 2*, vector transfected; *lane 3*, clone 1; *lane 4*, clone 2; *lane 5*, clone 3. This experiment demonstrates that the forced expression of annexin A2 in cells normally devoid of both annexin A2 and S100A10 results in the expression of S100A10. C. Transfection of HT1080 cells with annexin A2 siRNA results in the loss of S100A10. HT1080 cells were transfected with the pSUPER vector containing annexin A2 siRNA. Stable cells were isolated and the annexin A2 and S100A10 levels were analyzed in total cell lysates by Western blotting. *lane1*, untransfected; *lane 2*, vector transfected; *lane 3*, clone 1. This experiment shows that down-regulation of annexin A2 using an annexin A2-specific small interfering RNA (siRNA) also blocks the expression of S100A10. Collectively, these experiments show that S100A10 levels are regulated by annexin A2.

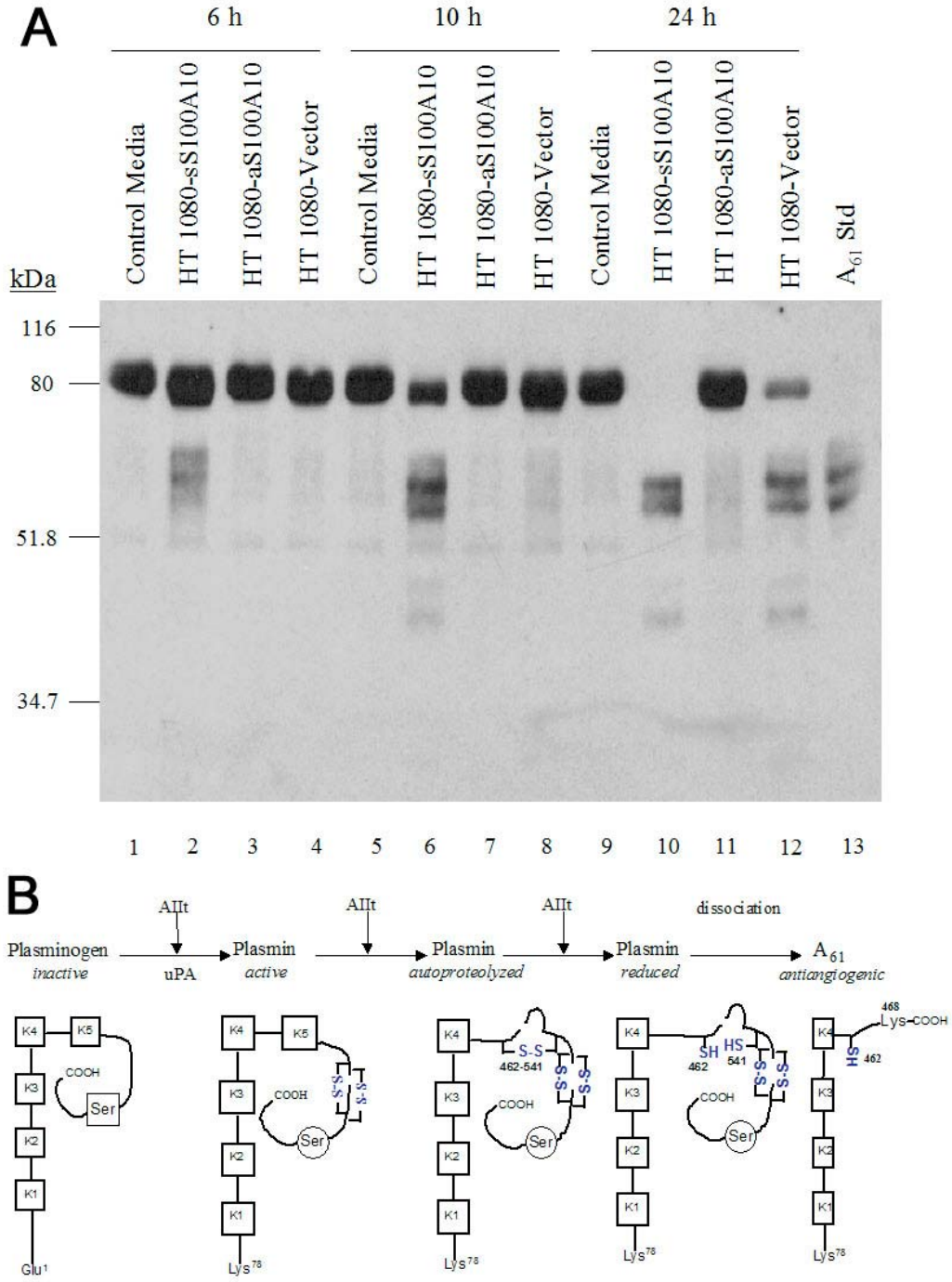

Figure 4. (A) Down-regulation of AIIt blocks A_{61} generation by HT1080 cells. Transfected HT1080 cells were incubated with DMEM containing 2 micromolar [Glu]-plasminogen. After the indicated time of incubation, the medium was analyzed by reduced SDS-PAGE followed by Western blot with monoclonal anti-human plasminogen kringle 1-3 antibody. As a control, each protein was incubated with DMEM in the absence of cells (*lane 1, 5, 9*). The following HT1080 clones were used; S100A10 sense-transfected cells (*lane 2, 6, 10*); S100A10 antisense-transduced cells (*lane 3, 7, 11*); control vector-transfected cells (*lane 4, 8, 12*). A_{61} standard is also shown (*lane 13*). (B) Diagrammatic illustration of the mechanism of A_{61} generation. AIIt stimulates the uPA-catalyzed cleavage of plasminogen to plasmin at Arg^{561}-Val^{562} peptide bond. This reaction is promoted by the C-terminal lysines of the S100A10 subunit on HT1080 cell surface. Plasmin then catalyzes the cleavage of the Lys^{77}-Lys^{78} and Lys^{468}-Gly^{469} peptide bonds of plasmin by autoproteolysis. AIIt then cleaves the Cys^{462}-Cys^{541} disulfide bond. This reaction is catalyzed by the Cys^{334} residue of the annexin A2 subunit. The indications are K, kringle domain of plasminogen, S-S, disulfide bond, and SH, free thiols.

