A 14-kDa cathepsin L-derived carboxyl IGFBP-2 fragment is sequestered by cultured rat ileal crypt cells

Phillip V. Gordon, Jessica B. Paxton, John F. Kuemmerle, and Nena S. Fox. A 14-kDa cathepsin L-derived carboxyl IGFBP-2 fragment is sequestered by cultured rat ileal crypt cells. *Am J Physiol Gastrointest Liver Physiol* 289: G79–G87, 2005. First published February 10, 2005; doi:10.1152/ajpgi.00384.2004.—IGF-II gut drives fetal mucosal growth during gestation. IGF binding protein-2 (IGFBP-2) has a high affinity for IGF-II and tightly regulates IGF-II availability during fetal and early neonatal growth. We have previously demonstrated that glucocorticoids alter IGF homeostasis in the neonatal ileum, but the mechanism(s) by which this occurs is poorly understood. We hypothesized that dexamethasone alters proteolytic regulation of IGFBP-2 in ileal crypt cells. To test this, ileal crypt (ileal epithelial (IEC)-18) cells were cultured in serum-free media and used to study IGFBP-2 catabolism by immunohistochemistry, gene array analysis, and pharmacological perturbation with dexamethasone. In addition, isolated human IGFBP-2, IGF-II, and cathepsins B, D, and L were utilized for in vitro protease assays. We found IGFBP-2 to be highly abundant in IEC-18 culture, and sequestration of carboxyl IGFBP-2 antigen was seen within vesicular bodies of some cells. Dexamethasone significantly decreased the number of these cells and decreased IGFBP-2 in the media. On gene array analysis, cathepsin L’s message abundance was significantly increased by dexamethasone, and, by in vitro assay, cathepsin L created a 14-kDa carboxyl fragment that corresponded to the sole antigen detected in IEC-18 cell lysates as well as a 16.5-kDa fragment found in the media. The sequestered fragment size was formed preferentially when IGF-II was present, whereas the larger fragment size was formed preferentially when IGF-II was absent. Cathepsins B and D did not produce these fragments in vitro and were not detected in IEC-18 media. We conclude that dexamethasone alters IGFBP-2 catabolism through its effects on cathepsin L.

dexamethasone; insulin-like growth factor; ileum; gut development

FETAL GROWTH IS PREDOMINANTLY regulated by an autocrine-paracrine growth factor known as IGF-II (9, 33). Syndromes of fetal overgrowth result from the disruption of IGF-II homeostasis, either by overexpression of IGF-II (13, 15) or by decreased IGF-II degradation (23, 41a). We postulate that altered catabolism of IGF binding proteins (IGFBPs) might be a potential regulatory mechanism as well. The fetal gut is particularly dependent on IGF-II and is disproportionately affected in these fetal overgrowth syndromes. Gut crypt cells utilize IGF-II to sustain proliferation in the postnatal periods (3). This arrangement holds true throughout life, as IGF-II is also a crucial growth factor for gastrointestinal cancers (10, 30, 31).

IGFBP-2 has a high affinity for IGF-II and is coexpressed with IGF-II in the fetal gut (19, 24, 38, 41). While IGFBP-2 inhibits IGF-II-mediated proliferation in cell culture models (21, 22), there is no intestinal phenotype in IGFBP-2 knockout mice (where overgrowth of the fetal gut might have been anticipated) (40), suggesting that its effects on IGF homeostasis are complex and balanced.

IGFBP-2 has been found in a wide variety of vertebrates, including zebrafish, and is universally abundant in the developing gut (14). However, compared with mammalian IGFBP-2, zebrafish IGFBP-2 does not have the putative proteoglycan-binding site. This sequence is thought to mediate cell surface binding and is not found in any other IGFBP family member. Also, zebrafish IGFBP-2 does not exhibit cell membrane binding. The function of IGFBP-2 cell surface binding is not known, but it has been speculated that this capacity facilitates IGF-II-mediated signaling (20). IGFBP-2 has a highly specific affinity for heparin (an integral component of all proteoglycans) when IGF-II is bound to it, and this affinity is lost when IGF-II is absent (2, 8). Cell surface binding may be relevant to IGFBP-2 catabolism. IGFBP-2 has a relatively high affinity for IGF-II compared with other members of the IGFBP family, and cell surface proteolysis of IGFBP-2 should augment IGF-II availability to the IGF receptor (5, 39).

