Cancer invasion and metastasis is a process requiring a coordinated series of (anti-)adhesive, migratory, and pericellular proteolytic events involving various proteases such as urokinase-type plasminogen activator (uPA)/plasmin, cathepsins B and L, and matrix metalloproteases. Novel types of double-headed inhibitors directed to different tumor-associated proteolytic systems were generated by substitution of a loop in chicken cystatin, which is nonessential for cysteine protease inhibition, with uPA-derived peptides covering the human uPA receptor binding sequence uPA-(19–31). The inhibition constants of these hybrids toward cysteine proteases are similar to those of wild-type cystatin (K_i, papain (pM), 1.9–2.4; K_i, cathepsin B (nM), 1.0–1.7; K_i, cathepsin L (pM), 0.12–0.61). FACS analyses revealed that the hybrids compete for binding of uPA to the cell surface-associated uPA receptor (uPAR) expressed on human U937 cells. The simultaneous interaction of the hybrid molecules with papain and uPAR was analyzed by surface plasmon resonance. The measured K_i value of a papain-bound cystatin variant harboring the uPAR binding sequence of uPA (chCys-uPA-(19–31)) and soluble uPAR was 17 nM (K_i value for uPAuPAR interaction, 5 nM). These results indicate that cystatins with a uPAR binding site are efficient inhibitors of cysteine proteases and uPA/uPAR interaction at the same time. Therefore, these compact and small bifunctional inhibitors may represent promising agents for the therapy of solid tumors.

Tumor invasion and metastasis to distant loci requires directed migration of cancer cells. Such a migration/invasion process depends on the concerted action of various proteolytic systems (1, 2) allowing the tumor cells to detach from the extracellular matrix and to degrade the surrounding tissue and tumor stroma. A major proteolytic system involved in these processes is the plasminogen activation system, with components like the serine protease plasmin, its activator urokinase-type plasminogen activator (uPA), the cell surface-associated uPA receptor uPAR (CD 87), and the two inhibitors PAI-1 and PAI-2 (2, 3). The zymogen of uPA, pro-uPA, is activated by different proteases such as serine (e.g. plasmin, kallikrein) and cysteine proteases (e.g. cathepsin B or L). Via uPA/uPAR interaction, the proteolytic activity of the uPA system is focused on the tumor cell surface, inducing signal transduction, cell proliferation, chemotaxis, and cell migration (2, 4). Cell surface-bound uPA efficiently activates plasminogen to plasmin, which not only degrades components of the extracellular matrix, but also converts pro-uPA to uPA as well as some of the pro-forms of matrix metalloproteases to active matrix metalloproteases (1–4). The cysteine proteases cathepsin B and L are essential for intracellular protein degradation in the lysosomes (5). In tumor cells, in addition to their intracellular localization, cathepsin B and L are located at the tumor cell surface and may activate pro-uPA to uPA and degrade extracellular matrix components (6).

The interplay of different proteolytic systems (serine, cysteine, and matrix metalloproteases) enables tumor cells to detach from the primary tumor, penetrate the extracellular matrix, invade blood and lymphatic vessels and eventually, form metastases (7). Consistent with the biological role of proteases in tumor invasion and metastasis, high antigen levels of uPA and uPAR, as well as cathepsin B and L, were shown to correlate with poor patient prognosis in different cancer diseases (2, 8, 9).

Given the involvement of the plasminogen activation system and its increased expression in malignant solid tumors, this system represents an attractive target to attack tumor invasion and metastasis. Several strategies have been employed to efficiently reduce the expression or to affect the proteolytic activity of uPA. These approaches include the use of active site inhibitors, antibodies to uPA or uPAR, as well as oligodeoxynucleotides or RNA directed against uPA and uPAR (4). Other attempts are aimed at abrogation of the uPA/uPAR interaction employing synthetic uPA-derived peptides encompassing the binding region of uPA to uPAR (10) or recombinant soluble uPAR (suPAR) as a scavenger for uPA (11). Application of such
reagents in in vitro and in vivo studies led to the suppression of uPA expression, a reduction in proteolytic activity, or reduced uPA binding to cell surface-associated uPAR, and consequently to a reduction of tumor cell invasion and metastasis (2, 3).

Equally importantly, cathepsins B and L also contribute to the invasive phenotype of solid malignant tumors (6, 12). Human cystatin C, the most potent inhibitor of mammalian cysteine proteases, as well as several other cysteine protease inhibitors, prevent tumor cell invasion and metastasis in vitro and in vivo (13, 14). For instance, the invasive capacity of murine SCC-VII squamous carcinoma cells was significantly reduced by addition of the synthetic inhibitor E-64, an effective inhibitor of lysosomal cysteine proteases and also by CA-074, a specific inhibitor of cathepsin B. Transfection of these cells with a cDNA expression vector encoding cystatin C also resulted in a significant reduction of their invasive potential (14).

