Urokinase (u-PA)-mediated cell surface plasminogen activation is required for cellular tissue invasion. This invasion occurs in environments rich in plasminogen activator inhibitors (PAIs), which efficiently inhibit receptor-bound two-chain u-PA. Single-chain u-PA (scu-PA) was recently found to efficiently initiate cell surface plasminogen activation, and we herein describe the interaction of scu-PA with PAI type 2 (PAI-2). In the fluid phase (no cells), the plasminogen-activating activities of both scu-PA and Glu<sup>188</sup>-scu-PA (a plasmin non-activatable variant of scu-PA) were inhibited in a concentration-dependent manner by recombinant human PAI-2. This inhibition occurred with both forms of scu-PA remaining as single-chain molecules throughout the interactions. Although scu-PA did not form SDS-stable complexes with PAI-2, preincubation of scu-PA with <sup>125</sup>I-PAI-2 demonstrated a dose-dependent inhibition of SDS-stable complex formation between <sup>125</sup>I-PAI-2 and subsequently added two-chain u-PA. This indicates that although a "stable intermediate" type complex between scu-PA and PAI-2 was not detected, there was a physical association between the two molecules that shared at least some determinants with the two-chain u-PA-PAI-2 complex. In contrast, Glu<sup>188</sup>-scu-PA bound to u-PA receptors on monocytes was only minimally inhibited by a large molar excess of PAI-2. These data suggest that the initiation of cell surface plasminogen activation may involve the partitioning of scu-PA between PAI-2 (a "negative modulator") and the u-PA receptor (a "positive modulator") and that the enzymatic activity of receptor-bound scu-PA may allow initiation of cell surface proteolysis even in PAI-2-rich environments. A model along these lines is presented.

Cellular invasion requires cell surface-associated proteolysis that is potent but properly controlled. A proteolytic "cascade" of several enzymes and inhibitors performs this function with the initial enzyme being urokinase-type plasminogen activator (u-PA) (<sup>1</sup>). By analogy to intracellular metabolic enzyme cascades, u-PA may be considered to be the rate-limiting enzyme for plasminogen-dependent cell surface-associated proteolysis, in that its activity determines the activity of the remainder of the pathway (<sup>2</sup>). The initiating step of this enzymatic pathway involves the binding of single-chain u-PA (scu-PA) to its cell surface receptor (u-PA receptor, u-PAR) and the binding of plasminogen to its cognate binding sites on the same cell (<sup>3</sup>, <sup>4</sup>). This colocalization results in approximately 100-fold more efficient activation of plasminogen than if the reactants are in solution (<sup>5</sup>), suggesting it is much more likely that plasminogen will be activated at the cell surface than in the fluid phase. The proteolytic process is further restricted to the cell surface by the fact that plasmin on its cellular binding sites is protected from inhibition by its primary inhibitor, α<sub>2</sub>-antiplasmin (<sup>3</sup>, <sup>4</sup>). Cell surface plasmin being thus protected rapidly cleaves receptor-bound scu-PA to the more proteolytically active tcu-PA (<sup>6</sup>), which in turn might activate plasminogen more rapidly. This appears to be a positive feedback loop and raises the question of how the process, once started, is turned down. A potential mechanism for limiting such positive feedback lies in the observation that cell-bound tcu-PA is efficiently inhibited by plasminogen activator inhibitors (PAIs) (<sup>7</sup>). However, this inhibition does not seem to significantly favor plasminogen activation at the cell surface compared with reactants in solution and would seem to inhibit cell invasion through PAI-rich tissues.

Because it has recently been shown that scu-PA rather than tcu-PA is the enzyme that initiates cell surface plasminogen activation (<sup>5</sup>) and because the effect of PAI on scu-PA is unknown, we utilized human monocytes to investigate this regulatory interaction. We demonstrated that whereas scu-PA in solution interacts with PAI-2, scu-PA bound to cell surface u-PAR is protected from PAI-2 inhibition, serving to favor plasminogen activation at the cell surface rather than in solution. We propose a model for regulation of cell surface plasminogen activation and, by analogy to intracellular enzyme cascades, propose that u-PA plays the role of a "pacemaker" enzyme (<sup>2</sup>), with PAI-2 being the "negative modulator" and u-PAR being the "positive modulator."

