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Regulation of cathepsin B activity by 2A2 monoclonal antibody

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Cathepsin B (EC 3.4.22.1) is a lysosomal cysteine protease with both endopeptidase and exopeptidase activity. The former is associated with the degradation of the extracellular matrix proteins, which is a process required for tumour cell invasion and metastasis. In the present study, we show that 2A2 monoclonal antibody, raised by our group, is able to regulate cathepsin B activity. The EPGYSP sequence, located between amino acid residues 133–138 of cathepsin B in the proximity of the occluding loop, was determined to be the epitope for 2A2 monoclonal antibody using SPOT analysis. By surface plasmon resonance, an equilibrium dissociation constant (K_d) of 4.7 nM was determined for the interaction between the nonapeptide CIAEPGYSP, containing the epitope sequence, and 2A2 monoclonal antibody. 2A2 monoclonal antibody potentiated cathepsin B exopeptidase activity with a activation constant (K_a) of 22.3 nM, although simultaneously inhibiting its endopeptidase activity. The median inhibitory concentration values for the inhibition of hydrolysis of protein substrates, BODIPY FL casein and DQ-collagen IV were 761 and 702 nM, respectively. As observed by native gel electrophoresis and gel filtration, the binding of 2A2 monoclonal antibody to the cathepsin B/cystatin C complex caused the dissociation of cystatin C from the complex. The results obtained in the present study suggest that, upon binding, the 2A2 monoclonal antibody induces a conformational change in cathepsin B, stabilizing its exopeptidase conformation and thus disabling its harmful action associated with its endopeptidase activity.

Introduction

Lysosomal cysteine proteases, or cysteine cathepsins, are involved in a variety of physiological processes, such as protein turnover within lysosomes, hormone processing, antigen presentation and bone resorption [1]. Of the 11 human cysteine cathepsins (B, C, H, L, S, K, O, F, X, V and W), cathepsin B (EC 3.4.22.1) is the most abundant and the most exhaustively studied. In addition to its role in normal cellular processes, several pathophysiological states have been attributed to its increased activity, including arthritis [2,3],

Abbreviations
Abz, ortho-aminobenzoic acid; AMC, 7-amino-4-methylcoumarin; Dnp, 2,4-dinitrophenyl; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HRP, horseradish peroxidase; Ig, immunoglobulin; K_a, activation constant; K_d, equilibrium dissociation constant; NHS, *N*-hydroxysuccinimide; SPR, surface plasmon resonance; Z, benzyloxycarbonyl.

Alzheimer's disease [4,5], pancreatitis [6,7], muscular dystrophy [8] and tumour progression [9,10]. The enzyme is also involved in the regulation of cell growth through degradation of internalized growth factors and their receptors [11], as well as in the pathways of programmed cell death [12,13].

Increased levels of cathepsin B protein and activity are found in tumour tissues and have been suggested as prognostic markers in patients with breast, lung, colon and ovarian carcinomas, as well as gliomas and melanomas [9,14]. The localization of cathepsin B in transformed and tumour cells has been shown to change from the perinuclear vesicles, as found in normal cells, to the peripheral cytoplasmic regions. Moreover, in tumour cells, cathepsin B can be secreted into the extracellular environment or be associated with the cell surface [15]. The secreted cathepsin B can activate other proteases acting downstream in the catalytic cascade [16] or directly degrade the extracellular matrix (ECM) proteins. However, ECM degradation depends on the activity of both extracellular and intracellular proteases, and cathepsin B plays an active role in these processes [17,18].

Cathepsin B acts not only as endopeptidase, as do most of the other cysteine cathepsins, but also as an exopeptidase (i.e. as a dipeptidyl carboxypeptidase that removes dipeptides from the C-terminus of proteins and peptides) [19]. This activity depends on a structural element unique to cathepsin B, the occluding loop that partially blocks the active site cleft and positions a positively-charged imidazole group of a histidine residue (His111) to accept the negative charge at the C-terminus of the substrate [20]. Furthermore, the occluding loop is suggested to be flexible and therefore to adopt a conformation allowing the enzyme to act as an endopeptidase [21]. Thus, cathepsin B is able to participate in both the early and late stages of protein breakdown.

The activity of cathepsin B is regulated in many ways, ranging from the pH of the environment to the presence of endogenous inhibitors (i.e. the cystatins). The balance between the inhibitors and cathepsin B is critical for normal functioning of cellular processes, and cystatins have been shown to block the enzyme's activity effectively at both acidic and neutral pH [22]. The latter act as competitive inhibitors, binding reversibly into the active site of the enzyme. Cystatins, including human cystatin C, are general inhibitors of cysteine proteases. For cathepsin B, their K_i value is in nanomolar range [23]. Access of these inhibitors to the enzyme's active site is partially hindered by the occluding loop and occurs by a two-step mechanism in which the N-terminus of the inhibitor first binds to the enzyme, displacing the occluding loop, followed by the binding of another two loops of the inhibitor [24]. Besides protein inhibitors, the irreversible epoxysuccinyl inhibitor E-64 and other cathepsin B specific epoxide containing synthetic inhibitors, such as CA-074, have been used to inhibit cathepsin B *in vitro* [18].

The natural and synthetic protease inhibitors have been used to impair the excess activity of proteases in preclinical studies [25]; however, they lack specificity and are toxic at higher concentrations [26]. The alternative approach is to use monoclonal antibodies (mAbs) that bind specifically to the protease and neutralize its biological activity. In the last decade, mAbs have become an important part of the modern biopharmaceutics repertoire and were shown to be safe and effective therapeutic agents [27,28]. A murine 2A2 neutralizing mAb against cathepsin B has been raised by our group and shown to be effective in decreasing tumour cell invasion [18].

The present study aimed to identify the epitope in the vicinity of the enzyme's active site to which 2A2 mAb binds, to determine the interference with the binding of substrates and other inhibitors and to identify the mechanism by which it regulates the activity of cathepsin B.

Results

Preparation and characterization of 2A2 mAb and its Fab fragments

2A2 mAb (41.3 mg) was isolated and purified from hybridoma cell medium. Fab fragments prepared by papain degradation of 2A2 mAb were purified by affinity chromatography on a Protein A Sepharose (Pharmacia, Uppsala, Sweden). On SDS/PAGE, the biologically active Fab fragment was identified as two band protein at 25 kDa corresponding to the heavy and light chains of the immunoglobulin (Fig. 1A). The yield of Fab fragment preparation was 20.4%. 2A2 mAb corresponds to the immunoglobulin IgG2a subclass, as determined by indirect ELISA.