In line with these data, transfection of cystatin C cDNA into B16 melanoma cells resulted in a decrease of the metastatic capacity of these cells, whereas the growth rate of the transfected cells was unaltered (15).

Both the plasminogen activation system and the cysteine proteases cathepsins B and L represent selective therapeutic targets for the inhibition of tumor invasion and metastasis. Here, we report on the design and characterization of a novel type of bifunctional inhibitor, which reduces the enzymatic activity of cysteine proteases and prevents interaction of uPA with the receptor uPAR. For this dual activity to occur, a loop that is not essential for cysteine protease inhibition in cystatin was replaced by the uPAR binding sequence of uPA. Bifunctional inhibitors of this type were tested for their capacity to simultaneously inhibit cysteine proteases and to suppress uPA binding to uPAR.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Cystatin/uPA Variants—
Synthetic genes of chicken cystatin (chCys(M29F, M89L)) or human cystatin C, cloned into pUC plasmids (16, 17), were used as templates in reverse long range PCR (High Fidelity Expand PCR, Roche Diagnostics, Penzberg, Germany) for deletion of the DNA sequence encoding aa 71–83 in chicken cystatin (Swiss-Prot accession no. P01038) and aa 73–83 in human cystatin C (Swiss-Prot accession no. P01034), respectively. The deleted regions were substituted by the coding sequences of peptide uPA-(19–31) and uPA-(21–30) [S21C, H29C], respectively, which were introduced by the PCR primers. Subsequently, the coding sequence of the cystatin variants as well as those of chCys(M29F, M89L) (from now on referred as chCys-wt) and hCys-wt were ligated into the bacterial expression vector pQE-30 (Qiagen, Hilden, Germany). The resulting constructs encode cystatins/cystatin variants with an amino-terminally located (histidine)6 tag.

The Escherichia coli strain SG10003(pREP4) (Qiagen) was transformed with the vectors and recombinant protein expression induced overnight by the addition of isopropyl-β-D-thiogalactosidase (final concentration 2 mM). The bacteria were lysed in 8 M urea, 0.1 M NaHPO4, 0.01 M Tris, pH 8.0, the debris removed by centrifugation (12,000 × g, 15 min, 4 °C), and the supernatant subjected to Ni2+-NTA-agarose affinity chromatography. After two washing steps with 6 M guanidinium hydrochloride, 0.1 M NaHPO4, 0.01 M Tris, pH 8.0 and pH 6.0, respectively, the proteins were eluted with the same buffer but at pH 4.3. Dithiothreitol was added at a final concentration of 2 mM and the solution stirred overnight at 4 °C. For refolding, the proteins were dialyzed against 6 M urea, 50 mM Tris, 0.02% NaN3, pH 8.0, for 8 h, followed by two dialysis steps against 2 M urea, 50 mM Tris, 300 mM NaCl, 2.5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.02% NaN3, pH 8.0, for 2 days each. Finally, the proteins were dialyzed against phosphate-buffered saline, pH 7.6, for 24 h and then stored at −20 °C until used.

Determination of Kinetic Constants and Active Concentrations—
Continuous fluorimetric inhibition assays were performed and evaluated as described in detail (18, 19). Inhibition of papain (10 pm, Roche Molecular Biochemicals), cathepsin B (25 pm, Calbiochem), and cathepsin L (10 pm, Calbiochem) were assayed at 30 °C with the fluorogenic substrate Z-Phe-Arg-7(4-methyl)coumarylamide (Bachem, Heidelberg, Germany) in 250 mM sodium acetate buffer, pH 5.5, 2 mM EDTA, 0.015% Brij 35, 1 mM dithiothreitol (added freshly) and 1% Me2SO. For cathepsin B, the equilibrium inhibition constants (Ki) were determined directly from the initial slope (υo) and the final slope (υf) of the enzyme with inhibitor in the steady state. For papain and cathepsin L, Ki values were calculated from the rate constants (K = koff/kon), which were obtained from pre-steady-state analysis. Ki values were corrected for competition with the substrate (20). Inhibitorily active concentrations of chicken and human cystatin and their variants were determined by titration with E-64 standardized papain (4 nM) (20) using the substrate Z-Phe-Arg-7(4-methyl)coumarylamide (10 μM, Bachem).