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Glu<sup>188</sup>-scu-PA, tcu-PA, and goat anti-human u-PA were kindly supplied by the individuals previously noted (<sup>5</sup>). Glu<sup>188</sup>-scu-PA was treated as previously described to ensure that the plasmin-cleavable form of scu-PA was present (<sup>5</sup>). Human scu-PA was generously supplied by Dr. J. Henkin of Abbott, recombinant human PA, scu-PA where glutamic acid has been substituted for lysine at amino acid 158; u-PAR, u-PA receptor; PAI-2, plasminogen activator inhibitor type 2; TBS, Tris-buffered saline; serpin, serine protease inhibitor; PAGE, polyacrylamide gel electrophoresis.
PAI-2 was generously supplied by Dr. E. Schüler of Behring, plasminogen-free fibrinogen was kindly supplied by Dr. M. Mosseson, Milwaukee, WI, ovalbumin was from Sigma, and plasminogen was prepared as previously described (5, 8). Sepharcl S-200 was from Pharmacia LKB Biotechnology Inc.

Methods—Iodination of proteins. 125I-fibrin plate assays, and SDS-PAGE were carried out exactly as described (5). Figures of autoradiograms indicate molecular mass markers in kDa. The direct, plasmin-independent assay, which monitors cleavage of 125I-plasminogen to 125I-plasmin, was carried out exactly as described by Mussoni et al. (9), except phenylmethylsulfonyl fluoride-treated bovine serum albumin was substituted for human albumin. In this assay the relative amount of 125I-plasmin light chain on autoradiograms of SDS-polyacrylamide gels is an excellent measure of plasminogen activation or the inhibition of activation (9).

Inhibition of u-PA in Solution—The indicated form of u-PA was incubated 30 min at 37 °C in fibrin plate assay buffer (0.1 M Tris, pH 7.4, 0.1% gelatin) with the indicated molar ratio of either PAI-2 or ovalbumin. The mixture was then added to an 125I-fibrin plate assay and inhibition determined by comparing to u-PA preincubated with buffer alone.

Interaction between scu-PA and PAI-2—Interaction between 125I-PAI-2 and various forms of u-PA was performed by incubating 75 ng of 125I-PAI-2 with buffer, 75 ng of the indicated form of u-PA, or 150 ng of plasminogen in a final volume of 30 μl of TBS for 30 min at 37 °C, boiling in gel buffer containing 2-mercaptoethanol, and analyzing by 10% SDS-PAGE and autoradiography. Detecting competition between scu-PA and tcu-PA for interaction with 125I-PAI-2 was carried out by incubating the indicated amount of scu-PA (or plasminogen as a control) with 75 ng of 125I-PAI-2 in 10 μl of TBS for 10 min at 37 °C. tcu-PA (75 ng) was then added in 5 μl of TBS to each reaction for a further 5 min at 37 °C, followed by SDS-PAGE under reducing conditions and autoradiography.

Inhibition of Receptor-bound u-PA—Human monocytes were isolated from leukocyte-rich by-products of plateletpheresis preparations in the presence of cycloheximide and utilizing an acid wash step exactly as previously described (5). Such monocytes have all detectable endogenous u-PA removed from their surface u-PA receptors and synthesis of a large excess of Trasylol is monitored by SDS-PAGE and autoradiography to detect SDS-stable complexes between the two molecules. However, in contrast to tcu-PA (12, 13), scu-PA did not form SDS-stable complexes with 125I-PAI-2 (not shown). This implies the "stable intermediate" state described for serpin-serine protease complexes (12, 14) does not form between scu-PA and PAI-2 and agrees with the observations of Estreicher et al. (15).

The interaction between a serpin and its target protease proceeds in two stages, first a reversible association between the two molecules that does not require the active site serine, and second, formation of the SDS-stable complex, which does (13, 14). To determine whether the interaction between scu-PA and PAI-2 might be similar to the initial (first stage) interaction between tcu-PA and PAI-2, we investigated whether scu-PA could specifically interfere with the formation of complexes between tcu-PA and 125I-PAI-2. Increasing concentrations of scu-PA were preincubated with 125I-PAI-2 for 10 min, at which time a constant amount of tcu-PA was added to each reaction for 5 min. The reactions were then analyzed by SDS-PAGE and autoradiography to detect SDS-stable complexes between...
tEU-PA and 125I-PAI-2. As shown in Fig. 3, scu-PA, in a concentration-dependent manner, interfered with the ability of tEU-PA to form complexes with 125I-PAI-2. This interference was specific, as plasminogen preincubated with 125I-PAI-2 did not interfere with formation of the complexes. Interestingly, however, if the above experiment was repeated but the incubation with tEU-PA was extended to 30 min (i.e., allowing tEU-PA more time to compete with scu-PA for interaction with 125I-PAI-2), there was no longer an inhibitory effect of scu-PA on the formation of SDS-stable complexes between tEU-PA and 125I-PAI-2 (Fig. 3, bottom). This suggests that the interaction between scu-PA and PAI-2 is specific and requires some of the same determinants utilized in the interaction between tEU-PA and PAI-2. Furthermore, the interaction between scu-PA and PAI-2 seems to be reversible. Upon dissociation from scu-PA, PAI-2 was still active, as tEU-PA could form an SDS-stable complex with PAI-2 that had previously been associated with scu-PA. The affinity of the interaction between scu-PA and PAI-2 remains to be determined and compared with that between tEU-PA and PAI-2. It will be important to know if the competitive advantage tEU-PA holds in forming complexes with PAI-2 over time is due to the formation of essentially irreversible complexes or whether the interaction of PAI-2 with each of the u-PAs, despite using common determinants, differs in affinities.