On IEF, a set of isoforms of 2A2 mAb with p*I* values in the range 6.5–7.0 was observed (Fig. 1B), confirming the monoclonality of the mAb. As reported previously [29], these isoforms exhibit micro-heterogeneity most probably as a result of the diverse glycosylation profile.

Equilibrium dissociation constant (K_d) between 2A2 mAb and cathepsin B

The K_d between cathepsin B and the neutralizing antibody was determined using a method proposed by

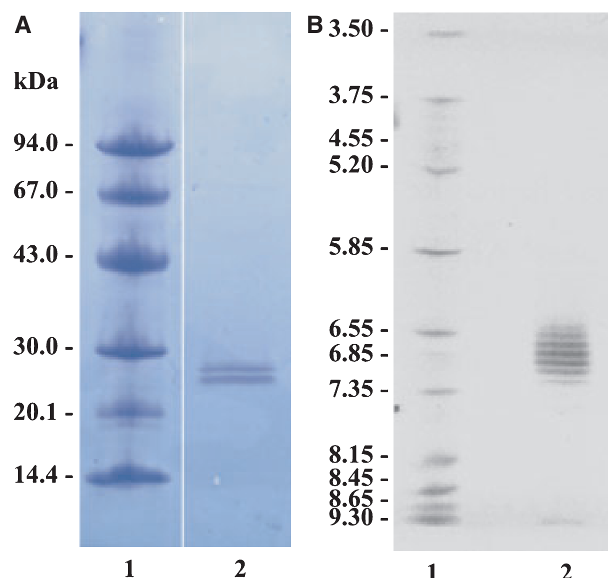

Fig. 1. Characterization of cathepsin B neutralizing 2A2 mAb and its Fab fragment. (A) SDS/PAGE of the Fab fragment (lane 2); low molecular weight standards (lane 1). (B) IEF of 2A2 mAb (lane 2); IEF standards (lane 1).

Friguet *et al.* [30] and was found to be 2.7 ± 1.8 nM, depicting a strong interaction between 2A2 mAb and cathepsin B.

Determination of the 2A2 mAb binding site on cathepsin B

The binding site of 2A2 mAb on cathepsin B was determined by SPOT analysis (SPOTs System; Zeneca, Cambridge, UK). In the first step, 36 decapeptides overlapping the amino acid sequence of mature cathepsin B (Fig. S1) were synthesized on the spots of cellulose membrane. After incubation of the membrane with 2A2 mAb, followed by the detection with secondary goat anti-(mouse IgG) conjugated with horseradish peroxidase (HRP) and peroxidase substrate, a positive, dark coloured reaction was observed at the spot with the sequence ICEPGYSPTY (Fig. 2A). To define the position of the epitope more precisely, five additional decapeptides overlapping that amino acid sequence were synthesized. Decapeptides 1, 2 and 3, all possessing the EPGYSP sequence, reacted positively with 2A2 mAb (Fig. 2B). In control experiments, primary antibody was omitted from the assay (data not shown).

The epitope sequence EPGYSP is located at the exposed part of the cathepsin B molecule, between amino acid residues 133–138 in the proximity of the occluding loop (Fig. 2C).

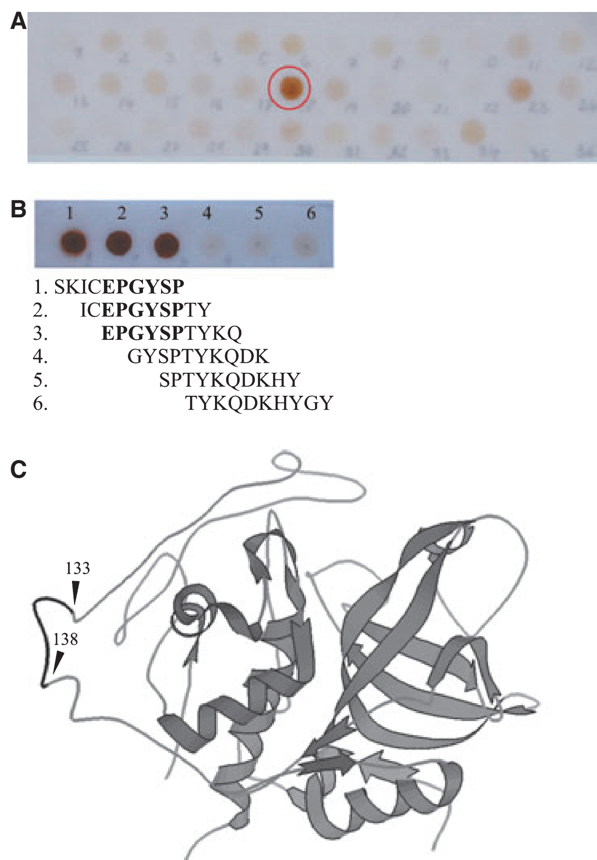

Fig. 2. Determination of the 2A2 mAb binding site on cathepsin B using SPOT analysis. (A) 2A2 mAb reacted positively with ICEPGYSPTY decapeptide in the first step (marked with a circle). (B) Individual amino acids comprising the binding site were determined on five additional decapeptides synthesized in the second step. Decapeptides 1 (SKICEPGYSP), 2 (ICEPGYSPTY) and 3 (EPGYSPTYKQ) at spots 1, 2 and 3, respectively, possessing the common EPGYSP motif reacted positively with 2A2 mAb. (C) Structure of human cathepsin B (Protein Databank code 1 HUC) represented by a ribbon diagram in the standard view. Arrows indicate the position of the 2A2 mAb epitope with EPGYSP motif at the occluding loop of cathepsin B molecule between amino acids 133–138.

Surface plasmon resonance (SPR)

The kinetics of binding of 2A2 mAb was tested on CIAEPGYSP nonapeptide, mimicking the epitope for the antibody on cathepsin B. Different concentrations of the 2A2 mAb (0.5–2.0 nM) were applied to the CM5 sensor surface, which was immobilized with the nonapeptide (Fig. 3A). The K_d of 4.7 nM ($\chi^2 = 8$) obtained by fitting the curves according to the Langmuir binding model (1 : 1) was in accordance with both the results obtained by SPOT analysis, which revealed the amino acid sequence motif EPGYSP as the epitope for 2A2 mAb, and the K_d for the interaction between 2A2