RESULTS

Design, Cloning, Expression, and Purification of Cystatin/uPA Variants—Cystatins, the natural inhibitors of cysteine proteases, as well as uPA-derived peptides, which abrogate uPA/uPAR interaction are well known to reduce the invasive capacity of tumor cells in vitro and in vivo. For the design of a bifunctional molecule that combines both functional activities, a loop in human cystatin C and that of chicken cystatin, which is nonessential for cysteine protease inhibition, was substituted by the binding sequence of uPA to uPAR (uPA-(19–31)) or a variation of this sequence (uPA-(19–31) and chCys-wt, respectively) in uPAR-bearing promyeloid human U937 cells. The still viable cells were incubated with a mixture consisting of FITC-pro-uPA (16 ng) and refolded chCys-uPA-(19–31) and chCys-wt, respectively, and the cell-associated fluorescence was determined by flow cytometry (21).
The yield of functional active human cystatin variants was rather low compared with the chicken cystatin variants. Therefore, most of the experiments were performed only with chicken cystatin/cystatin variants.

**Kinetic Constants and Cellular Binding Characteristics of Recombinant Cystatins/Cystatin Variants**—The recombinant chicken variants (chCys-wt, chCys-uPA-(19–31), and chCys-uPA-(21–30)[S21C, H29C]) display $K_i$ values for inhibition of papain, cathepsin L, and cathepsin B close to that of natural chicken cystatin from egg white (Table I), indicating that the amino-terminal histidine tag has no negative impact on inhibitory activity. Similar kinetic results were obtained with recombinant human cystatin C and two of its variants, with $K_i$ values between 2.5 and 3.0 pM toward papain compared with about 0.5 pM for mature cystatin C (23).

To investigate whether chCys-uPA-(19–31) inhibits binding of uPA to cell surface-associated uPAR, PMA-stimulated U937 cells were incubated with a mixture of FITC-labeled pro-uPA and purified, refolded chCys-uPA and then subjected to flow cytometry. The uPAR-directed binding epitope of uPA closely interacted with binding of FITC-uPA to the cells, whereas chCys-wt without a uPA-peptide uPA-(19–31) did not affect pro-uPA/uPAR interaction (Fig. 2). From these results it can be concluded that chCys-uPA-(19–31) inhibits cysteine protease proteolytic activity in solution and blocks binding of uPA to cellular uPAR.

**Simultaneous Binding of chCys-uPA-(19–31) to Papain and suPAR**—The ability of the cystatin/cystatin variants to inhibit cysteine proteases and of chCys-uPA-(19–31) to suppress uPA binding to cellular uPAR on U937 cells were determined in independent experiments. These results did not prove that a single molecule displays both characteristics simultaneously.

To address this question, surface plasmon resonance technology (BIAcore 2000) was used. For this purpose, the cysteine protease papain was covalently bound to a CM-5 chip (Fig. 3). After binding cystatin variants chCys-uPA-(19–31) or chCys-uPA-(21–30)[S21C, H29C] to the immobilized papain, the resulting papain-cystatin variant complex was washed vigorously. No significant dissociation of the cystatin variants from the immobilized papain was observed. suPAR was added to the immobilized papain/chCys-uPA-(19–31), papain/chCys-uPA-
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(21–30)[S21C, H29C], or papain/chCys-wt complexes. suPAR bound to both of the papain-cystatin complexes containing the cystatin/uPA variants but not to papain/chCys-wt or to papain alone. The amount of suPAR binding to the chCys-uPA-(21–30) [S21C, H29C]-papain complex was lower compared with suPAR binding to papain/chCys-uPA-(19–31). This result corresponds to the results obtained by FACS analysis, demonstrating that cyclo-(19–31)uPA-(19–31) is an ~3-fold better antagonist of uPA/uPAR interaction than cyclo-(21,29)uPA-(21–30) [S21C, H29C] (data not shown).

A concentration-dependent binding of suPAR to chCys-uPA-(19–31)/papain complex was observed (Fig. 3). The binding curves were used for calculation of a $K_D$ value for the interaction of suPAR with chCys-uPA-(19–31). Simulation of the binding curves using the BIAcore evaluation software indicated that mass transport seems to be of no importance for this interaction (not shown). The $K_D$ value of the interaction of chCys-uPA-(19–31) with suPAR was 17 nM, which is in the range of that of uPA binding to suPAR, which was also measured using surface plasmon resonance ($K_D = 5$ nM, data not shown), indicating that the binding sequence of uPA to uPAR in chCys-uPA-(19–31) is correctly folded and functionally active.

