Defective Acidification of Intracellular Organelles Results in Aberrant Secretion of Cathepsin D in Cancer Cells

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Aberrant secretion of lysosomal hydrolases such as (pro)cathepsin D (proCD) is a common phenotypic change in many human cancers. Here we explore the underlying molecular defect(s) and find that MCF-7 breast and CaCo-2 colorectal cancer cells that are unable to acidify their endosomal compartments secreted higher amounts of proCD than did acidification-competent cancer cell types. The latter secreted equivalent amounts of proCD only after dissipation of their organelar pH gradients with NH₄Cl. Assessing the critical steps that resulted in proCD secretion revealed that the Golgi-associated sorting receptor for CD, i.e. the cation-independent mannose-6-phosphate receptor (MPR300), was aberrantly distributed in acidification-defective MCF-7 cells. It accumulated mainly in late endosomes and/or lysosomes as a complex with its ligand (proCD or intermediate CD), as evidenced by its co-localization with both CD and LAMP-2, a late endosome/lysosome marker. Our immunoprecipitation analyses also showed that MCF-7 cells possessed 7-fold higher levels of receptor-enzyme complexes than did acidification-competent cells. NH₄Cl induced similar receptor redistribution into LAMP-2-positive structures in acidification-competent cells but not in MCF-7 cells. The receptor also recovered its normal Golgi localization upon drug removal. Based on these observations, we conclude that defective acidification results in the aberrant secretion of proCD in certain cancer cells and interferes mainly with the normal disassembly of the receptor-enzyme complexes and efficient receptor reutilization in the Golgi.

Like many other lysosomal hydrolases, cathepsin D (CD) is synthesized in the endoplasmic reticulum as an inactive (52–54 kDa) glycosylated propeptide (1). In the Golgi, proCD is tagged with a phosphomannosyl targeting signal (mannose-6-phosphate) that mediates its cation-independent mannose-6-phosphate receptor (MPR300)-dependent sorting into the endosomal compartments (2, 3). The other receptor, the cation-dependent receptor (MPR46), is thought to bind a distinct set of lysosomal enzymes (4–6) and appears not to be involved in the sorting of CD (5, 7). After transport to endosomes, MPR300-ligand complexes are disassembled and the receptor is recycled back to the Golgi/TGN via a retromer-assisted transport system (8, 9), whereas proCD is further cleaved into a 44-kDa intermediate form (10, 11) prior to its final maturation in lysosomes into a two-chain catalytic enzyme consisting of ~30- and ~14-kDa fragments (1).

Many breast and colorectal cancer cells anomalously secrete increased amounts of cathepsins B and D into the extracellular space where these enzymes have been shown to contribute to the invasive and metastatic properties of cancer cells (12, 13). Previously, a number of possibilities have been put forward and tested as potential causes for CD secretion in cancer cells. These include e.g. increased expression of CD (7), its altered binding to the MPR46 (7), a failure of cancer cells to synthesize the phosphomannosyl tag (14), and the absence or inactivating/missense mutations in the MPR300 itself (15). In addition, it has also been reported that the secretion of mature CD directly from pericellular lysosomes contributes to CD secretion by metastatic cancer cells (16). However, none of these observations have provided a satisfying explanation for the secretion of proCD by multiple cancer cell types.

It has also been well documented that efficient processing and lysosomal targeting of proCD requires the existence of proper pH gradients between the cytoplasm and the Golgi/TGN or the endosomal compartments. For example, the binding of proCD to MPR300 in the Golgi occurs normally at pH 6.4–6.5 (17), whereas dissociation of the complex requires more acidic pH (18–20). Perturbation of these pH gradients, e.g. with chloroquine or ammonium chloride (NH₄Cl), results in delayed intracellular maturation of proCD (7) and its increased secretion from the cells (21, 22).

pH alterations are commonly associated with malignant transformation and growth (23–26) as well as multidrug resistance (27, 28). pH-dissipating drugs also do not increase the secretion of CD in cancer cells, in contrast to normal cells (20), suggesting that an altered pH homeostasis might be an additional cause for the secretion of proCD in various cancer cell lines. Here we explore this possibility by using, for the first time, cell lines that either can or cannot acidify their intracellular organelles properly. Previous work (27, 28) has shown that such breast cancer cell lines exist (MCF-7 and MCF-7/AdR cells) and that their inability/ability to acidify correlates well with their sensitivity/resistance to chemotherapeutic drugs. Because MCF-7 cells are also known to secrete CD (20), they provide a convenient drug-free system to assess the pH dependence and the molecular details in CD sorting that are affected by defective acidification. Utilizing these two breast cancer cell lines and three randomly selected colorectal cancer cell lines with unknown acidification potential we now report that an acidification defect correlates with increased CD secre-
tion and impairs normal lysosomal sorting of proCD by reducing the amount of the free receptor in the Golgi.