Antibodies that block cell surface plasminogen binding can be used to establish their antigens as putative plasminogen receptors. Annexin A2 was identified on the surface of RAW264.7 macrophages using a commercially available monoclonal antibody (Zymed Laboratories, Clone Z014), and it was shown that this antibody inhibited the binding of ^{125}I-Lys-plasminogen to the cell surface by 35% (215). The same antibody was also employed recently to investigate the role of annexin A2 in plasmin generation by cerebral microvascular endothelial cells (216). Unfortunately, there is an inherent difficulty in interpreting the results of these studies. Since it has been established that annexin A2 on the cell surface exists primarily as the heterotetramer complex with S100A10, one must ask whether the observed effects of the anti-annexin A2 are due to the blockage of a plasminogen binding site on annexin A2 or on the adjacent S100A10 subunit. It is entirely possible that the binding of anti-annexin A2 to the annexin A2 tetramer obscures the carboxyl-terminal lysine residues of the S100A10 subunit, and it might even cause the dissociation of S100A10 from annexin A2. In the studies conducted thus far, it is simply not possible to distinguish between an effect upon annexin A2 monomer, or upon the S100A10 and annexin A2 subunits of annexin A2 tetramer.

Further complicating matters is the fact that attempts to inhibit plasminogen binding with antibodies to annexin A2 have met with mixed results. For instance, the inhibition of plasminogen binding to human umbilical vein endothelial cells (HUVECs) by polyclonal antisera to human annexin A2 was reported a decade ago (144). More recently, a variety of antibodies to annexin A2 (including the aforementioned Z014 clone) were found to have no effect upon plasminogen binding to thrombin-treated HUVECs (217). This group demonstrated that both annexin A2 and S100A10 were significantly increased on the surface of human umbilical vein endothelial cells (HUVECs) after treatment with thrombin or the thrombin receptor-activating peptide. The increase in both proteins resulted in the enhanced binding of plasminogen to the cells and an approximate 6-fold increase in plasmin generation. Interestingly, they observed that intracellular S100A10 was increased but annexin A2 remained unchanged after thrombin treatment. They concluded from this observation that S100A10 might be necessary for the extracellular transport of annexin A2. Of particular interest was their observation that an antibody to S100A10 blocked plasminogen binding to the HUVECs. Hence, this data support the notion that S100A10 is a key plasminogen regulatory protein on the HUVEC cell surface and that annexin A2 plays an ancillary role, possibly by promoting the export of S100A10 to the cell surface.