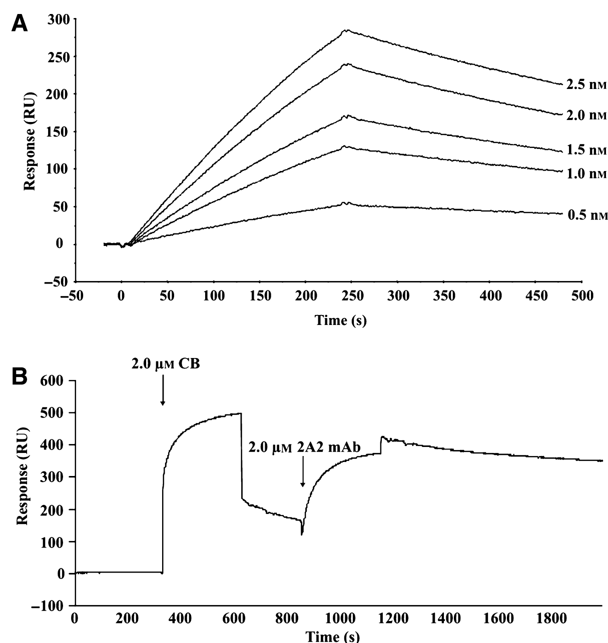

Fig. 3. SPR sensograms depicting the interaction between 2A2 mAb and its epitope on cathepsin B. (A) Increasing concentrations of 2A2 mAb were allowed to flow over a CM5 sensor chip immobilized with nonapeptide CIAEPGYSP (300 RU), mimicking the epitope for 2A2 mAb. The obtained sensograms were fitted according to the Langmuir binding model (1 : 1), yielding a K_d of 4.7 nM. (B) Cathepsin B was pre-bound to immobilized cystatin C (3000 RU) and 2A2 mAb was allowed to flow over the sensor surface. 2A2 mAb bound to cathepsin B.

mAb and intact cathepsin B (2.7 nM). Cathepsin B specific 3E1 mAb was used as a control and showed no binding in the same concentration range (data not shown).

Additionally, two octapeptides, KCSAICEP and SAICEPGY, were tested for binding to 2A2 mAb. They contain the EP and EPGY sequences, respectively, of the predicted epitope sequence EPGYSP. 2A2 mAb showed no binding to either octapeptide (Figs S2 and S3), revealing that these short sequences alone do not represent the epitope.

Effect of 2A2 mAb on cathepsin B activity and ECM degradation

Using endopeptidase substrate benzyloxycarbonyl-RR-7-amino-4-methylcoumarin (Z-RRAMC) (Merck, Darmstadt, Germany), only partial inhibition of cathepsin B endopeptidase activity was obtained by 2A2 mAb (data not shown). This is in line with previous studies [31], demonstrating that this substrate is not the most appropriate for assessing cathepsin B activity, which depends on the conformation of the occluding loop because it occupies the S_3–S_1' subsites of cathepsin B. To determine the full effect of 2A2 mAb on cathepsin B endopeptidase activity, we used protein substrates BODIPY FL casein and DQ-collagen IV. In both cases, 2A2 mAb significantly inhibited cathepsin B endopeptidase activity, as was evident from the median inhibitory concentration values: 761 ± 12 nM for BODIPY FL casein degradation and 702 ± 20 nM for DQ-collagen IV degradation. When we used an exopeptidase substrate ortho-aminobenzoic acid GIVRAK[2,4-dinitrophenyl OH [Abz-GIVRAK(Dnp)-OH] [32], activation and not inhibition of cathepsin B exopeptidase activity was observed with an activation constant (K_a) of 22.6 ± 6.8 nM. These results show that 2A2 mAb inhibits cathepsin B endopeptidase activity at the same times as potentiating its exopeptidase activity.

The antibody also successfully inhibited ECM degradation, as shown by fluorescence microscopy using DQ-collagen IV as substrate, which gives bright green fluorescence upon hydrolysis. We show that MCF-10A neoT cells degrade DQ-collagen IV both intra- and pericellularly (Fig. 4A) and that the addition of 2A2 mAb to the medium significantly reduces degradation of DQ-collagen IV (Fig. 4B). 3E1 mAb non-neutralizing antibody to cathepsin B did not inhibit DQ-collagen IV degradation (Fig. 4C).

Interaction of intact 2A2 mAb and its Fab fragment with the cathepsin B/cystatin C complex

The interaction between 2A2 mAb and the epitope was studied on the cathepsin B/cystatin C complex. Cathepsin B/cystatin C complex was incubated in the presence of various concentrations of 2A2 mAb. The binding of 2A2 mAb was followed by native gel electrophoresis. Increasing the concentration of 2A2 mAb at a fixed molar ratio of cathepsin B and cystatin C resulted in weaker bands of the cathepsin B/cystatin C complex and stronger bands corresponding to a newly-formed complex with 2A2 mAb (Fig. 5A). The experiment was repeated with Fab fragments of 2A2 mAb and the results obtained were the same as those for intact mAb (Fig. 5B).

In the reverse experiment, adding an increasing concentration of cystatin C to the cathepsin B/2A2 mAb complex did not alter the stability of the complex (Fig. 5C). Additionally, the results obtained using SPR revealed that 2A2 mAb retains its ability to recognize cathepsin B even after the enzyme is bound to cystatin C (Fig. 3B), confirming that 2A2 mAb and cystatin C do not compete for the same binding site on cathepsin B. However, the binding of 2A2 mAb did not release

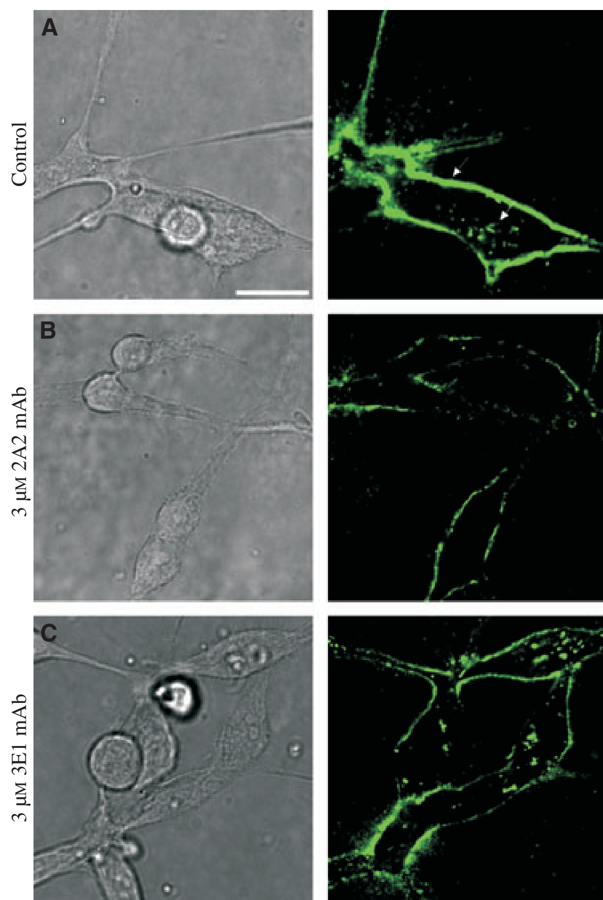

Fig. 4. Inhibitory effect of 2A2 mAb on ECM degradation. MCF-10A neoT cells were incubated for 24 h on Matrigel mixed with DQ-collagen IV. Images were obtained in the presence of NaCl/P_i (A), 3 μM 2A2 mAb (B) and 3 μM 3E1 mAb (C). In the control experiment (A), degradation products are visible intracellularly and pericellularly (white arrow). Addition of the 2A2 mAb to the assay medium reduced the degradation of DQ-collagen IV (B). Degradation products are visible intracellularly and pericellularly after the addition of a non-neutralizing 3E1 mAb, raised against cathepsin B (C). Left panels are differential interface contrast images; right panels are images of green fluorescence after hydrolysis of DQ-collagen IV. Scale bar = 20 μm.