EXPERIMENTAL PROCEDURES

Cell Lines and Culturing—Human breast cancer cells (MCF-7), African green monkey kidney cells (COS-7), and human colorectal adenocarcinoma cell lines (HT-29, SW-48, and CaCo-2) were all obtained from the American Type Culture Collection (Manassas, VA). Multidrug-resistant MCF-7/AdrR cells were kindly provided to us by Dr. Myles Cabot (Santa Monica, CA). All cell lines were maintained at 5% CO2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (PromoCell, Heidelberg, Germany), Glutamax, and antibiotics (penicillin-streptomycin).

Antibodies—The cathepsin D polyclonal antibody used for Western blotting and immunostaining was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The polyclonal anti-cathepsin D antibody used for immunoprecipitations was purchased from Calbiochem. The monoclonal anti-MPR300 antibody and polyclonal antibodies against EAA-1 (early endosomal antigen 1) were purchased from Affinity Bioreagents (Golden, CO). The monoclonal anti-α-tubulin (clone DM1A) and -α-actin (clone DM1A) were from Sigma-Aldrich, and the monoclonal antibody against GM130 (Golgi matrix protein of 130 kDa) was from BD Biosciences-Transduction Laboratories. The monoclonal antibody against LAMP-2 (H4B4) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Alexa-488- and Alexa-594-conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated anti-rabbit and anti-goat secondary antibodies were purchased from P. A. R. I. S. (Compiegne, France) or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

Indirect Immunofluorescence—Cells were grown on glass coverslips overnight at normal cell culture conditions. Culture medium was then replaced with fresh medium containing 2 μM LysoSensor Yellow/Blue DND-160 (Molecular Probes). Cells were incubated for an additional 5 min at 37°C with or without NH4Cl prior to fixation (4% paraformaldehyde). After blocking (1% bovine serum albumin and 0.1% saponin in phosphate-buffered saline (PBS)), the cells were double- or triple-stained using both monoclonal and polyclonal anti-MPR300 antibodies simultaneously with the anti-cathepsin D antibody and organelle marker antibodies (GM130, EEA-1, and LAMP-2). Alexa Fluor-conjugated secondary antibodies were used for visualization. Cells were embedded with ImmunoMount (Shandon, Pittsburgh, PA), and stained specimens were examined and photographed using an Olympus epifluorescence microscope and a CCD camera.

Staining of Acidic Organelles with the LysoSensor Dye—Cells were grown on glass coverslips overnight at normal cell culture conditions. Culture medium was then replaced with fresh medium containing 2 μM LysoSensor Yellow/Blue DND-160 (Molecular Probes). Cells were incubated for an additional 5 min at 37°C with or without NH4Cl before analyses by SDS-PAGE. After size separation, proteins were transferred onto the Protran® nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked with 5% milk powder (Blotto) or 0.5% bovine serum albumin, 0.1% Tween 20, and 1% glycerol in PBS for antibodies against human cathepsin D and MPR300. Membranes were washed with the same buffer and incubated with appropriately diluted primary and 1:10 000-diluted horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized using chemiluminescence substrates (ECL, Amersham Biosciences) and exposure to Hyperfilm ECL-films (Amersham Biosciences) for 1–30 min before quantification of the bands with the PhosphorImager software (ImageQuant version 5.2, Amersham Biosciences).

RESULTS

MCF-7 and CaCo-2 Cells Are Unable to Acidify their Intracellular Organelles—We first determined the ability of the different cancer cell lines to acidify their intracellular organelles by using the LysoSensor Yellow/Blue indicator dye (Fig. 1). This dye accumulates in acidic organelles (the trans-Golgi, endosomes, and lysosomes) and emits yellow/blue light depending on the acidity of a given organelle. Microscopical examination of stained cells (Fig. 1) demonstrated a large number of bluish (acidic) vesicular structures in COS-7, MCF-7/AdrR, HT-29, and SW-48 cells, consistent with their normal acidification potential. Some SW-48 cells also possessed larger yellowish (i.e. less acidic) vesicular structures whose exact identity is currently not known. In contrast, (Fig. 1), very few stained vesicular structures were detected in MCF-7 breast cancer cells, in accordance with previous observations (27, 28). Interestingly, only a few dye-positive structures were detected also in CaCo-2 cells, showing that defective acidification is not restricted only to MCF-7 breast cancer cells. The specificity of the dye for acidic organelles was demonstrated by showing that there were no stained organelles present if the cells were treated for 4 h with 100 mM bafilomycin A1, a vacuolar H+-ATPase inhibitor, prior to staining (data not shown).

