Cathepsin Inhibition Prevents Autophagic Protein Turnover and Downregulates Insulin Growth Factor-1 Receptor–Mediated Signaling in Neuroblastoma

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ABSTRACT

Inhibition of the major lysosomal proteases, cathepsins B, D, and L, impairs growth of several cell types but leads to apoptosis in neuroblastoma. The goal of this study was to examine the mechanisms by which enzyme inhibition could cause cell death. Cathepsin inhibition caused cellular accumulation of fragments of the insulin growth factor 1 (IGF-1) receptor. The fragments were located in dense organelles that were characterized as autophagosomes. This novel discovery provides the first clear link between lysosomal function, autophagy, and IGF-1–mediated cell proliferation. A more in-depth analysis of the IGF1 signaling pathway revealed that the mitogen-activated protein kinase (MAPK) cell-proliferation pathway was impaired in inhibitor-treated cells, whereas the Akt cell survival pathway remained functional. Shc, an adapter protein that transmits IGF-1 signaling through the MAPK pathway, was sequestered in autophagosomes; whereas

IRS-2, an adapter protein that transmits IGF-1 signaling through the Akt pathway, was unaffected by cathepsin inhibition. Furthermore, Shc was sequestered in autophagosomes as its active form, indicating that autophagy is a key mechanism for down-regulating IGF-1-induced cell proliferation. Cathepsin inhibition had a greater effect on autophagic sequestration of the neuronal specific adapter protein, Shc-C, than ubiquitously expressed Shc-A, providing mechanistic support for the enhanced sensitivity of neuronally derived tumor cells. We also observed impaired activation of MAPK by epidermal growth factor treatment in inhibitor-treated cells. The Shc adapter proteins are central to transducing proliferation signaling by a range of receptor tyrosine kinases; consequently, cathepsin inhibition may become an important therapeutic approach for treating neuroblastoma and other tumors of neuronal origin.

Introduction

Neuroblastoma is the most common solid tumor of childhood and the most common cancer of infancy. Despite progress in the treatment of other pediatric cancers, such as lymphoma and leukemia, the survival rate for high-risk neuroblastoma remains less than 40% (Maris, 2010). Understanding the molecular pathways that are specifically involved in the survival and proliferation of neuroblastoma cells may provide insight into developing novel therapies.

A specific, irreversible inhibitor of cathepsins B and L, Fmoc-Tyr-Ala-CHN₂ (FYAD), induces cell-cycle arrest and selective apoptosis of neuroblastoma cell lines. The apoptosis and cell-cycle arrest was preceded by accumulation of dense multilayered vesicles (Colella et al., 2010). Appearance of these vesicles coincides with processing of LC-3 I to LC-3 II, a hallmark of autophagy (Cartledge et al., 2013). Furthermore, FYAD slowed the growth of tumors in mice, indicating that inhibition of cathepsins B and L may provide a new approach

to the treatment of neuroblastoma (Cartledge et al., 2013). Inhibitors related to FYAD have been shown to cause neuronal developmental defects in rodent embryos (Ambrosio and Harris, 1994), and genetic deletion of both cathepsins B and L causes neuronal degeneration in newborn mice (Felbor et al., 2002), but the inhibitors are well tolerated by mature mice, which show no evidence of neurotoxicity (Komatsu et al., 1986; Anagli et al., 2008; Desmarais et al., 2008; Doyle et al., 2010). At birth and in utero, rodent neuronal cells are still in a proliferative state, indicating that proliferating neuronal cells are uniquely sensitive to the inhibition of cathepsins B and L. In humans, the proliferative phase of neuronal development in the developing embryo is essentially complete by the second trimester of pregnancy; consequently, treating neuroblastoma with drugs that specifically target proliferating neuronal cells could be less toxic to the patient than conventional chemotherapy that targets all proliferation cells.

The mechanism by which cathepsin inhibition leads to neuroblastoma cell death is less clear. Induction of autophagy provides a mechanism by which tumor cells can survive in the hostile microenvironment of established tumors, where poor vascularization can result in nutrient deprivation and hypoxia. In addition, autophagy prevents excessive cell growth and proliferation, thereby reducing bioenergetic needs during

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ABBREVIATIONS: ALK, anaplastic lymphoma kinase; FYAD, Fmoc-Tyr-Ala-CHN₂; EGF, epidermal growth factor; IGF-1, insulin-like factor 1; LC-3, microtubule-associated protein 1A/1B-light chain; MAPK, p44/42 mitogen-activated protein kinase (or Erk1/2); PBS, phosphate-buffered saline.

nutrient deprivation (Rabinowitz and White, 2010). Autophagy is part of the mechanism that leads to cancer-cell dormancy, and its prevention may prevent formation of new dormant cancer cells and relapse (Amaravadi, 2008; Lu et al., 2008; White, 2012). Immunohistochemical analyses of several tumor specimens consistently show increased levels of the autophagy marker LC-3 in the center of tumors compared with normal tissue, correlating with tumor progression and poor patient prognosis, (Fujii et al., 2008; Miao et al., 2010). Thus, autophagy is generally considered a cancer-cell survival pathway rather than a step toward cell death, and its prevention may prevent cancer cell survival and dormancy; however, it is proteolysis of damaged proteins and cell organelles by lysosomal proteases during autophagy that provides the bioenergetic resources to allow cancer-cell survival; consequently, inhibition of the proteases would block this resource supply. Thus, accumulation of LC-3 II during cathepsin inhibition may be a marker of autophagy inhibition rather than induction (Tanida et al., 2005; Jung et al., 2015). Nevertheless, bioenergetic resources are unlikely to be limiting in cell culture, where cathepsin inhibition still results in accumulation of autophagic vesicle and cell death in neuroblastoma. Cancer cells of neural origin may be more sensitive to autophagy inhibition (Nixon, 2013).