cathepsin B/2A2 mAb complex from the immobilized cystatin C, in contrast to the results obtained by native gel electrophoresis.

Size exclusion chromatography

The dissociation of cystatin C from the cathepsin B/cystatin C complex by 2A2 mAb was tested by size exclusion chromatography. 2A2 mAb, cathepsin B and cystatin C were applied individually to a size exclusion column and eluted as peaks corresponding to molecular weights of 161.8, 28.2 and 13.2 kDa, respectively. The formation of the cathepsin B/cystatin C complex was seen as a shift of the elution peak of cathepsin B (Fig. 6A). The addition of 2A2 mAb to the preformed cathepsin B/cystatin C complex resulted in the disappearance of the complex, which was replaced by a peak corresponding to a molecular weight of 220.1 kDa. Western blotting (Fig. 6B) showed that cystatin C was absent and that cathepsin B and 2A2 mAb were present in this complex. The molecular weight corresponds to a complex of one 2A2 mAb with two molecules of cathepsin B (calculated molecular weight of 218.2 kDa). The molar ratio between cystatin C to cathepsin B determined by ELISA in the fraction eluted at 15.84 mL (cathepsin B/cystatin C complex) was 1.3 ± 0.2, which is consistent with the tight-binding nature of the inhibitor. The ratio was reduced to 0.3 ± 0.1 in the fraction eluted at 11.28 mL (cathepsin B/cystatin C complex, incubated with 2A2 mAb), confirming that this peak contains only cathepsin B and 2A2 mAb, and that cystatin C has been dissociated from cathepsin B by the action of the antibody.

Discussion

Cathepsin B is unique among cysteine proteases in its ability to cleave protein substrates as both an endopeptidase and an exopeptidase [33]. The endopeptidase activity is associated with the degradation of proteins of the ECM, a process required for tumour cell invasion and metastasis [34,35]. In the present study, we show that the 2A2 mAb binds cathepsin B in the proximity of the active site, which causes inhibition of cathepsin B endopeptidase activity and activation of its exopeptidase activity.

The dual activity of cathepsin B is a consequence of the occluding loop, a flexible structure that can adopt different conformation states. In mature cathepsin B, the occluding loop is held to the enzyme body by two salt bridges, His110-Asp22 and Arg116-Asp224, which limits the access of substrates to the primed sites of the active-site cleft and thereby reduces the enzyme's endopeptidase activity. Additionally, His111, which is positioned at the tip of the occluding loop, forms interactions with the C-terminal carboxylate group of the substrate, potentiating the exopeptidase activity of cathepsin B [20,36]. Consequently, cathepsin B is a poor endopeptidase relative to other cysteine proteases (e.g. papain and cathepsin L) [31]. Removal of the occluding loop contacts results in a dramatic increase in endopeptidase activity, suggesting that the binding of endopeptidase substrate is possible when the occluding loop moves away from the enzyme's body, thus

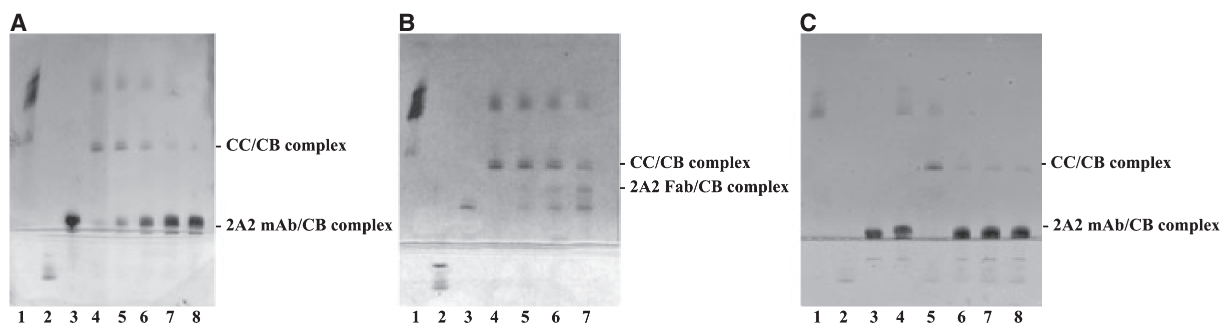

Fig. 5. Interaction between cathepsin B/cystatin C complex and 2A2 mAb and its Fab fragment, studied by native gel electrophoresis. (A) Increasing concentrations of 2A2 mAb added to the pre-formed cathepsin B/cystatin C complex (molar ratio 2 : 3) resulted in a decreased concentration of the cathepsin B/cystatin C complex and an increased concentration of 2A2 mAb complex as detected by stronger bands in lanes 5–8. Lane 1, cathepsin B (CB); lane 2, cystatin C (CC); lane 3, 2A2 mAb (mAb); lane 4, CB/CC (2 : 3) complex; lane 5, CB/CC/mAb (2 : 3 : 0.25); lane 6, CB/CC/mAb (2 : 3 : 0.5); lane 7, CB/CC/mAb (2 : 3 : 1.0); lane 8, CB/CC/mAb (2 : 3 : 1.5). (B) Similar to 2A2 mAb, the increased concentration of its Fab fragment resulted in a decreased concentration of the cathepsin B/cystatin C complex and an increased concentration of complexes formed between the Fab fragment and cathepsin B. Lane 1, cathepsin B (CB); lane 2, cystatin C (CC); lane 3, Fab fragment (Fab); lane 4, CB/CC (2 : 3) complex; lane 5, CB/CC/Fab (2 : 3 : 0.25); lane 6, CB/CC/Fab (2 : 3 : 0.5); lane 7, CB/CC/Fab (2 : 3 : 1.0). (C) The increasing concentrations of cystatin C added to pre-formed cathepsin B/2A2 mAb complex did not change the concentration of cathepsin B/2A2 mAb complex. Lane 1, cathepsin B (CB); lane 2, cystatin C (CC); lane 3, 2A2 mAb (mAb); lane 4, CB/mAb (1 : 0.5); lane 5, CB/CC (1 : 1); lane 6, CB/mAb/CC (1 : 0.5 : 1); lane 7, CB/mAb/CC (1 : 0.5 : 1.5); lane 8: CB/mAb/CC (1 : 0.5 : 2).