The IGFBP-2 antigen has been immunolocalized within the cytoplasm of colon cancer cells (28, 27). Because all IGFBPs have a signal peptide sequence that targets them for rapid excretion through the cell membrane, nascent IGFBPs are generally not visualized within the cytoplasm by immunolocalization. Therefore, we presumed that this localization pattern represented endocytosis of an IGFBP-2 fragment. Such a marker would offer unique opportunities for experimental exploration of IGF homeostasis at the cellular level.

Our laboratory has previously demonstrated that dexamethasone (Dex) shifts IGFBP-2 immunolocalization within ileal epithelial cells (IEC-18) of the newborn mouse (18), potentially providing a tool by which to modulate IGF homeostasis at the cellular level. Rat IEC-18 cells are a nontransformed lineage that can be cultured in serum-free conditions and demonstrate a pleiotropic response to Dex (32). We hypothesized that Dex would alter IGFBP-2 catabolism in IEC-18 culture through a specific protease(s).

Cathepsin L has been reported to be a specific endopeptidase for IGFBP-3, the IGFBP family member with the closest sequence homology to IGFBP-2 (42), prompting us to specu-
late that cathepsin L might also regulate IGFBP-2. This idea is further bolstered by the knowledge that secreted cathepsins can be transiently associated with the cell surface and have short half-lives (4, 6, 12), making them theoretically ideal for IGFBP regulation near the IGF receptor.

METHODS

Cell culture. Rat IEC-18 cells (American Type Culture Collection, Rockville, MD) from aliquots of passages 6–8 were grown to confluence in DMEM with 10% FBS and 0.01% insulin. The time period for complete epithelial cell confluence was 24–48 h. Confluent cells were incubated for 72 h in DMEM with 10–6 M Dex or fresh DMEM as control. After 72 h, the media were aspirated, and the cells were harvested for immunocytocchemistry or Western blot analysis. Alternatively, six human cell culture lines (Caco-2, KM-12, HCT-15, DLD-1, Colo 205, and SW 620 lineages) were cultured according to the supplier’s instructions (American Type Culture Collection) until confluent and were utilized for carboxyl IGFBP-2 immunolocalization or harvested for Western blot analysis.

Media washout experiments. To determine where cathepsin L was derived from (a soluble pool with a weak association to the cell surface or a covalently anchored pool), confluent cells were switched derived from (a soluble pool with a weak association to the cell surface or a covalently anchored pool), confluent cells were switched from conditioned DMEM with 10% FBS and 0.01% insulin to new surface or a covalently anchored pool), confluent and were utilized for carboxyl IGFBP-2 immunolocalization or harvested for Western blot analysis.

Western blots and gel densitometry. Media samples were collected for each condition after the 72-h incubation was complete. Loading samples for media were diluted 1:4 with loading buffer and run on 12% acrylamide gels. Cell lysate samples were collected from 150-mm dishes, washed with PBS, and recovered in 700 μl of lysis buffer by scraping the dish. Cells were spun down for 10 min in a prechilled centrifuge. The supernatant was diluted 1:4 with loading buffer and run on 12 or 15% acrylamide gels. For detection of IGFBP-2 fragments and cathepsin peptides, fresh media or lysates from control and Dex-treated dishes were harvested from 150-mm dishes and concentrated to minimum volumes with centricron tubes (YM-10 pore size, Millipore, Billerica, MA) in parallel, and these were then diluted 1:4 with loading buffer and run on 15% acrylamide gels. After the gel had been transferred onto a nitrocellulose membrane, 0.5% Ponceau S stain was applied to confirm equitable protein transfer across all lanes.

All anti-IGFBP antibodies used for Western blotting were goat polyclonal antisera generated against the COOH-terminus of each protein, except for the IGFBP-2 NH2-terminus monocal antibody, and all were obtained from the commercial stock available from Santa Cruz Biotechnology (Santa Cruz, CA). All of these antibodies have been used previously by the authors and react appropriately to positive controls derived from rodents (18). Western blot membranes were incubated in 5% milk block for 1 h at room temperature before primary antibody was applied for 1 h at room temperature. Primary antibody was washed off with TBS-Tween, and secondary antibody was placed on the membranes for 1 h and then washed again. Avidin-biotin complex (ABC) was placed on the membrane for 1 h and then washed and visualized with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) using radiographic film, as specified by the manufacturer.

Densitometry of radiographic films was performed with a Molecular Dynamics densitometer using Image Quant 5.0 (Amersham Pharmacia Biotech, Cambridge, MA). For paired comparisons between serum-free media (SFM)- and Dex-treated conditions, mean values for SFM were assigned values of 100%, and Dex-treated conditions were then calculated as a fraction of the control, and significance was determined by using a one-tailed t-test for unequivocal variance. For proteolytic fragment mapping, actual pixel volume values were recorded for each of the generated fragments in three separate experiments, and two-tailed paired t-tests were performed to test for the effects of IGF-II addition at fixed concentrations of cathepsin L. For all densitometric analyses, statistical differences were defined as a P value <0.05.