In this study, we tested a hypothesis that cathepsin inhibition causes cell death in neuroblastoma by dysregulation of cell signaling pathways required for tumor cell growth and survival. Specifically, we examined the role of cathepsins in regulation of the insulin growth factor-1 (IGF-1) receptor signaling pathway. IGF-1 signaling is involved in proliferation of cells in the nervous system during development (Gould, 2007; Joseph D'Ercole and Ye, 2008; O'Kusky and Ye, 2012, and components of IGF signaling pathways are elevated in tissues from a variety of cancers, including neuroblastoma (Martin et al., 1992; Samani et al., 2007). IGF-1 stimulates growth and proliferation and prevents apoptosis of neuroblastoma cells in an autocrine and paracrine manner (Martin et al., 1993; Meghani et al., 1993; Singleton et al., 1996; Van Golen et al., 2000; Van Golen and Feldman, 2000), activating MAPK and Akt pathways (Kim et al., 1997; Kurihara et al., 2000). The growth stimulatory and prosurvival effects of IGF-1 signaling in neuroblastoma make IGF-1 receptor signaling an attractive target for developing therapies against neuroblastoma.

Materials and Methods

Cell Lines and Culture. SK-N-SH and IMR-32 cells were from American Type Culture Collection (Manassas, VA). NB-1691 cells were a gift from Peter Houghton (St. Jude's Children's Hospital, Memphis, TN). GM-11027 cells were from the Coriell Cell Repository (Camden, NJ).

All cell lines were maintained in minimum Eagle's medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% none essential amino acids (Mediatech Inc., Manassas, VA). IMR-32 cells were cultured in 75 cm² flasks and passaged upon reaching 80%–90% confluence every 3–4 days by mechanical disruption. Other cell lines were cultured in 100-mm tissue culture plates and passaged by treating with 0.25% trypsin (Mediatech Inc.) upon reaching 80%–90% confluence every 2–4 days. Cells were incubated at 37°C in a humidity saturated chamber containing 95:5; v/v air: CO₂.

Antibodies. Primary cathepsin B antibody was obtained from D. J. Buttle, University of Sheffield, UK (Buttle et al., 1988). Antibodies

against the following proteins were purchased from manufacturers as listed below: actin (Sigma-Aldrich, St. Louis, MO) (Zhou et al., 2005), calnexin (Millipore, Carrigtwohill, Ireland) (Methner and Mayfield, 2010), EGF receptor (Cell Signaling Technologies, Danvers, MA) (Kawahara et al., 2010), Grb-2 (Cell Signaling Technologies) (Benitez et al., 2011), IGF-1R (Cell Signaling) (Michels et al., 2013), IRS-2 (Cell Signaling) (Gao et al., 2014), Lamp-1 (Cell Signaling), LC-3B (Cell Signaling) (Tong et al., 2012), MAPK (Cell Signaling) (Michels et al., 2013), Akt (Cell Signaling), p-IGF-1R β (Cell Signaling), p-MAPK (Cell Signaling), p-Akt (Cell Signaling) (Michels et al., 2013), p-Shc A (Cell Signaling) (Krall et al., 2011), RAS (Cell Signaling), Shc A (Cell Signaling), N-Shc (BD Biosciences, San Jose, CA) (Tarr et al., 2002). The secondary goat fluor-568 anti-rabbit antibody was from Invitrogen. Antibodies were selected based on published data that showed reaction with proteins of the expected mobility in SDS-PAGE and reaction with the correct sized proteins was confirmed in this study.

Inhibitor and Growth Factor Treatment. FYAD (Bachem, King of Prussia, PA) and Pepstatin A (Sigma-Aldrich) stock solutions were made in ethanol at 2 mM and 1 mM concentrations, respectively. To treat cells with inhibitors, media was removed, and cells were washed once with phosphate-buffered saline (PBS). FYAD and Pepstatin A were diluted in media to final concentrations of 5 and 10 μ M, respectively. Vehicle controls with the same levels of ethanol were used. Treatment times were 72 hours unless noted otherwise.

Before growth factor treatment, medium was removed from cells and the cells were washed three times with PBS, followed by 4 hours of incubation with serum-free medium. The cells were then treated with 50 ng/ml IGF-1 or EGF (Cell Signaling Technologies) for 5 minutes. The medium was removed, and cells were put on ice in ice-cold PBS for 5 minutes. The PBS was then removed, and cells were homogenized in 1% Triton-X-100 in PBS containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich).