pH-dependent Intracellular Processing and Secretion of proCD in Cancer Cells—To compare the intracellular processing and secretion of CD in the above cell types, we metabolically labeled cells for 6 h with the Pro-MixTM 35S-labeling reagent. After chase (12 and 24 h) in the presence and the absence of NH4Cl, different CD forms in total cell lysates and culture media were immunoprecipitated before analyses by SDS-PAGE and autoradiography (Fig. 2A). In each cell line, proCD (52 kDa), intermediate CD (mainly the 44 kDa band), and mature CD (30 kDa) were detected. The 52-kDa form, i.e. proCD, was the major form secreted into the culture medium. Quantification revealed (Table I) that the majority (60–74%) of the different CD bands consisted of the mature CD (30 kDa band) in all cell types except for MCF-7 and CaCo-2 cells, in which this form represented 37 and 46% of the total CD in the cells, respectively. The immature CD pool (including intracellular proCD and intermediate CD pools) comprised between
13–34% of the total CD found in the cells and the medium (Fig. 2A). The amount of the secreted proCD totalled only 8% in COS-7 cells, whereas in cancer cells secreted proCD ranged from 9 to 44% of the total CD. Highest secretion levels were detected in acidification-defective MCF-7 and CaCo-2 cells (44 and 22%, respectively). Compared with acidification-competent COS-7 and breast cancer MCF-7/AdrR cells, ~5- and 3-fold higher amounts of proCD were secreted by acidification-defective MCF-7 and CaCo-2 cells, respectively.

Treatment of cells with NH$_4$Cl (10 mM) during the 12 h-chase period changed the relative proportions of different CD forms in all cell types examined (Fig. 2B). The proportion of the mature CD (30 kDa) decreased in all cell types, whereas the level of the immature CD was either decreased (COS-7), roughly equal (HT-29), or increased by ~2-fold (MCF-7/AdrR, MCF-7, SW-48, and CaCo-2) relative to the levels observed in untreated cells. The amount of the secreted proCD changed most dramatically, totalling between 26 and 35% of the total CD in the cells. The highest increase was observed in COS-7 cells (~4-fold increase) and SW-48 cells (~3-fold increase; Fig. 2B). In contrast, in acidification-defective MCF-7 and CaCo-2 cells CD secretion was either slightly lower (MCF-7, 0.8-fold) or slightly higher (CaCo-2, 1.2-fold) than the secretion level observed in the absence of the drug (Fig. 2B). Thus, drug treatment increased the secretion of proCD mainly in acidification-competent cell lines and not in acidification-defective MCF-7 and CaCo-2 cells, demonstrating the strict pH-dependence of CD secretion.

**Table I**

|          | Mature | Immature | Secreted |
|----------|--------|----------|----------|
| COS-7    | 72.6   | 19.0     | 8.5      |
| MCF-7/AdrR | 59.9   | 25.6     | 14.5     |
| MCF-7    | 36.5   | 19.4     | 44.1     |
| SW-48    | 73.7   | 13.0     | 13.2     |
| HT-29    | 57.4   | 33.5     | 9.1      |
| CaCo-2   | 45.9   | 31.9     | 22.3     |

**Defective Acidification Impairs Dissociation of the Receptor-CD Complexes in MCF-7 Cells**—To assess whether the increased secretion of CD in acidification-defective MCF-7 cells is simply due to the absence of the functional MPR300, we performed comparative Western blot analyses with the anti-MPR300 antibody and found that COS-7, MCF-7, and MCF-7/AdrR cells all expressed equal amounts of the receptor (100, 102 and 103%) with respect to the amount of expressed α-tubulin (data not shown). This was also confirmed by indirect immunofluorescence (Fig. 3) with the MPR300 antibody, which
stained specific vesicular structures near the nuclei in each of the three cell lines examined. In COS-7 and MCF-7/AdrR cells, these structures were distinct from the vesicular structures stained simultaneously with the anti-CD antibody. However, the receptor and CD showed extensive co-distribution in MCF-7 cells, suggesting that the two proteins predominantly reside in the same intracellular compartments in these cells.