enabling the binding of the extended substrate [31]. This is actually the case with procathepsin B, where the propeptide folds on the enzyme's surface, shielding the active site, whereas the occluding loop is lifted above the body of the enzyme [37,38]. A similar mechanism applies to the binding of cystatin C to cathepsin B, which takes place in two steps: an initial weak interaction with N-terminal region of the inhibitor inducing a conformational change (i.e. the dislocation of the occluding loop), which leads to tighter binding of the whole inhibitor, stabilizing the endopeptidase conformation [24,39].

Cathepsin B contributes to both intracellular and pericellular degradation of ECM proteins, both *in vitro* (i.e. type IV collagen, laminin, fibronectin) and *in vivo* (i.e. type IV collagen) [18,34,40], implicating its role in malignant disease by facilitating tumour invasion and metastasis. It was suggested that the enzyme possesses exopeptidase activity at pH values below 5, corresponding to the acidic environment in lysosomes and in other acidic compartments [41], whereas endopeptidase activity prevails at a pH above 5.5 [42], with a pH optimum at 7.4 [31], suggesting its extracellular involvement. However, at neutral or alkaline pH, purified cathepsin B undergoes irreversible denaturation [43,44], and this process is slowed down by the presence of glycosaminoglycans. Almeida *et al.* [45] revealed that heparan sulfate binding to cathepsin B not only inhibited exopeptidase activity, at the same time as retaining its endopeptidase activity, but also protected the enzyme against alkaline pH induced inactivation, suggesting that heparan sulfate might help prevent inactivation of the enzyme at the cell surface and potentiate its endopeptidase activity, thereby enabling pericellular degradation of ECM proteins. Whether the binding of heparan sulfate to cathepsin B changes the conformation of the occluding loop is not known.

There is a need for novel specific cathepsin B inhibitors that would effectively inhibit endopeptidase activity because the existing synthetic inhibitors of cathepsin B (e.g. CA-074) primarily impair its exopeptidase activity [46] and are not as effective as inhibitors at higher pH values, where cathepsin B behaves as an endopeptidase [47]. As shown in a previous study [18], 2A2 mAb significantly reduced tumour cell invasion, which depends on the degradation of ECM proteins that are possible substrates for cathepsin B endopeptidase activity. The specificity of the antibody, its internalization into tumour cells and the ability to retain its inhibitory activity at neutral and acid pH [18] make feasible its application in the treatment of cancer and other diseases that have increased cathepsin B endopeptidase activity.

Using SPOT analysis, the amino acid sequence EPGYSP was identified as the epitope for 2A2 mAb on cathepsin B. This was confirmed using SPR where the interaction between 2A2 mAb and CIAEPGYSP, a nonapeptide mimicking the epitope on cathepsin B, resulted in strong binding with a K_d of 4.7 nM. The

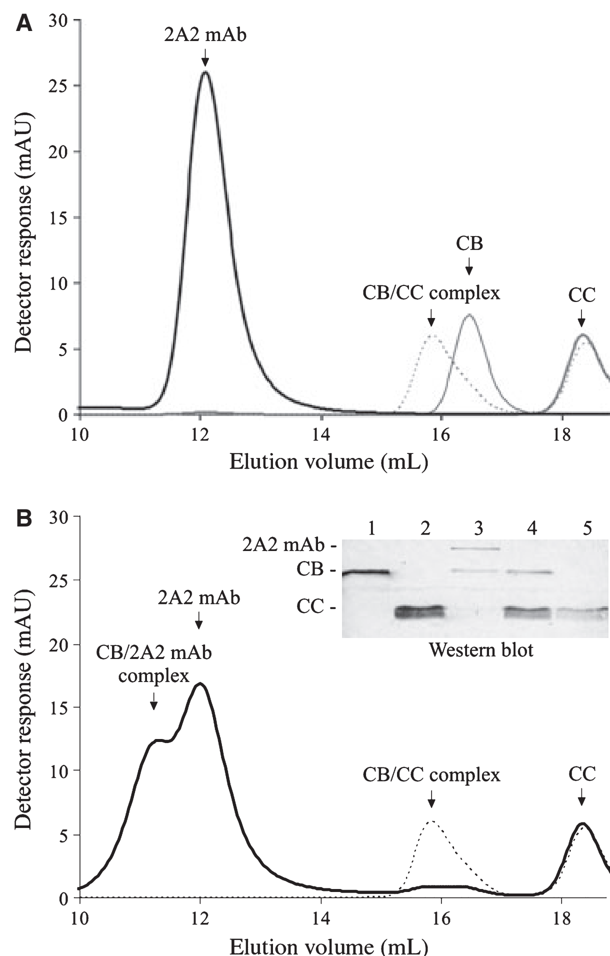

Fig. 6. Dissociation of cystatin C from the cathepsin B/cystatin C complex by 2A2 mAb as shown by size exclusion chromatography and western blot analysis. (A) One hundred microliters of sample: cathepsin B (thin black line), cystatin C (thick grey line) and 2A2 mAb (thick black line), respectively were applied on a Superdex 200 10/300GL column and eluted with 50 mM phosphate buffer containing 150 mM NaCl (pH 6.5) at a flow rate 0.8 mL·min^{-1}. Cathepsin B and cystatin C (1 : 3 molar ratio) were incubated in elution buffer for 2 h at room temperature prior to application to a Superdex 200 column (dashed black line). (B) Incubation of the cathepsin B/cystatin C complex with 2A2 mAb for a further 2 h in a 1 : 3 : 1 molar ratio (thick black line) resulted in the disappearance of the peak at 15.84 mL corresponding to cathepsin B/cystatin C complex and the appearance of a new peak at 11.28 mL. The western blot (insert) shows the absence of cystatin C and the presence of cathepsin B and 2A2 mAb in this peak, corresponding to a cathepsin B/2A2 mAb complex. Lane 1, recombinant cathepsin B; lane 2, recombinant cystatin C; lane 3, fraction eluted at 11.28 mL (cathepsin B/cystatin C complex incubated with 2A2 mAb); lane 4, fraction eluted at 15.84 mL (cathepsin B/cystatin C complex); lane 5, fraction eluted at 18.35 mL (cystatin C).

latter is in agreement with the K_d of 2.7 nM that was obtained for the interaction between 2A2 mAb and the intact cathepsin B. The possibility that shorter sequences, such as EP or EPGY, should represent the epitope was also excluded by SPR. EPGYSP is located between amino acids 133–138 at the exposed part of the cathepsin B molecule near the occluding loop (Fig. 2C). The location of the epitope indicates that the binding of 2A2 mAb might change the conformation of the loop, which is known for its flexibility [37], and, in this way, stabilize the exopeptidase conformation. Our hypothesis is supported by the enzyme kinetics, which shows an increase in exopeptidase activity of cathepsin B in the presence of 2A2 mAb. Furthermore, 2A2 mAb also inhibited cathepsin B endopeptidase activity, as determined by the degradation of DQ-collagen IV and BODIPY FL casein.