Immunoblotting. At the termination of experiments, cells were scraped, centrifuged, and washed three times with PBS, followed by lysis with urea lysis buffer (7 M urea, 2 M Thiourea, 1% CHAPS 30 mM Tris, pH 8.5) or 1% Triton-X-100 in PBS, containing 2 $\times$ protease and phosphatase inhibitor cocktail. Protein concentrations were subsequently determined, and samples with equal amounts of protein were made in 8 $\times$ sample buffer (0.2 M Tris, pH 6.8, 20% glycerol, 10 mM β mercaptoethanol, 10% SDS) and denatured by heating at 95°C for 3 minutes. Proteins were separated by SDS-PAGE using precast 10% (w/v), or 4%–15% (w/v) polyacrylamide gel in tris-glycine buffer (Biorad, Carlsbad, CA). The gels were then transferred onto a polyvinylidene fluoride membrane (Whatman International Ltd., Maidstone, England) using a wet transfer system in transfer buffer for 1.5–2 hours at 110–120 V. The membranes were then blocked for 1 hour at room temperature with blocking buffer [5% nonfat dry milk in Tris-buffered saline (TBS)] and incubated with primary antibody typically diluted at 1:1000–2000 in incubation buffer [5% bovine serum albumin in TBS/Tween 20 overnight at 4°C. The blots were then rinsed twice and washed three times with TBSTween 20 and then probed with appropriate secondary horseradish peroxidase–conjugated antibody for 1 hour at room temperature. The blots were washed and developed using enhanced chemiluminescent technology. Relative intensities were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunoprecipitation and Triton Fractionation. Cells were lysed in ice-cold lysis buffer (1% Triton-X-100 in PBS) containing 2 $\times$ phosphatase and protease inhibitor cocktail. The lysate was centrifuged at 10,000g for 15 minutes at 4°C to separate insoluble material. The Triton soluble fraction was transferred to a fresh tube, and the Triton insoluble fraction was rinsed twice with ice-cold PBS and dissolved in urea lysis buffer and subjected to immunoblotting. For immunoprecipitation, primary antibody was added to the Triton soluble fraction and incubated overnight at 4°C. Protein G beads (Thermo Scientific, Waltham, MA) were then added to vials, followed by 1 hour incubation at 4°C. The beads were then separated and washed, and the proteins solubilized in SDS-PAGE sample buffer, followed by immunoblotting.

Primary Antibody Conjugation. A primary antibody against LC-3 was conjugated to Alexa fluor 488 using Apex antibody labeling kit (Life Technologies, Carlsbad, CA) according to the manufacturer's guidelines. To remove small interfering buffer components, the antibody carrier buffer was replaced by PBS using centrifugal filter units (Millipore) before labeling. Antibody concentration and fluorescent labeling was measured by nano-drop 2000C spectrophotometer (Thermo Fisher Scientific) before and after labeling.

Immunofluorescent Staining. For colocalization studies, a sequential immunofluorescence staining procedure was needed when primary antibodies were from the same species. Cells were plated on glass coverslips in six-well plates at 50%–80% confluence. The next day, cells were treated with or without inhibitors for 18 hours. Then the media was removed and cells were washed thoroughly with ice-cold PBS, followed by fixation in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, CA) on ice for 30 minutes. The cells were then washed with ice-cold PBS and permeabilized with methanol at -20°C for 15 minutes. Cells were then washed and blocked in 10% goat serum in PBS for 1 hour at room temperature. The fixed cells were incubated with primary IGF-1R antibody overnight at 4°C . Cells were then washed and incubated with goat fluor-568 anti-rabbit secondary antibody. After washing, fixation with paraformaldehyde was repeated to prevent dissociation of antibody, followed by a wash and 1-hour incubation with 10 mM glycine to block autofluorescence from paraformaldehyde. Cells were then washed and incubated overnight at 4°C with the Alexa fluor488 conjugated LC-3 primary antibody in the presence of 4,6-diamidino-2-phenylindole. The double-labeled cells were

then washed and mounted in slow-fade mounting media (Molecular Probes, Eugene, OR). Samples were visualized by structured illumination microscopy on a Zeiss Elyra PS1 super-resolution microscope (Carl Zeiss Inc, Jena, Germany), and data were processed using Zen software (Applied Precision, Issaquah, WA). Further three-dimensional image processing was performed using Velocity Software (PerkinElmer Inc. Waltham, MA). The single labeling against LC-3 was performed using a similar procedure, eliminating the steps between the second paraformaldehyde fixation and mounting.

Percoll-Gradient Fractionation. Inhibitor-treated and -untreated cells were resuspended in ice-cold homogenization buffer (0.25 M Sucrose, 20 mM Tris, 10 mM EDTA, $2\times$ protease and phosphatase inhibitors, pH 7.4). Cells were broken in a Dounce homogenizer (Wheaton, Millville, NJ). The homogenate was then centrifuged twice at $1000g$ for 5 minutes to separate the nuclear pellet. The postnuclear supernatant was placed on the top of the Percoll gradient (35% Percoll; GE Healthcare, Uppsala, Sweden) on a 2.5 M sucrose cushion. Gradients were centrifuged at $35,000g$ for 1 hour in an angled rotor. Twelve fractions were then collected from each tube, and $100\ \mu\text{l}$ of each sample was mixed with SDS-PAGE sample buffer and heated to 95°C for 3 minutes. Samples were then centrifuged at $16,000g$ for 5 minutes, and the resulting supernatant was used for determination of protein concentrations with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Samples diluted with additional sample buffer to adjust protein concentrations and the separated on 4%–15% Tris-glycine gradient gels before Western blot analysis.

Fig. 1. FYAD and Pepstatin A induce accumulation of uncleaved active cathepsin B and accumulation of autophagic vesicles. (A) SK-N-SH cells were treated with protease inhibitors as described in *Materials and Methods*, and then whole lysates were harvested and subjected to immunoblotting for cathepsin B, LC-3, and actin, followed by densitometric analysis and quantification. Actin was used as loading control. Control levels of protein for each cell line were normalized to a value of 1.0 and compared with levels of proteins in treated cells (Student's *t* test, $*P < 0.05$, $**P < 0.005$). Error bars are S.D., and the error bar on the control is the average from the two cell lines. Data are from three separate experiments. (B) SK-N-SH cells were treated with inhibitors, and subjected to immunofluorescent staining against LC-3 (green) as described in *Materials and Methods*. The scale bar represents 1 μm .