Preincubation of suPAR with a 6-fold excess of uPA reduced binding of suPAR to the chCys-uPA-(19–31)/papain complex by 85%, providing further evidence that suPAR indeed interacts with chCys-uPA-(19–31) via the uPA-derived epitope.

These results demonstrate that chCys-uPA-(19–31) acts simultaneously with papain and suPAR and therefore functions as a tight-binding inhibitor of cysteine proteases as well as an effective uPA receptor antagonist.

**DISCUSSION**

The coordinated interaction of different proteolytic systems, e.g. serine proteases, cysteine proteases, matrix metalloproteases, and aspartyl proteases, is important for tumor cell invasion and metastasis (1). The invasive capacity of tumor cells can be lowered by synthetic inhibitors directed against these different proteases, but also as demonstrated for the plasminogen activator system by antagonists, which prevent focusing of the active proteases uPA to its cell surface receptor (4). We have developed and tested a novel type of bifunctional inhibitor directed against two different proteolytic systems. Inhibitors are based on cystatin, a naturally occurring cysteine protease inhibitor into which a uPA-derived binding sequence for uPAR was inserted. This novel type of inhibitor functions on one hand as an active site inhibitor of cysteine proteases and on the other hand as an antagonist of receptor/uPA interaction. Mutations in the region of aa 71–81 of chicken cystatin did not affect the binding of the mutated inhibitor to the target enzymes as expected from previous x-ray- and NMR structure analyses (24, 25) and as demonstrated previously for the chicken cystatin deletion variant (del—helix II), which lacks residues Cys$^{71}$-Met$^{80}$ (26). This mutated part of the molecule is not included in the contact region of the inhibitor with the cell surface uPAR.
cysteine protease (Fig. 1). As a result, the novel type of inhibitor reacts with cysteine proteases (e.g. papain and cathepsins B and L) and can bind simultaneously to uPAR, the cellular receptor of uPA, as demonstrated by surface plasmid resonance.

Various other bifunctional chimeric protease inhibitors have been generated. A fusion protein harboring the amino-terminal part of uPA (an 1–135) and human serum albumin (ATF-HSA hybrid) significantly reduced the invasive capacity of a breast cancer cell line in vitro (27). Similarly, a uPA-(1–137)/IgG chimera suppressed the metastatic capacity of human PC3 prostate carcinoma cells in nude mice (28). A significant suppression of B16 melanoma growth and neovascularization was observed after applying a fusion protein consisting of the growth factor-like domain of uPA, uPA-(1–48), fused to the Fc portion of human IgG (29). An inhibitory protein containing two functionally independent domains, which affects the uPA/plasmin system, has been generated by Kobayashi et al. (30). This bifunctional inhibitor consists of the ATF (uPA-(1–134)) and domain II of the serine protease inhibitor UTI and reduces experimental tumor invasion and metastasis by suppression of uPA/uPAR interaction via the ATF domain and by inhibition of the serine protease plasmin via UTI. Most importantly, a synergistic effect was observed, as a single application of UTI domain II or ATF resulted in only a relatively small therapeutic effect (30).

Other types of fusion proteins consisting of two functionally independent but highly homologous domains have also been developed. Hybrid molecules harboring different parts of the two serpins, \( \alpha_1 \)-proteinase inhibitor and thryroxine-binding globulin, inhibited human leucocyte elastase and bound to thryroxine with high affinity (31). Nevertheless, all of the chimeric proteins discussed here have been constructed by fusion of independent domains of different proteins (27, 30, 32–34) or by fusion of modules of structurally highly conserved proteins based on their modular architecture (31). In contrast, the presented cystatin variants have been generated, in a novel approach, by replacing 11 aa of the parent cystatin sequence with 13 aa of uPA, resulting in a biologically bifunctional active protein. The new bifunctional cystatin/uPA chimerae may have a broad field of action. They suppress two different tumor-relevant proteolytic systems (1, 2), cysteine and serine proteases, thereby leading to an effective inhibition of their matrix degrading proteolytic action. This antiproteolytic activity of the chimeric inhibitor is focused to the cell surface or, alternatively, may take place in the pericellular space.

This novel type of bifunctional protease/receptor inhibitor represents a candidate for the exploration of new therapeutic approaches in solid tumors. Both inhibitory functions of the cystatin/uPA variants are located on small and compact molecules, which are encoded by about 400 base pairs only. This may be useful in gene therapeutic approaches with viruses such as AAV (35) to achieve expression of such a molecule by tumor cells under the control of tissue-specific promoters.

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