We have also examined the role of the annexin A2 subunit of AIIt in plasminogen regulation. We reasoned that if annexin A2 played a role in plasminogen regulation when complexed with S100A10 then if we blocked the tPA and plasminogen binding sites of S100A10 component of the complex by removal of the carboxyl-terminal lysines, it would allow analysis of the plasminogen binding properties of annexin A2 that was complexed to S100A10. We observed that removal of the carboxyl-terminal lysine by carboxypeptidase treatment or by site-directed mutagenesis of S100A10, completely blocked the binding of tPA or plasminogen to this modified AIIt (204) (Figure 2A). This result established that annexin A2, complexed to S100A10 does not bind tPA or plasminogen. We also used this mutant AIIt to examine the role of complexed annexin A2 in plasminogen activation. We observed that compared to wild-type AIIt, the carboxyl-terminal deletion mutant retained about 12% of its activity. Therefore, this experimental approach revealed that even when complexed to S100A10, annexin A2 does not bind tPA or plasminogen and does not appreciably stimulate tPA-dependent plasminogen activation.

In conclusion, it is therefore likely that annexin A2 plays a major role in regulating plasmin activity and in the expression and transport of S100A10 to the cell surface. However, until the binding of plasminogen to native annexin A2 can be demonstrated, the experimental evidence does not support a role for annexin A2 as plasminogen receptor.

8. ROLE OF S100A10 IN PLASMINOGEN REGULATION AND CELLULAR INVASIVENESS

Studies from our laboratory have established that S100A10 can exist on the cell surface either complexed to annexin A2 as the heterotetramer, AIIt or as the S100A10 homodimer (125, 202, 203). It is therefore likely that annexin A2 serves to localize S100A10 to the cell surface. Possibly, the phospholipid-binding sites of annexin A2 function to tether the complex to the cell surface. The mechanism by which S100A10 can associate with the cell surface in the absence of its annexin A2 binding partner is not known but could involve the association of S100A10 with an unidentified plasma membrane protein. As discussed in Section 5, in order for a protein to be considered a *bone fide* plasminogen regulatory protein several critical criteria must be met. As discussed in this Section and shown in Figure 2, S100A10 has met these criteria and should therefore be considered an important plasminogen regulatory protein.

The first criteria for a putative plasminogen regulatory protein is that it binds plasminogen (Figure 2A). Surface plasmon resonance studies have established that S100A10 binds tPA (K_d of 0.45 micromolar), plasminogen (K_d of 1.81 micromolar) and plasmin (K_d of 0.36 micromolar). S100A10 possesses the prerequisite C-terminal lysine residues that had been shown to be essential for plasminogen activation at the cell surface. The carboxyl-terminus of S100A10 comprises the residues ([85]-Y-F-V-V-H-M-K-Q-K-G-K-K[96]). Removal of these carboxyl-terminal lysines from S100A10 attenuated tPA and plasminogen binding (204).

Another important criteria is that the binding of plasminogen to the regulatory protein converts plasminogen into the open, activation-susceptible conformation. The binding of plasminogen to S100A10 converts plasminogen into the open, activation-susceptible conformation thereby resulting in enhanced plasminogen