Data Analysis. All images are representative of at least three separate experiments, and quantitative data show mean and S.D. Control and treated samples are compared using Student's *t* test, and significant differences are noted in the figures.

Results

Treatment of neuroblastoma cell lines SK-N-SH and IMR-32 with FYAD, an inhibitor of cathepsins B and L, causes cell-cycle arrest and apoptosis in a dose-dependent manner (Colella et al., 2010). Induction of apoptosis was specific to neuroblastoma cell lines and was not observed in other cancer cells and nonmalignant fibroblastic cell lines (Colella et al., 2010). Apoptosis and cell-cycle arrest was preceded by accumulation of dense autophagic vesicles within inhibitor-treated cells (Colella et al., 2010).

The molecular mechanism that leads to cell-cycle arrest and apoptosis of neuroblastoma cells treated with inhibitors of lysosomal enzymes is not known. Lysosomes are known to play important roles in degradation of cell-surface receptors, especially receptor tyrosine kinases. Receptor tyrosine kinases are key regulators of growth, proliferation, and death of many cell types, including cancer cells. The central hypothesis of this study is that cathepsin inhibitors disrupt receptor-mediated cell signaling, which this leads to death of neuroblastoma cancer cells. In this study, we explored the IGF-1 receptor signaling system as a model to determine how inhibition of lysosomal function can lead to death of tumor cells.

Cathepsin Inhibition Blocks Processing of Cathepsin B. SK-N-SH and IMR-32 cells were treated with vehicle

control or protease inhibitors for 72 hours as described in *Materials and Methods*. Whole-cell lysates were collected and subjected to immunoblotting. In untreated cells, two major forms of mature cathepsin B are detected that correspond to a single-chain active form of the enzyme and the heavy chain of a cleaved active form of the enzyme (Fig. 1A). Inhibition of cathepsins B, L, and D significantly decreased levels of the cleaved active form of cathepsin B in both cell lines (Fig. 1A), indicating that these enzymes are responsible for the processing/turnover of cathepsin B in these cells.

Cathepsin Inhibition Increases Cellular Levels of LC-3 II. In untreated SK-N-SH cells, the primary form of LC-3 corresponds to the uncleaved cytosolic form (LC-3 I), whereas in treated cells, the cleaved form that is conjugated to phosphatidylethanolamine (LC-3 II) and recruited to membranes to initiate the formation of autophagic vesicles is predominant (Fig. 1A). Accumulation of autophagic vesicles was confirmed by immunofluorescent staining for LC-3 (Fig. 1B). In the vehicle-treated cells LC-3 staining was diffuse, with a few small LC-3-positive granules, consistent with a predominantly cytosolic location of protein. In inhibitor-treated cells, staining was punctuated with some donut-like structures, consistent with the formation and accumulation of a high number of large autophagic vesicles (arrows, Fig. 1B).

Cathepsin Inhibition Results in Accumulation of Fragments of IGF-1 Receptor. After treatment of SK-N-SH and IMR-32 cells with cathepsin inhibitors, levels of full-length IGF-1 receptor did not show a biologically relevant change, but two fragments of IGF-1 receptor approximately 50 and 70 kDa appeared (Fig. 2A). The antibody recognizes the

Fig. 2. Two fragments of IGF-1 receptor accumulate in inhibitor treated neuroblastoma cell lines. SK-N-SH and IMR-32 cells were treated with protease inhibitors or the vehicle control as described in *Materials and Methods*, and then whole lysates were harvested and subjected immunoblotting with an antibody against the C-terminus of IGF-1 receptor β subunit, followed by densitometric analysis and quantification (A). Other neuroblastoma cell lines GM11027 and NB1691, along with SK-N-SH and IMR-32, were similarly treated and subjected to immunoblot analysis (B). 20 μ g protein was loaded onto each well, and each experiment was repeated three times (significantly different from control, **P* < 0.05 using Student's *t* test).

C terminus of the β -chain of the receptor; consequently, the fragments correspond to the cytosolic and possibly the trans-membrane portion of the receptor.

Two other neuroblastoma cells lines, GM11027 and NB1691, also showed accumulation of fragments of the IGF1-R after treatment with protease inhibitors, but to a lower extent (Fig. 2B). Accumulated fragments account for 40% of the total protein in SK-N-SH cells, 5% in IMR-32 and GM11027 cells, and less than 1% in NB1691 cells after 3 days of inhibitor treatment. In these experiments, treatment with FYAD and Pepstatin alone were compared with treatment with both inhibitors. Fragments appeared only when FYAD was added to cells, and Pepstatin resulted in the appearance of an additional higher-molecular-weight band. These results demonstrate that the lysosomal proteases play a direct role in turnover of the IGF1-R in these cells.

Cathepsin Inhibition Causes Accumulation of Activated Adapter Proteins. Neither total IRS-2 nor any of the Shc isoforms (p46, p52, p66) showed any significant changes on inhibitor treatment (Fig. 3A). Steady-state levels of RAS and Grb-2, which bridges between Shc and RAS, were also unchanged. Although the Grb-2 level in inhibitor-treated IMR-32 cells appears lower in the blot shown, the difference was not significant after quantitation of multiple replicates. Inhibitor treatment selectively leads to the accumulation of activated, phosphorylated forms of Shc (Fig. 3A). Further

quantification of phosphorylated Shc isoforms revealed that the predominant phosphorylated isoform that accumulates within inhibitor-treated cells to an almost 4-fold level is the p52 Shc. This finding is consistent with the known role of this form of Shc as a signal transducer between IGF-1 receptor and downstream pathways. IMR-32 cells showed a similar pattern of accumulation of activated Shc (Fig. 3A).