In experiments studying the effect of 2A2 mAb on the stability of the cathepsin B/cystatin C complex, we demonstrated that increasing concentrations of 2A2 mAb or its Fab fragment caused a decrease in the level of the cathepsin B/cystatin C complex, whereas the level of the complex formed between cathepsin B and the antibody or its Fab increased. This suggests that the binding of the antibody can displace the occluding loop from its endopeptidase position, which is required for the binding of cystatin C to cathepsin B [24,39], stabilizing its exopeptidase conformation. The result is a dissociation of cystatin C from the complex (Fig. S4). In a reverse experiment, increasing concentrations of cystatin C did not cause a decrease in the level of the cathepsin B/2A2 mAb complex, suggesting that the dissociation of cystatin C is not the result of simple competition with 2A2 mAb for the same binding site on cathepsin B. The latter was supported by SPR, which showed that 2A2 mAb still binds to cathepsin B bound to cystatin C on a sensor chip (Fig. 3B), again suggesting that 2A2 mAb and cystatin C occupy different binding sites on cathepsin B. However, cathepsin B remained bound to the immobilized cystatin C in the SPR experiment despite 2A2 mAb binding. To clarify whether the binding of 2A2 mAb to the cathepsin B/cystatin C complex in free solution results in a ternary complex, as evident by SPR, or in the dissociation of cystatin C and the formation of the cathepsin B/2A2 mAb complex, as suggested by native gel electrophoresis, size exclusion chromatography was employed. It clearly showed that the addition of 2A2 mAb caused the disappearance of the peak corresponding to the cathepsin B/cystatin C complex and the appearance of a higher molecular weight peak corresponding to the newly-formed cathepsin B/2A2 mAb complex. The analysis of the peaks by western blot analysis and ELISA confirmed that cystatin C is dissociated from its complex with cathepsin B after binding 2A2 mAb. The lack of dissociation of cathepsin

B/2A2 mAb from cystatin C in the SPR experiment remains to be elucidated; however, we can assume that it was attributable to the more rigid structure of cystatin C as a result of covalent linking to the CM5 sensor chip compared to its counterpart in solution.

In conclusion, 2A2 mAb is shown to inhibit cathepsin B endopeptidase activity and simultaneously potentiate its exopeptidase activity. Although further studies, including structural ones, are required to confirm the conformational changes of the active site of cathepsin B, the results obtained in the present study provide a specific mechanism for the regulation of the activity of cathepsin B, which can be triggered in diseases associated with its harmful action.

Experimental procedures

Cell culture and reagents

Hybridoma cells were grown in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 13% fetal bovine serum (HyClone, Logan, UT, USA), glutamine (Sigma, St Louis, MO, USA) and antibiotics. MCF-10A neoT cell line was provided by Bonnie F. Sloane (Wayne State University, Detroit, MI, USA). MCF-10A neoT were cultured in DMEM/F12 (1 : 1) medium (Gibco Invitrogen) supplemented with 5% fetal bovine serum, 1 μg·mL^{-1} insulin (Sigma), 0.5 μg·mL^{-1} hydrocortisone (Sigma), 50 ng·mL^{-1} epidermal growth factor (Sigma), glutamine and antibiotics.

Preparation of 2A2 mAb and its Fab fragments

Cathepsin B specific mouse 2A2 mAb capable of inhibiting its proteolytic activity was prepared as described previously [18]. The hybridoma cell lines were obtained by the fusion of splenocytes from BALB/c mice immunized with recombinant human cathepsin B [48] with NS1/1-Ag4-1 myeloma cells according to the method of Köhler and Milstein [49]. Screening for clones producing the most potent inhibitory antibodies was performed with the substrate Z-RR-AMC. mAbs were purified from the hybridoma culture medium using affinity chromatography on Protein A Sepharose.

2A2 mAb Fab fragments were prepared by proteolytic cleavage with papain (Sigma). Papain (2 mg·mL^{-1}) was activated by incubation in 0.1 M Tris–HCl buffer (pH 8.0), containing 2 mM EDTA and 1 mM dithiothreitol for 15 min at 37 °C. 2A2 mAb (1.4 mg·mL^{-1}) was added in a 1 : 100 molar weight ratio and incubated for 1 h at 37 °C. The mixture was then placed on ice and protected from light before iodoacetamide (Serva, Heidelberg, Germany) (20 mM final concentration) was added to stop the reaction. After overnight dialysis against NaCl/P$_i$ (pH 7.2), Fab fragments were purified by affinity chromatography on protein A Sepharose. Undegraded IgGs and Fc fragments bound to the column with 0.14 M phosphate buffer (pH 8.2), unbound Fab fragments were pooled, dialyzed against NaCl/P$_i$ (pH 7.2), and concentrated by ultrafiltration. The samples were checked for molecular weight and homogeneity by SDS/PAGE.

Characterization of 2A2 mAb

The IgG subclass of purified 2A2 mAb was determined by indirect ELISA. Microtiter plates were coated with 100 μL of recombinant human cathepsin B (2 μg·mL^{-1}) and incubated overnight at 4 °C. After washing and blocking, 100 μL of 2A2 antibody solution (0.5 μg·mL^{-1}) was added and incubated for 2 h at 37 °C. After washing, 100 μL of goat anti-(mouse IgG1, IgG2a, IgG2b or IgG3) sera conjugated to HRP (Nordic Immunology, Tilburg, The Netherlands) diluted 1 : 1000 in blocking buffer was added and the plate incubated for 2 h at 37 °C. The immune complexes were detected using 3,3′,5,5′-tetramethylbenzidine (Sigma) and H_2O_2 as substrate.

The monoclonality of the antibody was assessed by IEF using the PhastSystem (Pharmacia).