activator-dependent conversion of plasminogen to plasmin. For example, S100A10 or AIIt caused a quenching of the fluorescence of plasminogen that was FITC-labeled at its active site. This meant that S100A10 promoted an open activatable conformation of plasminogen. *In vitro* kinetic studies have shown that recombinant S100A10 stimulates the rate of tPA-dependent activation of plasminogen about 46-fold compared to an approximate 2-fold by the recombinant annexin A2 subunit and 77-fold by recombinant AIIt (Figure 2B). The stimulation of tPA-dependent activation of plasminogen by S100A10 or AIIt was inhibited by EACA. A deletion mutant of S100A10, missing the two carboxyl-terminal lysine residues retained only about 15% of the activity of the wild-type S100A10. Similarly, a mutant AIIt composed of the wild-type annexin A2 subunit and the S100A10 subunit deletion mutant possessed about 12% of the wild-type activity. Also, a peptide to the carboxyl-terminus of S100A10 ([85]-Y-F-V-V-H-M-K-Q-K-G-K-K[96]) inhibited the S100A10-dependent stimulation of tPA-dependent plasminogen activation. We also made a truncated AIIt by forming a complex between a peptide consisting of the amino-terminal 15 amino acids of annexin A2 (the S100A10-binding portion of annexin A2 (218)), and wild-type S100A10. Interestingly, this complex displayed similar stimulation of plasminogen activation to that of wild-type AIIt. This suggests that only the amino-terminal region of annexin A2 plays a role in plasminogen activation by binding to and influencing the conformation of S100A10 (212).

We have also shown that S100A10 is a high affinity substrate for several carboxypeptidases (Figure 2D) (205). Carboxypeptidase B has been shown to block cellular plasminogen activation by cleaving the carboxyl terminal lysines of plasminogen regulatory proteins (82, 85). We have demonstrated that in addition to the pancreatic carboxypeptidase B (CpB), physiologically relevant concentrations of plasma carboxypeptidase N (CpN) and thrombin-activated fibrinolysis inhibitor (TAFI) are capable of completely ablating the enhancement of plasminogen activation by AIIt and S100A10 (205). We have confirmed that the mechanism by which the carboxypeptidases abrogated stimulation of plasminogen activation was by removal of carboxyl-terminal lysine residues from S100A10. These studies have an additional ramification. S100A10 is such a potent stimulator of plasminogen activation that it makes physiological sense that a mechanism of down-regulating S100A10 activity exists. Our observation that S100A10 was a substrate for physiologically relevant carboxypeptidases suggested that a physiological mechanism, such as plasma carboxypeptidase-mediated loss of cellular plasminogen binding, may exist to down-regulate plasmin production and thereby protect cells from the deleterious effect of plasmin overproduction.

Another criteria for a plasminogen regulatory proteins is that if it binds plasminogen activators or plasmin, it must protect these enzymes from inactivation by their inhibitors. We have also shown that S100A10 and AIIt (but not annexin A2) protected tPA and plasmin from inactivation by PAI-1 and $alpha_2$-antiplasmin, respectively (Figure 2C) (212).

Plasmin can also utilize itself as a substrate, a reaction called autoproteolysis. This self-destruct mechanism is thought to be important to prevent collateral tissue damage by the accumulation of plasmin in the tissues. Our *in vitro* studies suggested that in addition to stimulating plasmin production, S100A10 and AIIt also stimulate plasmin autoproteolysis (126). The involvement of S100A10 in plasmin production and destruction suggests that S100A10 could produce a transient pulse of plasmin at the cell surface.

Another important criterion for a plasminogen regulatory protein is that it must directly bind a plasminogen activator or colocalizes with a plasminogen activator on the cell surface. Since S100A10 binds tPA, S100A10 can function as a template to colocalize tPA and plasminogen at the cell surface. However, S100A10 does not bind uPA or uPAR. We have shown that S100A10 colocalizes with uPAR. We have also shown the presence of uPAR in S100A10 immunoprecipitates and we have observed the colocalization of uPAR and S100A10 by immunofluorescence microscopy (Figure 2E) (125, 202, 203). Thus, S100A10 is intimately associated with the two key plasminogen activators; it directly binds tPA and colocalizes with the uPA-uPAR complex.