The observed co-localization of the CD and the MPR300 in MCF-7 cells also suggested that the two proteins may exist as receptor-ligand complexes. To demonstrate this possibility directly, we immunoprecipitated the receptor from the total lysates (pH 7.5) of the cells metabolically labeled for 16 h and quantified the amount of the co-precipitated cathepsin D in the immunoprecipitates (Fig. 4A). In each cell line, both proCD and intermediate CD (but not mature CD, data not shown) were detected in the precipitates. However, the membranes of the double CD band (proCD and intermediate CD) was low in COS-7 and MCF-7/AdrR cells, giving an intensity ratio of 1.2 between the two CD forms and the MPR300 (Fig. 4B). The proportion of the proCD and intermediate CD forms that co-precipitated with the receptor was much higher in MCF-7 cells. Based on the ratio (3.5:1), the relative levels of MPR300-CD complexes were roughly 7-fold higher in MCF-7 cells than in COS-7 and MCF-7/AdrR cells.

To verify that these receptor-CD complexes can be normally dissociated by acidic pH, we also performed immunoprecipitation analyses with slightly acidic pH buffer (pH 6 instead of the pH 7.5 buffer). We found that, at pH 6, the same amount of the receptor protein was precipitated in each cell line (Fig. 4A) without affecting markedly the amounts of the co-immunoprecipitated CD forms in COS-7 and MCF-7/AdrR cells. In MCF-7 cells, however, the receptor-CD complexes dissociated at pH 6, as evidenced by the absence of the two CD forms in the immunoprecipitates. We also treated microsomal membranes prepared from the two breast cancer cell lines with the same pH buffers (Fig. 4C) prior to immunoprecipitation of the different CDs from the high speed supernatant with the anti-CD antibody. Western blot analyses of the precipitates revealed that, in MCF-7 cells, acidic pH (pH 6) resulted in dissociation of the existing receptor-ligand complexes, as both proCD- and intermediate CD bands (52 kDa and 44 kDa) were detected in the supernatant fractions of these cells. Both of these bands were undetectable in MCF-7/AdrR cells in either of the pH buffers used, thus confirming that acidifica-

![Fig. 3. Subcellular distribution of cathepsin D and the MPR300 in acidification-competent (COS-7 and MCF-7/AdrR) and acidification-defective (MCF-7) cells.](http://www.jbc.org/)

![Fig. 4. Co-immunoprecipitation of CD with the MPR300 and its pH dependence.](http://www.jbc.org/)
localize extensively with the Golgi marker (GM130). In contrast, the receptor did not co-localize markedly with the early endosomal antigen (data not shown) or with the late endosome/lysosome marker (LAMP-2) (Fig. 5), which is consistent with its predominant residence in the Golgi membranes. In MCF-7 cells, however, the receptor showed a distinct localization from the Golgi marker antibody (Fig. 5) and co-localized most extensively with the late endosome/lysosome marker, the two proteins showing almost identical distribution in MCF-7 cells. These findings demonstrate that in MCF-7 cells the majority of the MPR300 proteins localize in late endosomes/lysosomes, together with bound CD (Fig. 3).

To confirm the pH dependence of the differential localization of the receptor in MCF-7 and MCF-7/AdrR cells, we treated cells with NH₄Cl. In acidification-competent MCF-7/AdrR cells, this treatment resulted in marked redistribution of the receptor from the Golgi into LAMP-2-positive structures (Fig. 6). In MCF-7 cells, however, the drug was without effect, as evidenced by the extensive co-localization of the receptor with the late endosome/lysosome marker, despite drug-treatment (Fig. 6). Similarly, drug removal did not cause any redistribution of the receptor in MCF-7 cells (data not shown), whereas in acidification-competent MCF-7/AdrR cells the receptor recovered its normal Golgi localization upon drug removal, as assessed by its co-localization with the Golgi marker (Fig. 6). Thus, it is shown that the existing pH gradients clearly affect the steady state distribution of the MPR300 between the Golgi and the endosomal compartments and that the receptor, under acidic conditions but not under neutral conditions, efficiently recycles back to the Golgi, consistent with its reutilization in the TGN (8).

**DISCUSSION**

Efficient vesicular trafficking and the correct sorting of proteins, including CD, are known to be dependent on proper ion and pH homeostasis within the secretory and endocytic compartments. Specific sorting or transport steps that have turned out to be the most sensitive to pH alterations include transport between the endoplasmic reticulum and the Golgi (31–33), within the Golgi stack (34), or between the TGN and post-Golgi organelles (35–37). However, very little is known about the
molecular switches that are regulated by pH and result in altered vesicular trafficking between the organelles. In this report, we extend these findings and report for the first time that defective acidification in certain cancer cells is the primary cause for their anomalous secretion of proCD. We also provide evidence to show that this secretion results from an altered intracellular trafficking of the MPR300 and from its prolonged association with proCD, whereby the receptor-enzyme complexes accumulate in endosomal/lysosomal compartments. On the basis of these observations it is suggested (Fig. 7) that, in the virtual absence of the free MPR300 in the Golgi, the newly synthesized proCD can freely enter constitutive secretory vesicles and be secreted out of the cell. 