Gene array analysis. IEC-18 cells were treated and processed, as described in Cell culture. Total RNA was harvested for each treatment...
condition in triplicate experiments and then used for gene chip analysis per the manufacturer’s protocol (rat gene chip no. 230A, Affymetrix, Santa Clara, CA). Subset analyses were used to assess treatment effects on individual mRNA abundances within all available members of the cathepsin family, as well as three well-known glucocorticoid-responsive genes that were included as positive controls for induced expression (with significant treatment differences defined as a P value <0.05 in these subsets, using two-tailed paired t-test analysis, provided as part of the standard analysis by the University of Virginia Biomedical Research Facility and the Dept. of Health Evaluation Sciences; www.healthsystem.virginia.edu/internet/biomolec/microarray.cfm).

Immunocytochemistry. Immunocytochemistry was performed for IGFBP-2 as well as cathepsins B, D, and L. After being fixed in formalin overnight, the cells were washed in DIG buffer (4% 1 M Tris base, 6% 5 M NaCl, 16% 1 M Tris-HCl) five times and then put in blocking solution (1% wt/vol BSA in DIG buffer) for 1 h at room temperature. This was followed by a 1-h incubation with goat polyclonal primary antibody for IGFBP-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody was washed off with DIG × 5, and secondary antibody (donkey anti-goat; Jackson Immunoresearch Laboratories, West Grove, PA) was placed on the slides for 1 h. ABC (Elite Series, Vector Laboratories) was prepared and applied per the manufacturer’s instructions, washed as above, and visualized with diaminobenzidine solution (Sigma, St. Louis, MO). The slides were counterstained with hematoxylin and coverslipped.

In vitro assays for cathepsin L mediate proteolysis. Human IGFBP-2, IGF-II, and cathepsins B, D, and L were utilized to generate antigenic carboxyl fragments in vitro at a temperature of 37°C and an activation pH of 5.5 to minimize nonspecific protease activity at lower concentrations of protease. Parallel reactions were run with 10× and 100× concentrations to detect nonspecific protease activity in samples where endopeptidase activity was absent. All samples were assessed by Western blot using the same IGFBP-2 polyclonal antibody that was used for immunolocalization. Comparisons between proteolysis conditions with and without IGF-II (added in twofold molar excess to IGFBP-2) were also performed simultaneously. All experiments were repeated in triplicate, and representative Western blots were chosen for presentation.

Statistics. A P value of <0.05 was required for significance in all statistical tests. For one gel densitometry study, a one-tailed t-test for unequivocal variance was used to compare IGFBP-2 abundance in SFM vs. Dex conditions because the data were transformed, such that each SFM value equaled 100% and its paired Dex value was transformed into a percentage of that value. All other statistical analyses were performed with a two-tailed, paired t-test.

RESULTS

Characterization of the IGFBPs in IEC-18 cell culture by Western blot analysis. The abundances of IGFBP-1 through -6 in IEC-18-conditioned media was analyzed by Western blot

### Table 1. Gene array data illustrating the effect of dexamethasone on mRNA abundance within all available members of the cathepsin family that had at least one value above background (defined as 10)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SFM Value</th>
<th>Dex Value</th>
<th>P Value</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3,522.7</td>
<td>3,725.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>8.3</td>
<td>10.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2,705.8</td>
<td>3,348.8</td>
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<tr>
<td>E</td>
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<td>55.7</td>
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<td>H</td>
<td>50.9</td>
<td>62.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
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</tr>
<tr>
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<td>+1.42</td>
</tr>
<tr>
<td>Q</td>
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<td>15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>88.7</td>
<td>73.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid (Glc) responsive genes</td>
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<tr>
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<td>1,312.5</td>
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Values are presented as means of arbitrary units of luminescence from triplicate experiments with all P values ≤0.05 reported. SFM, serum-free media; Dex, dexamethasone. Three glucocorticoid responsive genes are also included as positive internal controls.
technique in both control and Dex-treated conditions in a minimum of three separate experiments, with a representative experiment shown for each (Fig. 1A). In this model, Dex treatment was used as a means to promote IEC-18 pleiotropisms associated with differentiation (32), as well as catabolic alterations of IGFBP abundances (7, 29), thereby permitting us to discern IGFBPs with constitutive metabolism vs. those with more dynamic regulation. IGFBP-1, -3, -4, and -6 were not detected in the media. IGFBP-5 was equally detected in both control and Dex-treated conditions, whereas IGFBP-2 was highly abundant in both conditions but was diminished with Dex treatment compared with controls. IGFBP-2 also had a