K_d between 2A2 mAb and cathepsin B

The K_d between 2A2 mAb and cathepsin B was determined with ELISA according to the method of Friguet *et al.* [30]. Human recombinant cathepsin B at concentrations from 10 pM to 200 nM was mixed with 0.1 nM 2A2 mAb in NaCl/P$_i$, containing 10 mg·mL^{-1} of BSA. After 15 h of incubation at 4 °C, 100 μL of each mixture was transferred into wells of a microtiter plate precoated with human recombinant cathepsin B (2.5 μg·mL^{-1}) and incubated for 2 h at 37 °C. One hundred microliters of goat anti-(mouse IgG) conjugated to HRP (Dianova, Hamburg, Germany) at 1 : 5000 dilution was added after the washing step and incubated for 2 h at 37 °C. One hundred microliters of 2,2′-azinobis(3-ethylbenzthiazoline)sulfonic acid (1 mg·mL^{-1}) (Sigma) and 0.0012% H_2O_2 was added and incubated for 30 min at 37 °C. Absorbance was measured at 405 nm. The K_d was calculated with an equation proposed by Friguet *et al.* [30], using a Scatchard plot. The K_d was recalculated using a modified equation proposed by Stevens [50].

Determination of the 2A2 mAb binding site on cathepsin B

The 2A2 mAb epitope on cathepsin B molecule was determined using the SPOTs System and its associated software (SPOTSALOT) according to the manufacturer's instructions. Thirty-six overlapping decapeptide amino acid sequences

were selected from the amino acid sequence of mature human cathepsin B (Swiss-Prot database: P07858). The corresponding decapeptides were synthesized from their C-terminus on the pre-indicated spots on derivatized cellulose membrane according to the synthesis protocol prepared by the SPOTSALOT software. In each cycle, the corresponding Fmoc amino acid derivatives were dispensed to the spots and, after washing with dimethylformamide (Merck), all residual amino acid groups on the membrane were blocked by acetylation. Removal of Fmoc protecting groups generated free amino acid groups capable of binding Fmoc amino acids in the next cycle. After the final cycle, peptides were N-terminally acetylated, followed by deprotection of the side chain. After synthesis, the membrane with bound peptides was blocked with 50 mM Tris (pH 8.0), containing 140 nM NaCl, 3 mM KCl, 0.05% Tween 20 and 2% BSA (Sigma) at 4 °C overnight and subsequently incubated with 2A2 mAb (10 $\mu g \cdot mL^{-1}$) for 2 h at room temperature. The immune complexes were detected with secondary goat anti-(mouse IgG) conjugated to HRP (Dianova) at 1 : 1000 dilution in blocking buffer and the membrane incubated for 2 h at room temperature. 0.05% (Sigma) and 0.09% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.5) were used to visualize the spots.

SPR

The binding kinetics of 2A2 mAb to cathepsin B were determined by the SPR-based biosensor Biacore X (Biacore, Uppsala, Sweden). Cathepsin B specific 3E1 mAb (Krka, d.d., Novo mesto, Slovenia) was used as a control.

The nonapeptide CIAEPGYSP, mimicking the epitope for 2A2 mAb, was immobilized on the CM5 sensor chip according to the manufacturer's recommended ligand thiol coupling protocol. The flow rate of the HBS running buffer [10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, pH 7.4 containing 0.005% (v/v) P-20 surfactant] was 5 $\mu L \cdot min^{-1}$. The CM5 sensor chip surface was activated with a 2 min injection pulse of 1 : 1 *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). A reactive disulfide group was introduced with a 4 min injection pulse of 80 mM 2-(2-pyridinyldithio)ethaneamine in 0.1 M borate buffer (pH 8.5). CIAEPGYSP (50 $\mu g \cdot mL^{-1}$ in immobilization buffer, 10 mM citric buffer, pH 3.8) was flowed over the sensor surface for 7 min. Unreacted disulfide groups were deactivated with a 4 min injection pulse of 50 mM cysteine, 1 M NaCl in 0.1 M acetate buffer (pH 4.0). In the second flow cell of the sensor chip, used as a reference, injection of the nonapeptide was omitted. After immobilization, a 20 µL of 2A2 or 3E1 mAb in the concentration range 0.5–2.5 nM in HBS was injected. At the end of the sample plug, HBS buffer was flowed over the sensor surface enabling dissociation. Sensor surface was regenerated using 50 mM glycine-NaOH (pH 9.5). Kinetic data were obtained using BIAEVALUATION software (Biacore). Similarly, octapeptides SAICEPGY and KCSAICEP, containing only four or two amino acid residues of the predicted epitope sequence EPGYSP, were immobilized on the CM5 sensor chip using the amine coupling protocol. The flow rate of the HBS buffer was 5 $\mu L \cdot min^{-1}$. The CM5 sensor chip surface was activated with a 7 min injection pulse of 1 : 1 NHS and EDC. SAICEPGY and KCSAICEP (400 $\mu g \cdot mL^{-1}$ in 10 mM acetic buffer (pH 3.0) and 200 $\mu g \cdot mL^{-1}$ in 10 mM citric buffer, pH 3.8, respectively) were then introduced onto the sensor surface. Unreacted sites on the sensor surface were blocked with a 7 min injection pulse of 1 M ethanolamine (pH 8.5). In the reference flow cell, the injection of the octapeptide was omitted. After immobilization of the peptides 2A2 mAb (0.5–5000 nM in HBS) was tested for binding. Sensor surface was regenerated using 50 mM glycine-NaOH (pH 9.5).

To determine whether cystatin C and 2A2 mAb compete for the same binding site on cathepsin B, cystatin C was covalently bound to a CM5 sensor chip via primary amino groups using the manufacturer's protocol. The carboxymethylated surface was activated using a 7 min injection pulse of 1 : 1 NHS and EDC at a flow rate 5 $\mu L \cdot min^{-1}$. Cystatin C in HBS was then flowed over the activated surface. In a reference cell, the injection of cystatin C was omitted. Unreacted sites on the sensor surface were blocked with a 7 min injection pulse of 1 M ethanolamine (pH 8.5). Cathepsin B at a concentration of 2 µM was then applied and tested for binding the 2A2 mAb (2 µM). NaOH at a concentration of 50 mM was used for the regeneration.