![Diagram](image)

**Fig. 7.** Schematic presentation of the misorting of mannose-6-phosphate (M6P) ligands in acidification-defective breast cancer cells. Under normal conditions (1) MPR300 associates with its ligand in the TGN at pH 6.4–6.5. Bound ligand is then routed to endosomes in which the ligand dissociates from the receptor at acidic pH (below pH 6). Free receptor is transported back to the TGN, whereas the vesicle with the ligand is fused with the lysosome. In the absence of an acidic pH gradient (2) the ligand cannot dissociate from the receptor, and the complex accumulates mainly in late endosomes/lysosomes. As a result, the receptor is unable to recycle back to the Golgi, where its absence allows M6P-ligands to enter constitutive secretory vesicles and be secreted out of the cell.

absence of the MPR300 or its complete inability to bind proCD in the Golgi, as was evidenced by the presence of newly formed receptor-proCD complexes in these cells (Figs. 3 and 4). Previous work has also shown that the mannose-6-phosphate-containing ligands are also able to bind to MPR300 at pH 6.9–7 (17), i.e. at the pH of the Golgi/TGN in MCF-7 cells. The observed binding of CD to its receptor in MCF-7 cells also excludes the possibility that the lack of phosphomannosyl addition is responsible for aberrant secretion of CD in the cells, consistent with previous observations (14). Rather, our indirect immunofluorescence and immunoprecipitation data showed that defective acidification increases CD secretion mainly by interfering with the receptor-ligand dissociation and receptor recycling (Figs. 4–6). According to our results, the MPR300 co-localized extensively with both CD and LAMP-2, a late endosome/lysosome marker, in acidification-defective MCF-7 cells. Similarly, NH4Cl-induced extensive co-localization of the MPR300 and LAMP-2 in acidification-competent cells (Fig. 6), which is consistent with this redistribution being a pH-dependent process. In MCF-7 cells, drug treatment or its removal had no effect on receptor localization. This is exactly what one would expect if the pH of the endosomal compartments in these cells is at a steady state neutral enough (Fig. 1) to prevent the normal disassembly of the receptor-proCD complexes in these compartments. Our results thus seem to provide a logical explanation for the inability of pH-dissipating drugs to increase the secretion of CD also in other cancer cell types (7, 41) or to alter receptor distribution in these cells (Fig. 6).

Our findings are also compatible with recent studies showing that the knock down of one of the retromer subunits required for receptor recycling back to the Golgi results in receptor accumulation in late endosomes/lysosomes, perhaps indicating a shortened receptor half-life in these cells (9). Although not addressed here, this may also be the case in MCF-7 cells in which the receptor was found to accumulate in LAMP-2-positive structures as a complex with its ligands (Figs. 5 and 6). In addition to inhibiting receptor recycling, such prolonged complex formation may halt further proteolytic processing of proCD into mature CD and/or induce the degradation of the receptor-proCD complexes in lysosomes. Whether the accumulation of the receptor-enzyme complexes in late endosomes/lysosomes in MCF-7 cells represents their true end point or degradation or just a "bottle neck" in receptor recycling and reutilization in the TGN remains to be clarified in the future experiments.

The prevalence and the applicability of these findings to other CD-secreting cancer cell types remain to be clarified. However, the fact that one of three randomly selected colorectal cancer cell lines tested here was acidification-defective (CaCo-2) suggests that this defect may be more common than previously thought. In further support of this possibility, cells in solid tumors are known to be repetitively or continuously exposed to an acidic extracellular milieu due to hypoxia, altered energy metabolism, and a slow rate of waste removal (42, 43). In general, these changes give rise to a slightly more alkaline intracellular pH and a reversed pH gradient across cellular membranes. In addition, cytoplasmic alkalization or extracellular acidification have been implicated previously in several cancer-associated phenotypic alterations, including malignant growth, cellular transformation, altered expression of certain genes, acquisition of multidrug-resistance, and invasion and metastasis (23–26, 28, 44–48). Recently, we have also reported that such pH alterations in cancer cells may also be

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responsible for their lowered glycosylation potential (49). Collectively, these observations support the view that such pH alterations may be an inherent part of tumorigenesis and its progression. If true, the findings reported here emphasize the need to develop new approaches and interventions to restore normal acidification potential in cancer cells and to better control their invasive and metastatic growth. Our observation that artificial acidification was able to dissociate the preformed receptor-CD complexes in MCF-7 cells shows that such trials would be feasible.

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