Cathepsin Inhibition Impairs the IGF-1R Signaling Pathway. Total levels of MAPK were not significantly affected by protease inhibition in either SK-N-SH or IMR-32 cells; however, steady-state levels of p-MAPK were decreased by a factor of almost 5 in both cell lines (Fig. 3B). Effects of protease inhibition on activation of Akt were less pronounced, and although there was a decrease of p-Akt in the blot shown for SK-N-SH cells, the level of total Akt was also decreased, and the difference was not significant after quantitation of multiple replicates (Figs. 3B and 4, B and D). Whereas reduced activation of MAPK is consistent with reduced growth and survival of inhibitor-treated cells, it is counterintuitive to find that this is accompanied by increased levels of p-Shc, which would be expected to lead to activation of MAPK.

Cathepsin Inhibition Increases Levels of Lysosomal Markers. Lamp-1, a marker of lysosomal membranes, increased almost 2-fold in inhibitor-treated cells, consistent with decreased lysosomal proteolysis (Fig. 3C). Calnexin, a marker of endoplasmic reticulum, also increased in inhibitor treated cells, but to a lower extent (Fig. 3C).

Cathepsin Inhibition Does not Prevent IGF-1 Activation of its Receptor. Using immunoprecipitation and Western blotting, it was discovered that cathepsin inhibitor treatment did not block IGF-1-stimulated phosphorylation of the full-length receptor in SK-N-SH cells (Fig. 4A). Quantification of levels of activated receptor relative to total receptor showed that activation of the receptor is not impaired by protease treatment (Fig. 4C). The fragments of the receptor seen by direct Western blotting of treated cells were not detected by the immunoprecipitation procedure (results not shown). Before immunoprecipitation, insoluble material was removed by centrifugation. Solubilization of proteins in these pellets with SDS-PAGE sample buffer followed by Western blot analysis revealed the fragments of the receptor (Fig. 4A, lower panels). These fragments are phosphorylated, and no increased phosphorylation was noted in samples from cells treated with IGF-1 (Fig. 4A, lower panels). Our standard cell protein solubilization technique using urea and thiourea prevents loss of these triton-insoluble proteins. Proteins in the cytoskeleton, in lipid rafts and in protein aggregates, have all been reported to be poorly extracted by Triton-X-100 (Vale et al., 1985; Fujita et al., 2007; Aureli et al., 2016). We are not aware of prior reports on accumulation of Triton-insoluble fragments of the IGF-1 receptor after protease inhibition.

Cathepsin Inhibition Impairs IGF-1 Activation of Downstream Effectors. Although steady-state levels of activated MAPK were reduced in inhibitor-treated cells, added IGF-1 was still able to stimulate receptor activation in inhibitor-treated cells, indicating that the IGF-1 signaling pathway may still be intact. SK-N-SH cells were treated with protease inhibitors followed by serum starvation and IGF-1 treatment, as described in *Materials and Methods*. In vehicle-treated cells, both MAPK and Akt were phosphorylated upon treatment with IGF-1; however, in inhibitor-treated cells, MAPK activation was inhibited by 60% (Fig. 4, B and D). By

Fig. 3. Effect of proteases inhibition on components of IGF-1 signaling pathway. SK-N-SH and IMR-32 cells were treated with protease inhibitors as described in *Materials and Methods*, and then whole-cell lysates were harvested and subjected to immunoblotting, followed by densitometric analysis and quantification from three separate experiments. Representative blots show mediators of the signaling pathway, IRS-2, Grb-2, RAS, Shc, and phospho Shc (A), activators in the pathway, MAPK and Akt (B), and organelle markers, Lamp-1 and calnexin (C). (* $P < .05$, ** $P < 0.005$, compared with control, Student's t test).

Fig. 4. Activation of IGF-1 receptor and its signaling pathway in response to protease inhibition. SK-N-SH cells were treated with protease inhibitors and/or IGF-1 and immunoprecipitated with anti IGF-1R antibody followed by immunoblotting with anti-IGF-1R and anti-phospho-IGF-1R antibody (A, upper panels). Proteins not solubilized in the Triton-X-100 immunoprecipitation buffer were precipitated by centrifugation, dissolved in urea lysis buffer, followed by immunoblotting with anti-IGF-1R and anti-phospho-IGF-1R antibody (A, lower panels). Similarly treated cells were harvested, and Triton-X-100 soluble proteins were blotted to show the total and activated MAPK and Akt (B). Quantitation of levels of activated full-length receptor relative to total receptor (C), activated MAPK to total MAPK, and activated Akt to total Akt (D) after IGF-1 treatment were determined from three separate experiments (significantly different from control, $**P < 0.005$ using Student's *t* test). Samples were normalized by setting untreated samples to 1.0.

contrast, phosphorylation of Akt was not significantly affected (Fig. 4, B and D).