Lastly it must be shown that the loss of the candidate protein from the cell surface, results in a loss in cellular plasmin generation. To assess the role of S100A10 in cellular plasmin generation we used both retroviral-mediated gene transfer and lipofectamine-mediated transfection to transfect a model tumour cell, the HT1080 fibrosarcoma cell line, with a plasmid (pLIN) containing the *S100A10* gene in the sense (pLin-sS100A10) or antisense (pLin-aS100A10) orientation (202). Stable G418-resistant clones were propagated and used in the subsequent experiments. Surface labeling established that the pLin-sS100A10 clones expressed higher extracellular levels of S100A10 than the Vector control cell line, whereas S100A10 was virtually undetectable on the surface of the pLin-aS100A10 cell lines. Most importantly, our three antisense and four sense cell lines showed similar levels of annexin A2, uPA, tPA, uPAR, PAI-1 and PAI-2. We found that plasmin production by the pLin-aS100A10 cell lines was barely detectable, showing an approximate 95% reduction compared to the Vec cells (Figure 2F). In contrast, the pLin-sS100A10 cell lines showed a 2.5-fold increase in plasmin generation compared to the Vec cells. Consistent with their decreased plasmin production, the amount of extracellular matrix (SM-ECM) hydrolyzed by the pLin-aS100A10 clones was about 32% of the control cells. Furthermore, the pLin-sS100A10 cells showed an approximate 3-fold increase in SM-ECM hydrolysis compared to the control cells. We found no difference in the adhesive properties of any of the transfected cells. We also observed that although the control HT1080 cells formed tumors in SCID mice, the S100A10-antisense clones did not form tumors. Therefore, to test the metastatic potential (or more correctly their intravasation potential), the

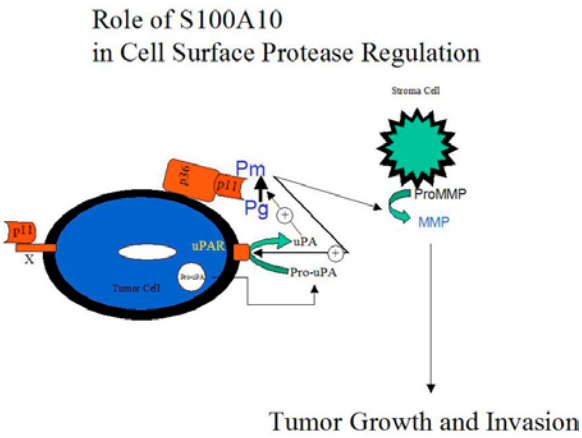

Figure 5. Diagrammatic illustration of the activation of plasminogen by cell surface S100A10. S100A10 is anchored to the cell surface via the phospholipid-binding sites of its binding partner, annexin A2, resulting in the formation of the heterotetrameric complex, AIIt. Alternatively, S100A10 can be associated with the cell surface in the absence of annexin A2, presumably bound to unidentified cell surface proteins (*X*). The formation of the plasminogen-S100A10 complex involves the interaction of the carboxyl-terminal lysines of S100A10 with the lysine-binding kringle domains of plasminogen. Once bound to S100A10, plasminogen undergoes a conformational change resulting in the transition of the protein to a more open, activatable conformation. Next, plasminogen is converted to plasmin by the action of trace amounts of active uPA which is bound to its receptor, uPAR. This reaction is potentiated by the colocalization of the plasminogen-S100A10 (the substrate complex) and uPA-uPAR complexes (the activator complex) at the cell surface. Subsequently, the plasmin-S100A10 and pro-uPA-uPAR complexes interact resulting in the conversion of pro-uPA to uPA by plasmin. The resultant uPA-uPAR complex interacts with the plasminogen-S100A10 complex, resulting in further production of plasmin, which activates additional pro-uPA, thus initiating a feed-forward activation cascade. Two distinct mechanisms are responsible for termination of plasmin production. Carboxypeptidases can remove the carboxyl-terminal lysines of S100A10 resulting in loss of plasminogen binding. Also, the formation of the S100A10-plasmin complex stimulates plasmin autoproteolysis and reduction resulting in the inactivation of plasmin and the generation of anti-angiogenic plasminogen fragments.

transfected cell lines were injected into the tail vein of SCID mice and the formation of lung metastatic foci was monitored. We found that the number of metastatic foci in the lungs decreased 3-fold for the S100A10-antisense cell line and increased 16-fold for the S100A10-sense cell line compared to Vec cell line. These results indicate that the ability of these tumor cells to extravasate and metastasize is directly related to extracellular S100A10 expression. Collectively our results with HT1080 fibrosarcoma cells establish S100A10 as a potential regulator of tumor cells growth invasiveness and metastasis.