Fig. 3. Immunolocalization and Western blots of cathepsins B, D, and L. A: representative Western blots detecting cathepsins B, D, and L in concentrated media and concentrated cell lysates from SFM- and Dex-treated IEC-18 cell cultures. Cathepsins B and D are detected in lysates but not in media, whereas L is detected in the media but not in the lysates. B: immunolocalization of cathepsin L in SFM- and Dex-treated cells following prolonged (10 min) detection with avidin-biotin complex horseradish peroxidase substrate. Two populations of cells are evident. A darker stained population is evident as a central strand in the SFM condition, whereas surrounding cells are less intensely stained in a pattern that is typical of prolonged staining. In the Dex-treated condition, this staining pattern persists, but the degree of staining on the cell strand is less intense, and there are fewer cells in the strand, reminiscent of the effect of Dex on C2 cells. C: double labeling of IEC-18 cells for C2 and cathepsin antigens (C2-positive and -negative cells are separated by one another by the dotted line). These studies reveal that cathespin L-stained cells and C2-positive cells are the same. In contrast, cathepsins B and D stain both C2-positive and -negative cells. The circles indicate comparable examples in double-labeled images, where a homogenous patch of C2-negative cells can be compared for staining among each of the three cathepsins.
IGFBP-2 FRAGMENT SEQUESTERED IN IEC-18 CELLS

much larger band size compared with IGFBP-5, suggesting the likelihood of greater abundance. We note that, on these gels, IGFBP-2 and IGFBP-5 do not comigrate (not shown), ruling out the possibility of cross reactivity as a confounder. Densitometric comparisons from the same three experiments demonstrate that there is a 15% reduction in IGFBP-2 abundance with Dex treatment \( (P < 0.05, \text{Fig. 1B}) \).

A 14-kDa carboxyl-terminal fragment of IGFBP-2 is found in IEC-18 cell lysates. We noted a single, small-sized, anti-carboxyl IGFBP-2-positive band in IEC-18 cell lysates, despite the absence of whole IGFBP-2. In contrast, no comparable bands were noted for IGFBP-5 (data not shown). To better characterize the IGFBP-2 antigen seen in cell lysates, IEC-18 cell lysates and media were concentrated, and Western blots were performed using 15% acrylamide gels to facilitate resolution of potential carboxyl antigenic fragments (Fig. 1C). To create a nomenclature for these fragments, we named them all with the prefix “C,” because they were carboxyl antigens, and then either 1 (for media), 2 (for IEC-18 lysates), or 3 (for colon cancer cell lysates), followed by a lowercase alphabetical designation in order of descending size if there was more than one of substantial prominence. In the media, we found whole IGFBP-2 and three proteolytic fragments of 18 kDa (C1a), 16.5 kDa (C1b), and 15 kDa (C1c) in size. We were unable to detect whole IGFBP-2 in the concentrated lysates with the anti-COOH-terminus antibody but found a 14-kDa size fragment as the sole detectable COOH-terminus antigen (C2). However, with an anti-NH2-terminus antibody and femtomolar sensitivity, we were able to identify a thin band of whole IGFBP-2 in cell lysates (Fig. 1C). In contrast, the IGFBP-2 NH2-terminus antigen is not detected in the media. This intriguing finding indicates that the anti-NH2-terminus antibody is specific for nascent protein and illustrates a posttranslational modification that obliterates the antigen with secretion (likely proteolytic clipping of the NH2-terminus signal sequence).

Characterization of C2 antigen in IEC-18 cell culture by immunocytochemistry. To further characterize the C2 fragment, we examined its cellular localization pattern by immunocytochemistry (Fig. 2). Our laboratory has previously demonstrated that staining by this anti-carboxyl IGFBP-2 antibody is highly specific and is abolished by blocking peptides, but we had not been able to identify the size of the intracellular antigen (18). We found that C2 is discretely localized in a vesicular pattern within the cytoplasmic space of some IEC-18 cells but was not localized in cells that flatten down and expand their cytoplasmic volume (which we called C2-negative cells for the purpose of this paper). This pattern of intracellular localization for IGFBP-2 antigen was unchanged by Dex treatment, although fewer cells appeared to have it. Based on these observations, we quantified the number of C2-positive and C2-negative cells in each treatment condition (Fig. 2) and found that Dex significantly decreased the number of C2-positive cells but did not change the total cell density, consistent with its ability to alter cell phenotype. We then used Dex treatment as a means to alter cell composition and then identify the protease responsible for generating C2 by gene array analysis.