Components of the IGF-1 Signaling Pathway Are Associated with Dense Organelles in Inhibitor-Treated Cells. The discovery that the C-terminal fragments of the IGF1 receptor were not soluble in Triton extraction buffer indicated that they are not likely to be associated with the full-length receptor on the cell membrane or in the cytosol of the cells. The Triton insoluble fraction was enriched for LC-3 II, whereas the lysosomal enzyme cathepsin B was mostly solubilized by Triton (Fig. 5). IRS-2 and total and activated forms of MAPK were also primarily solubilized by Triton. As found for the phosphorylated fragments of the IGF1-R, phosphorylated Shc was located primarily in the Triton-insoluble pellet (Fig. 5). This finding is consistent with the fragments of the receptor and activated Shc selectively accumulating with LC-3 II in autophagic vacuoles in inhibitor-treated cells.

To further clarify the subcellular localization of fragments of IGF-1R, LC-3 II, and phospho-Shc A in inhibitor-treated cells, a Percoll density gradient was used to fractionate cell components based on density, as described in *Materials and Methods*. Representative Western blots of fractions are shown in Fig. 6. The full-length IGF-1 receptor was clearly separated

from its fragments in the inhibitor-treated cells, with the fragments being in the dense fractions (Fig. 6A). LC-3 showed a similar distribution, with the LC-3 I colocalizing with the full-length receptor and LC-3 II colocalizing with the fragments of IGF1-R in the dense fractions (Fig. 6A). Cathepsin B was spread throughout the gradient but was more concentrated in the dense fractions, as would be expected for a lysosomal protein (Fig. 6A). In inhibitor-treated cells, levels that cleaved active cathepsin B were decreased, and levels of full-length active enzyme increased (Fig. 6A). IRS-2 and RAS also colocalized with LC-3 I and IGF1-R in the lighter fractions, consistent with a cytosolic or plasma membrane location of these proteins (Fig. 5A). No significant change was seen in the location of IRS-2 or RAS in cells treated with the cathepsin inhibitors (Fig. 6A).

Shc, Grb-2, PKB, and MAPK are distributed throughout the gradient, possibly owing to weak interactions with membranes of organelles of different densities; however, inhibitor treatment did not significantly alter this distribution (Fig. 6A). By contrast, the neuronal-specific adapter protein, N-Shc, is located primarily with soluble/plasma membrane proteins in untreated cells but shows a significant shift toward more dense fractions in inhibitor-treated cells (Fig. 6A). Phosphorylated p52 Shc is primarily located in dense fractions in

Fig. 5. Differential solubility of proteins of inhibitor-treated cells in Triton-X-100 lysis buffer. SK-N-SH cells with and without treatment with Pepstatin A and FYAD for 72 hours were lysed in cell lysis buffer (Triton-X-100 in PBS). After centrifugation, any insoluble material was solubilized in urea lysis buffer. Cells were also directly solubilized in urea lysis buffer. Equal quantities of protein were separated by SDS-PAGE and blotted with antibodies as indicated. In control cells, all proteins were solubilized by the cell lysis buffer (S). In treated cells, fragments of the IGF-1 receptor, LC-3 II, and phospho-Shc were located primarily in the insoluble material (I) that required urea lysis buffer for solubilization. All forms of the proteins were found in the total cell lysates (W). Similar results were found in three different experiments.

inhibitor treated cells, whereas in untreated cells the major phosphorylated form of Shc is p66 that is primarily located in less dense fractions (Fig. 6A).

Comigration in Percoll gradients indicates that proteins are located in subcellular sites that have similar densities but does not necessarily indicate that proteins are in the same subcellular compartments. To more closely examine the localization of LC-3 relative to the IGF1-R, we used structured illumination microscopy. Cells were treated with protease inhibitors or vehicle control and subjected to immunofluorescent labeling, as described in *Materials and Methods*. IGF-1 receptor showed endoplasmic reticulum and Golgi-like distribution pattern in both treated and untreated groups (Fig. 6B). LC-3 showed low-intensity, diffuse staining in the cytosol and a few very small granules in the vehicle-treated cells (Fig. 6B); however, in response to treatment, a great accumulation of several, large LC-3-positive granules were observed. Sequential Z sectioning as well as three-dimensional reconstruction was used to determine the relative localization of LC-3 and IGF-1 receptor. The level of resolution of this instrumentation revealed some images where IGF-1R colocalizes with LC-3 in the inhibitor-treated cells (Fig. 6B), but this was not observed in vehicle-treated cells, where large LC-3-positive vesicles are not present (Fig. 6B). Although this technique is not quantitative and the antibodies cannot distinguish between the

different molecular forms of LC-3 and receptor, the colocalization is consistent with LC-3 II and processed receptor colocalization as seen in Triton-insoluble fractions and dense particles of Percoll gradients.

Cathepsin Inhibition Impairs EGF Activation of Downstream Effectors. The effect of protease inhibition on the EGF receptor was examined, as it has an established role in several cancers, including neuroblastoma. SK-N-SH and IMR-32 cells were treated with inhibitors for 72 hours followed by immunoblot analysis using an antibody against the cytoplasmic domain of the receptor. The level of full-length EGF receptor did not show a biologically relevant change in either cell line (Fig. 7A); however, as seen for IGF-1-mediated signaling, cathepsin inhibition impaired EGF-mediated activation of MAPK but not activation of Akt (Fig. 7B).