We have also used siRNA to silence the *S100A10* gene and have developed an anti-S100A10-siRNA silencing vector (pSUPER) (203). Stable colorectal tumour cells with attenuated S100A10 were developed. We observed that the siRNA-mediated down-regulation of *S100A10* gene expression resulted in a major decrease in the appearance of extracellular S100A10 protein and correlated with a 45% loss of plasminogen binding, a 65% loss in cellular plasmin generation and a complete loss in plasminogen-dependent cellular invasiveness. Collectively, our data supports a key role for S100A10 in plasminogen regulation. A model summarizing these data is presented in Figure 5.

9. THE ROLE OF ANNEXIN A2 HETEROTETRAMER IN ANGIOGENESIS

Cleavage of plasmin(ogen) produces anti-angiogenic plasminogen fragments, typically consisting of the first three or four kringle domains. Since these fragments can inhibit primary tumor growth as well as angiogenesis-dependent growth of metastases, they have been referred to as angiostatin (219-221). Although angiostatin was originally identified as a 38 kDa plasminogen fragment from mouse urine and serum (220), it is now apparent that angiostatin is a collective term that describes a large family of anti-angiogenic plasminogen fragments. Since the first identification of 38 kDa-angiostatin, many different mechanisms of angiostatin generation have been proposed by various laboratories. Among these, a direct cleavage of plasminogen by proteinases such as metalloelastase (MMP-12), MMP-2, prostate-specific antigen, and cathepsin D appears to be the most probable mechanism to generate the 38 kDa fragment (222). On the other hand, angiostatin larger than 38 kDa can be generated via different mechanisms that include plasmin reduction and autoproteolysis, and the reduction of plasmin followed by cleavage by a serine protease. This suggests that plasmin reduction is important to generate the larger fragments (223-226).

As discussed in Section 8, our laboratory has shown that AIIt not only stimulates plasmin production but also promotes plasmin autoproteolysis. Importantly, fragments larger than 38 kDa were shown to be produced as a result of plasmin autoproteolysis (126). We demonstrated that the major form of angiostatin produced by plasmin autoproteolysis was a novel fragment of an apparent molecular mass of 61 kDa (on reduced SDS-PAGE) which we subsequently named A_{61} (227). We have also shown that non-transformed cells such as bovine capillary endothelial cells as well as cancer cells produce a similar molecule. Furthermore, a similar *A*-chain fragment is present in human serum from healthy volunteers as well as cancer patients, and in serum from normal and tumor-bearing mice, which suggests that A_{61} is one of the key angiostatin molecules that are produced under physiological conditions (227).

It was established from the N- and C-terminal sequencing that A_{61} was an internal fragment of plasminogen that encompasses the sequence Lys^{78}-Lys^{468} (227). Thus, in order for A_{61} to be released from

autoproteolyzed plasmin, the peptide bonds Lys^{77}-Lys^{78} and Lys^{468}-Gly^{469} of plasmin must be cleaved, and the Cys^{462}-Cys^{541} disulfide bond of plasmin must be reduced. In addition, the release of A_{61} will therefore result in the generation of a free sulfhydryl residue at Cys^{462}. To assess the role of AIIt in plasmin reduction *in vitro*, we used free sulfhydryl-reactive reagents such as 3-(N-maleimidylpropionyl)biocytin (MPB). The reaction of free sulfhydryl-containing proteins with MPB results in the biotinylation of the protein, which allows easy detection with horseradish peroxidase-conjugated streptavidin. We showed that the incubation of plasminogen and uPA with AIIt resulted in the generation of A_{61} (228). Furthermore, the A_{61} generated in these reactions reacted with MPB, confirming the presence of a free sulfhydryl in A_{61}. AIIt stimulated the dose- and time-dependent conversion of plasminogen to A_{61}. Since AIIt stimulated the generation of A_{61} in the absence of sulfhydryl donors, these data suggest that AIIt promotes the cleavage of a plasmin disulfide, presumably the Cys^{462}-Cys^{541} disulfide, resulting in the release of A_{61} from plasmin and the generation of a free sulfhydryl-containing cysteine (Cys^{462}) in A_{61}. We demonstrated that incubation of either the S100A10 or annexin A2 with plasminogen and uPA also stimulated the generation of A_{61} (228). However, AIIt was a more potent plasmin reductase than either subunit, suggesting that the interaction of the subunits potentiated the plasmin reductase activity of either subunit. The mutagenesis study also established that both Cys^{61} and Cys^{82} of the S100A10 subunit and Cys^{334} of the annexin A2 subunit were required to sustain the plasmin reductase activity of the protein. Since the Cys^{334} residue of annexin A2 is highly conserved among many of the annexin proteins (229), it was suspected that other annexins which possess this cysteine might also have plasmin reductase activity. However, among the seven different annexins tested only annexin A2 and AIIt possessed plasmin reductase activity (228). This result establishes that plasmin reductase activity is not a common feature of the annexins.