To then determine whether scu-PA bound to its cell surface receptor interacts with PAI-2, we employed the non-denaturing conditions of a fibrinolytic activity assay to detect inhibition of receptor-bound Glu158-scu-PA by PAI-2. This form of scu-PA was used in these experiments, because as previously shown, it remains in the single-chain form throughout the plasmin-dependent cell surface PA assay (5), and the results are thus not confounded by the generation and inhibition of tEU-PA. Fig. 4 demonstrates that whereas tEU-PA on monocyte surface receptors was indeed efficiently inhibited by PAI-2 (8, 15), Glu158-scu-PA on monocyte surface receptors was only minimally inhibited by PAI-2 at molar ratios that efficiently inhibited both soluble Glu158-scu-PA and receptor-bound tEU-PA. This failure to inhibit receptor-bound Glu158-scu-PA was due to an unanticipated inactivation of PAI-2, as PAI-2 preincubated with monocytes bearing Glu158-scu-PA, then recovered, retained full inhibitory activity against tEU-PA. In addition, protection from inhibition was specific for PAI-2, as neutralizing anti-u-PA IgG did inhibit receptor-bound Glu158-scu-PA (non-immune IgG showed no inhibition) (data not shown).

The minimal inhibition of receptor-bound scu-PA by PAI-2 is supported by previous data. Estreicher et al. (15) observed very little inhibition of plasmin-dependent cascinolysis when mono-
We cannot, however, reconcile our data with observations where it is reversibly bound and inhibited by PAI-2. We would hypothesize that the generation of tcu-PA yields a set of conditions as conducive to inhibition by PAI-2 as to accelerated activation of plasminogen. The data of Ellis et al. (3) support this; tcu-PA on U-937 surface receptors activates cell surface plasminogen with a catalytic efficiency of $1.65 \times 10^5 \, \text{m}^{-1} \text{s}^{-1}$, and PAI-2 inhibits surface receptor-bound tcu-PA with an apparent association constant of $3.3 \times 10^5 \, \text{m}^{-1} \text{s}^{-1}$ (7). The complexes between PAI-2 and tcu-PA on the cellular receptor are rapidly internalized (22), leaving the surface barren of PA activity and essentially halting the initiation of the proteolytic cascade. If further cell movement requiring proteolysis is needed, the cell must put more u-PAR on its surface, via either recycling or new synthesis.

The above hypothesis would help explain how initiation of cell surface PA is controlled. It also suggests that receptor-bound scu-PA is a relevant enzyme for activation of plasminogen and that receptor-bound tcu-PA may be more relevant to inhibition. The above hypothesis conforms to present data and predicts other events; direct experimental testing of this hypothesis will therefore be very important.

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FIG. 4. Glu$^{148}$-scu-PA on the monocyte surface receptor is not inhibited by PAI-2. Monocytes with either Glu$^{148}$-scu-PA or tcu-PA on surface u-PA receptors were prepared as outlined under "Experimental Procedures." Conditions were standardized to use the number of cells possessing approximately 5 ng of u-PA of either type on their surface(s). u-PA-bearing cells were incubated with various molar ratios of PAI-2 (as indicated on ordinate) for 30 min at 37°C and added to wells of an 125I-fibrin plate assay as described under "Experimental Procedures." The results are representative of those found in four separate experiments. FDP, fibrin degradation products.

FIG. 5. Representation of hypothesis for regulation of cell surface plasminogen activation. The proposed events are detailed in the text. Pg, plasminogen; REC, receptor; PA, plasmin.
REFERENCES