Discussion

The first unexpected finding in this study was that fragments of the IGF-1 receptor accumulate in neuroblastoma cells when cathepsins are inhibited. The fragments correspond to the C-terminal cytosolic domain of the protein, which is located on the cytosolic face of the plasma membrane in the intact protein. This result indicates that the cytosolic portion of the receptor must gain access to the lysosomal compartment for degradation by cathepsins B, L, and D. FYAD alone induced the appearance of a 50-kDa fragment, and an additional 70-kDa fragment appeared with combination treatment of both FYAD and Pepstatin A. Thus, cathepsin D is not essential for the turnover of the IGF-1 receptor, but it can process the 70-kDa fragment to a 50-kDa fragment in the absence of cathepsin B and L activity. The cytosolic and transmembrane portion of the IGF-1 receptor β -chain contains 432 amino acids, indicating that most, if not all, of the cytosolic domain is normally degraded by lysosomal enzymes. Although it has been proposed that the proteasome is responsible for turnover of the IGF-1 receptor (Girnita et al., 2003; Carelli et al., 2006), we did not see accumulation of higher-molecular-weight ubiquitinated forms of the receptor in inhibitor-treated cells. The cleavage site that gives the 70-kDa fragment is predicted to be in the extracellular domain of the receptor, indicating that the initial processing is by either a lysosomal protease not affected by FYAD or Pepstatin A or a cell-surface/extracellular protease. The identity of the responsible protease(s) is not yet known. These results emphasize the important role of lysosomes in degradation of the cytosolic portion of the IGF-1 receptor in neuroblastoma cell lines.

FYAD may inhibit other cathepsins, and we have shown that a related compound inhibits cathepsin S in spleen tissue (Mason et al., 1989); however, we showed that the major cathepsins inhibited in neuroblastoma cells are cathepsins B and L (Colella et al., 2010). Furthermore, several inhibitors of cathepsins B and L with different mechanisms of enzyme inactivation cause neuroblastoma cell death, whereas inhibitors of the individual enzymes are not effective (Colella et al., 2010; Cartledge et al., 2013). Cathepsins have to be inhibited by at least 90% to cause cell death (Colella et al., 2010). Although attempts to reduce cathepsin levels sufficiently by siRNA technology were not successful, genetic deletion of both enzymes in mice specifically cause apoptosis of proliferating neuronal cells in mice (Felbor et al., 2002), consistent with our data on chemical inhibition of both proteases.

Fig. 6. Fragments of IGF-1 receptor are sequestered within autophagic vesicles of inhibitor-treated cells. SK-N-SH cells were treated with protease inhibitors or vehicle control. Cells were harvested, postnuclear fractions prepared, and organelles separated by Percoll density-gradient fractionation. Fractions were immunoblotted as described in *Materials and Methods*. Representative blots from one of three separate experiments are shown (A). Cells were sequentially stained by immunofluorescent labeling for LC-3 (green), and IGF-1 receptor (red) and subcellular compartments were examined by structured illumination microscopy and three-dimensional image analysis (B).

Percoll-gradient fractionation showed that fragments of the IGF-1 receptor were localized separate from full-length IGF-1 receptor, colocalizing with LC-3 II, and activated Shc A in dense organelles. The IGF-1 receptor fragments and Shc A were phosphorylated, indicating that both the IGF-1 receptor and a key adaptor protein are sequestered in autophagic vacuoles for degradation after activation. Thus, autophagy appears to play a role in downregulation of IGF-1-mediated cell proliferation. Other adaptor proteins, IRS-2, Grb-2, and RAS, did not cosegregate with autophagic vesicles, indicating that the sequestration mechanism is specific for Shc proteins. The primary isoform of ShcA sequestered within autophagic vesicles was p52, the form known to be the principal signal transducer (Okada et al., 1995; Ravichandran, 2001). Shc A is ubiquitously expressed, but in neuronal cells, expression is

greatest during development and is dramatically lower in the nervous system of newborns when the proliferative phase of neuronal development is complete (Sakai et al., 2000). Thus, targeting Shc A in neuroblastoma may not affect normal differentiated neuronal cells. Shc-C (Rai/N-Shc) is a neuronal-specific adaptor protein and may be a better target for cancers of the neuronal system (O'Bryan et al., 1996; Pelicci et al., 1996; Nakamura et al., 1998; Tanabe et al., 1998). Shc-C mRNA levels are elevated in most neuroblastoma cell lines, especially those with N-myc or anaplastic lymphoma kinase (ALK) amplification, and are particularly elevated in tumor samples from patients with a poor prognosis (Miyake et al., 2005, 2009; Terui et al., 2005). We showed that cathepsin inhibitor treatment causes a greater shift of Shc-C into denser organelles than seen for Shc-A, indicating that this neuronal-specific

Fig. 7. Cathepsin inhibition impairs EGF signaling in neuroblastoma cells. SK-N-SH cells and IMR-32 were treated with protease inhibitors and subjected to immunoblotting for EGFR (A). SK-N-SH cells were similarly treated with protease inhibitors, followed by EGF treatment as described in *Materials and Methods*, and subjected to immunoblotting against total and activated MAPK and Akt (B). Quantitative data from three separate experiments are shown in graphs below the immunoblots. ** $P < 0.005$.

adapter protein is more affected by cathepsin inhibition. Inhibition of Shc-C function is likely to prevent the function of several RTKs that are important for neuroblastoma cell proliferation, including IGF-1 receptor, ALK, and TrkB, resulting in selective sensitization and enhanced apoptosis of neuroblastoma cells. High levels of phosphorylated Shc-C (but not IRS-2 or PLC- γ), have been observed in stable complexes with ALK in neuroblastoma cell lines and tissue samples with ALK amplification, indicating that this RTK acts primarily through the Shc/MAPK proliferative pathway (Miyake et al., 2005; Miyake et al., 2009).