In order to see whether AIIt could play an essential role in the generation of A_{61} at the cell surface, we developed a strategy to either increase or decrease the extracellular expression of S100A10 subunit. As discussed in Section 8, we transduced HT1080 fibrosarcoma cells with the retrovirus expressing the S100A10 gene in the sense (pLin-sS100A10) or antisense (pLin-aS100A10) orientation, and selected clones with increased (pLin-sS100A10) or decreased (pLin-aS100A10) surface expression of S100A10 (202). These stably transduced cells were incubated with plasminogen, and the generation of A_{61} was measured. As shown in Figure 4A, incubation of the pLin-sS100A10 cells with plasminogen resulted in enhanced A_{61} generation compared to the Vec cells. In contrast, the pLin-aS100A10 cells failed to produce A_{61} (15). Additionally, it was confirmed that A_{61} generated by HT 1080 cells was reduced, since it reacted with MPB. However, when plasmin was inactivated by prior treatment with the serine protease inhibitor, diisopropylfluorophosphate (DIFP) and then incubated with the HT1080 cells, the generation of A_{61} by the cells was not detected. Furthermore, the catalytically inactive plasmin that was incubated with the cells was not reduced, since it did not react with MPB. These results establish that plasmin autoproteolysis is required before plasmin reduction can occur. Collectively, these data suggest that the mechanism of A_{61} generation involves the uPA-dependent conversion of plasminogen to plasmin followed by plasmin autoproteolysis and reduction of autoproteolyzed plasmin, and all of three steps are stimulated by AIIt (Figure 4B).

Furthermore, HeLa cells transfected with S100A10 antisense also failed to convert plasminogen to A_{61} (228). As discussed in Section 8, we also developed Colo 222 colorectal cancer cells that showed decreased surface expression of S100A10 (203). Although annexin A2 is present intracellularly, it does not exist at the extracellular surface in these cells. Interestingly, we observed that A_{61} generation was not affected by the level of cell surface expression of S100A10 in these cells, suggesting that both subunits of AIIt are required to generate A_{61} (unpublished observation). Altogether, these results establish an essential role of AIIt in A_{61} generation at the cancer cell surface. The data therefore suggest that AIIt may play a role in angiogenesis by regulating the cellular release of an anti-angiogenic protein.

10. SUMMARY AND PERSPECTIVES

It is apparent that S100A10 is a key plasminogen regulatory protein (see model presented in Figure 5). Furthermore, S100A10 is not only involved in fibrin homeostasis and angiogenesis but is also involved in tumor growth, invasion and metastasis. One challenge for the future will be to examine the universality of S100A10-regulated plasminogen activation. Since S100A10 is a low molecular weight molecule, few proteomics studies have examined the levels of this protein during cancer development. It is therefore unclear if the enhanced proteolytic activity demonstrated by cancer cells is due to changes in S100A10 expression. It is possible, for example, that the changes in uPA/uPAR levels which are observed in many cancer cells are sufficient to enhance cellular proteolytic activity without changes in S100A10 levels. Therefore, S100A10 could play the role of a constitutive receptor. Still another challenge will be to elucidate the mechanism by which annexin A2 participates in S100A10-dependent plasminogen regulation. It is unclear how annexin A2 affects the S100A10 levels or how annexin A2 facilitates the transport of S100A10 to the surface. It is possible that the up-regulation of annexin A2 observed in many cancers serves to increase or localize S100A10 levels at the cell surface. Lastly, the observation that S100A10 plays a role in tumor cell invasiveness and metastasis makes S100A10 a possible target for therapeutic intervention. Since the plasminogen-binding site of S100A10 is known, it is possible that reagents that block this region may also be useful to halt tumor growth and metastasis.

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