Regulation of cathepsin B activity and ECM degradation by 2A2 mAb

The effect of 2A2 mAb on cathepsin B endopeptidase activity was assessed using protein substrates BODIPY FL casein and DQ-collagen IV. Thirty microliters of activation buffer (10 mM cysteine in Mes buffer, pH 6.0) and 20 µL of cathepsin B solution in Mes buffer (pH 6.0) were preincubated for 15 min at room temperature. Fifty microliters of mAb (10 µM) solution and 100 µL of BODIPY FL casein (10 $\mu g \cdot mL^{-1}$) were added and mixed gently for 1 h at room temperature. Fluorescence was measured at 485 nm excitation and 538 nm emission wavelengths. When using DQ-collagen IV as a substrate, the enzyme was activated in 400 mM phosphate buffer (pH 6.8) containing 0.1% poly(ethylene glycol), 1.5 mM EDTA and 5 mM dithiotheitol for 5 min at 37 °C. Five microliters of DQ-collagen IV (final concentration 10 $\mu g \cdot mL^{-1}$) and 10 µL of 2A2 mAb or NaCl/P_i were added to a well of a black microtiter plate and the reaction was initiated by adding 85 µL of activated cathepsin B (final concentration 200 nM). Fluorescence was monitored at 495 nm excitation and 515 nm emission wavelengths.

The inhibitory effect of 2A2 mAb on ECM degradation was observed using fluorescence microscopy. Wells of

precooled Lab-Tek™ Chambered Coverglass (Nalge Nunc International, Rochester, NY, USA) were coated with 50 μg·mL^{-1} of the quenched fluorescent substrate DQ-collagen IV suspended in 40 μL of 100% Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at 4 °C. DQ-collagen IV/Matrigel matrix was allowed to polymerize for 40 min at 37 °C. Four hundred microliters of MCF-10A neoT cells (4×10^4 per well) in growth medium containing 2% Matrigel and 3 μM 2A2 mAb, 3 μM 3E1 mAb or NaCl/P_i, respectively were plated onto gelled Matrigel. After 24 h of incubation at 37 °C with 5% CO_2, the samples were monitored for fluorescent degradation products using an Olympus IX 81 motorized inverted microscope and CELLR software (Olympus, Tokyo, Japan).

Exopeptidase activity of cathepsin B was evaluated using FRET substrate Abz-GIVRAK(Dnp)-OH (Bachem, Bubendorf, Switzerland). The Enzyme was activated in 60 mM acetate buffer (pH 5.0) containing 0.1% poly(ethylene glycol), 1.5 mM EDTA and 5 mM dithiotheitol for 5 min at 37 °C. Five microliters of Abz-GIVRAK(Dnp)-OH (final concentration 1 μM) and 10 μL of 2A2 mAb or NaCl/P_i were added to a well of a black microtiter plate and the reaction was initiated by adding 85 μL of activated cathepsin B (final concentration 0.5 nM). Fluorescence was monitored at 320 nm excitation and 420 nm emission wavelengths. Kinetic parameters were obtained using SIGMAPLOT software in conjunction with the ENZYME KINETICS MODULE add-on (Systat Software Inc., Chicago, IL, USA).

Interaction of intact 2A2 mAb and its Fab fragment with the cathepsin B/cystatin C complex

The effect of 2A2 mAb and its Fab fragment on the stability of the complex formed between recombinant human cathepsin B and recombinant human cystatin C [48,51] was assessed by native gel electrophoresis. Cathepsin B and cystatin C were preincubated in a 2 : 3 molar ratio in 0.01 M phosphate buffer (pH 6.5) for 1 h at room temperature. The cathepsin B/cystatin C complex was then incubated with increasing concentrations of 2A2 mAb or Fab for 1 h at room temperature. To test the effect of cystatin C on the stability of the cathepsin B/2A2 mAb complex, cathepsin B and 2A2 mAb were preincubated in a 2 : 1 molar ratio for 1 h at room temperature. Cystatin C was added to the solution in 1 : 1, 2 : 3 and 1 : 2 molar ratios relative to cathepsin B and incubated for 1 h at room temperature. Then 2.5 μL of each sample, mixed in a 1 : 1 ratio with native gel running buffer (20 mM Tris/HCl, pH 8.0, 2 mM EDTA, 5% SDS, 0.02% bromophenol blue) was loaded on a homogeneous 20% polyacrylamide gel (Pharmacia) and separated on the PHAST system (Pharmacia) using native buffer strips (0.88 M L-alanine, 0.25 M Tris, pH 8.8 in 3% agarose). After separation, gels were developed on the PhastGel system (Pharmacia) using Coomassie blue staining.

Size exclusion chromatography

The ability of 2A2 mAb to cause dissociation of cystatin C from cathepsin B was assessed by size exclusion chromatography on the ÄKTA™ FPLC™ (GE Healthcare) system. For all samples, 100 μL of sample was applied on a Superdex 200 10/300GL column (GE Healthcare, Milwaukee, WI, USA) and eluted with 50 mM phosphate buffer containing 150 mM NaCl (pH 6.5) at a flow rate 0.8 mL·min^{-1}. The molecular weights were calculated from the calibration curve: elution volume = $-5.76 \times \log MW + 42.07$ (R^2 = 0.9778), which was obtained with the calibration standards: aldolase (160 kDa), BSA (67 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa). First, cathepsin B, cystatin C and 2A2 mAb were analyzed individually. Then cathepsin B was incubated with cystatin C (1 : 3 molar ratio) in the elution buffer for 2 h at room temperature. The 2A2 mAb (1 : 1 molar ratio relative to cathepsin B) was added to the mixture and incubated for an additional 2 h at room temperature.

Western blot analysis and ELISA

The presence of cystatin C, cathepsin B and 2A2 mAb in eluted peaks obtained with size exclusion chromatography was determined by western blot analysis. Samples were boiled in reducing sample buffer for 10 min, separated by 12.5% SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4 °C with 0.5% Tween in PBS and incubated with mouse anti-cathepsin B 3E1 mAb (10 μg·mL^{-1}) and rabbit polyclonal sera [52] against cystatin C (5 μg·mL^{-1}) in 0.05% Tween 20 in NaCl/P_i for 1 h at room temperature. After washing with 0.05% Tween 20 in NaCl/P_i, the membrane was incubated with secondary goat anti-rabbit (1 : 1000) (Gibco Invitrogen) and goat anti-mouse (1 : 1000) (Dianova) sera conjugated to HRP in 0.05% Tween in NaCl/P_i for 45 min at room temperature. The spots on the membrane were visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 M Tris (pH 7.5).

The molar ratio of cathepsin B to cystatin C in eluted fractions was determined by a specific ELISA, as described previously [52].

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Supporting information

The following supplementary material is available:
Fig. S1. Amino acid sequence of mature cathepsin B with marked decapeptides.

Fig. S2. SPR sensogram presenting the interaction between 2A2 mAb and octapeptide SAICEPGY.
Fig. S3. SPR sensogram presenting the interaction between 2A2 mAb and octapeptide KCSAICEP.
Fig. S4. Proposed mechanism for the 2A2 mAb induced dissociation of cystatin C from the cathepsin B/cystatin complex.

This supplementary material can be found in the online article.

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