In contrast to higher levels of activated Shc in inhibitor-treated cells, levels of activated MAPK were significantly reduced. The activated Shc trapped in autophagic vesicles is not able to activate downstream cytosolic proteins such as MAPK, impairing proliferation in treated cells. Further activation of MAPK by IGF-1 was also greatly diminished in inhibitor treated cells. Inhibition of the Shc/MAPK activation appears to be specific as activation of Akt by IGF-1 was not impaired, consistent with the observation that cathepsin inhibition did not result in sequestration of IRS-2 or block phosphorylation of the intact IGF-1 receptor. Thus, cathepsin inhibition in neuroblastoma cells leads to accumulation of Shc in autophagolysosomes and impairs MAPK signaling, identifying a novel mechanism by which protease inhibitors can block cell proliferation and ultimately lead to tumor cell death (Fig. 8).

Initial preclinical animal studies and phase 1 and 2 clinical trials using antibodies that specifically inhibit the function of the IGF-1 receptor showed low toxicity, good tolerance, and improved patient outcome (Gualberto and Pollak, 2009); however, several phase 3 trials failed, partly owing to activation of alternative pathways that stimulated tumor regrowth

(Pollak, 2012b; Yee, 2012). Small-molecule inhibitors of the tyrosine kinase activity of the receptor appear to be more effective than the antibodies, possibly because of the inhibition of additional kinases (Dool et al., 2011; Pollak, 2012a,b). Reagents that target more than one signaling pathway may be more efficacious as they could prevent activation of alternative signaling pathways that enable tumor cell survival. Antibodies and small molecules show some efficacy against neuroblastoma, both in vitro and in vivo (Maloney et al., 2003; Houghton et al., 2010; You et al., 2014) but have not yet progressed to clinical trials in children. Cathepsin inhibition also blocks MAPK activation by EGF, indicating that impairment of the adaptor protein function of Shc can affect growth stimulation by other receptor tyrosine kinases. As seen for IGF-1, activation of Akt by EGF was not significantly affected by protease inhibition. Thus, although IGF1 signaling is impaired, it may not be the key mechanism that leads to cell death.

Chloroquine and related compounds are being studied as potential therapies for a variety of cancers, although the mechanism of action is not clear (Kimura et al., 2013). Chloroquine should have effects similar to those of FYAD and Pepstatin A as it raises the pH in lysosomes, impairing lysosomal function, including blocking proteolysis; however, chloroquine disrupts multiple pH-dependent processes, including iron transport, immune processes, and vesicular trafficking. Nevertheless, chloroquine has been used at high concentrations to treat malaria and is relatively safe.

A major challenge for in vivo efficacy in targeting cathepsin activity is delivery of sufficient drug to the tumor site. We showed that cathepsins B and L need to be inhibited by 90% or more to effect tumor cell death (Cartledge et al., 2013). Whereas chloroquine is able to completely inhibit lysosomal

Fig. 8. Role of cathepsins in autophagic turnover of receptor tyrosine kinase signaling. Receptor tyrosine kinase signaling stimulates cell survival and proliferation through the IRS/Akt and Shc/MAPK pathways (A). Endocytosis results in internalization of activated receptors, but signaling can continue (B). Capture of endosomes by autophagy disrupts signaling and in neuroblastoma cells selective traps Shc adapter proteins. IRS, Akt, and MAPK remain in the cytosol and are inactivated by either dephosphorylation or degradation by the proteasome (C). Fusion of autophagosomes with lysosomes enables digestion of Shc and the cytosolic portion of receptors by cathepsins B, D, and L in autophagolysosomes (D). Final degradation of the receptor can occur after dissolution of the internalized endosomal membrane (E). When cathepsins are inhibited, fragments of the IGF-1 receptor and Shc accumulate in the autophagolysosomes. In neuroblastoma cells, Shc-mediated MAPK signaling is impaired by cathepsin inhibition, identifying a novel mechanism by which protease inhibitors can block cell proliferation and ultimately lead to tumor cell death.

hydrolysis of lipoproteins in tissue extracts, it inhibits lysosomal hydrolysis by less than 60% in vivo (Stein et al., 1977). Furthermore, long-term treatment with chloroquine appears to increase lysosomal protease activity in mice after drug withdrawal (Ambroso and Harris, 1994). Thus, any potential benefits of chloroquine in cancer therapy may not be directly related to cathepsin inhibition.

FYAD is an irreversible inhibitor of cathepsins B and L; consequently, reactivation of proteases on drug withdrawal is not possible. The primary challenge is to develop drug formulations and treatment regimens that enable irreversible inhibitors to inhibit newly synthesized proteases to ensure tumor cell death in vivo (Cartledge et al., 2013). Nevertheless, lack of toxicity and negligible nonspecific interactions may make irreversible cathepsin inhibitors valuable compounds for the development as therapeutics to treat neuroblastoma and other nervous system tumors.

The discovery that cathepsin inhibition impairs Shc-mediated cell signaling provides alternative strategies to combine FYAD with drugs such as Sorafenib that inhibit kinases in the MAPK pathway (Wilhelm et al., 2008). A recent

study indicates that cathepsin inhibition may sensitize neuroblastoma to commonly used therapeutic agents (Gangoda et al., 2015). Agents with good safety profiles that target multiple growth-related pathways simultaneously should improve cancer treatments by preventing tumor cells surviving by activating alternative signaling pathways.

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Authorship Contributions

Participated in research design: Soori, Mason.
Conducted experiments: Soori, Lu.
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