Gene array analysis of cathepsin mRNA abundance following Dex treatment. To screen for cathepsins that might be involved in the formation of IGFBP-2-carboxyl fragments (C1s and C2), we utilized gene array subset analysis to approximate the change in mRNA abundance with Dex treatment for all available members of the cathepsin family (Table 1). We found that cathepsin L was the only family member with a significant increase in mRNA abundance (Table 1). We note that transcriptional changes do not necessarily equate to changes in protein abundance when the product is capable of autolysis and has a short half-life, so we also investigated cathepsins at the protein level.

Cathepsin immunochemistry and double labeling with anti-IGFBP-2 antibody. To further characterize the cathepsin family, we chose the three cathepsins that have been most commonly investigated within tissues of the distal intestine (1, 11, 36, 37) and coincidentally had the three highest mean mRNA abundances on our gene array analysis and performed Western blot and immunolocalization studies for each. Western blots of cathepsins B and D revealed each to be present in cell lysates, but neither was appreciably detected in concentrated media in either treatment condition (Fig. 3A). In contrast, cathepsin L was detected in concentrated media in both treatment conditions but was not detected in lysates and appeared to be more abundant with Dex treatment. We were unable to detect cathepsin L in unconcentrated media samples, despite aggressive protease inhibition (data not shown), confirming our suspicion that quantification of cathepsin L would be problematic.

Immunolocalization of cathepsin L required prolonged detection to optimize visualization of cell surface staining (10 min of ABC reaction), consistent with low-protein abundance. This revealed increased localization on strands of cells, reminiscent of those that sequester C2, whereas less staining was seen upon the surface of the surrounding cells (Fig. 3B). This pattern persisted but was less robust after Dex treatment, suggesting decreased cell surface association as well as a decrease in the number of robustly stained cells. To confirm that cathepsin L preferentially associates with the cell surface of C2 sequestering cells, double-labeling experiments were performed with anti-IGFBP-2 immediately followed by overlay with either anti-cathepsin B, D, or L (Fig. 3C). These...
experiments revealed that cathepsin L and IGFBP-2 stain the same cells. In contrast, cathepsins B and D showed light staining of all cells. These findings are consistent with a preferential cell surface association between cathepsin L and C2-positive cells. There are several reports in the literature that demonstrate cathepsin L cell surface membrane binding to be a weak association (25, 26). Our next step was to test this possibility in our model.

C2 fragment formation is dependent on the presence of conditioned media. To determine whether cathepsin L is weakly associated with the cell surface, media washout experiments were performed to dilute the soluble pool of cathepsin L, and then cells were allowed to sit in new media for 3 h. Western blot assessments of C2 uptake in cell lysates revealed that no C2 was endocytosed but that whole, nascent-sized IGFBP-2 was contained in lysates instead (Fig. 4). This simple experiment confirms that cathepsin L is only weakly associated with the cell surface. In other experiments, to determine whether genotypic variations in IGFBP-2 proteolysis and endocytosis exist, six colon cancer cell lineages were screened for the presence of intracellular COOH-terminus IGFBP-2 antigen localization. Two cell lines, Colo 205 and SW 620, were found to have the most intense intracellular staining (data not shown), and cell lysates from these cultures were utilized for IGFBP-2 Western blots (Fig. 4). Colo 205 exhibited whole IGFBP-2 in their lysates, and the majority of this was found to stain with the anti-NH2-terminus antibody, indicating that these cells retain nascent IGFBP-2. One possible interpretation of this finding, since these cells do not contain the C2 fragment and thus presumably do not have cathepsin L-dependent cleavage at the cell surface, is that cathepsin L is also required for cleavage of the signal peptide sequence. In contrast, lysates from the colon cancer cell line known as SW 620 contained two COOH-terminus fragments smaller than C2. These find-

Fig. 5. Western blots of human IGFBP-2 alone or with variable concentrations of cathepsin L (A), cathepsin B (B), and cathepsin D (C). These same assays were also run in the presence or absence of IGF-II (present at twice the molar quantity of IGFBP-2) to determine the effect of ligand binding on cleavage efficiency. Finally, IEC-18 cell lysates and conditioned media were run on the same gels for comparisons of fragment sizes. We find that at 1X cathepsin L, the 16.5-kDa fragment [the “C1b”-sized fragment (C1b-in vitro)] predominates but that IGF-II inhibits cleavage at this site. At 10X, the 14-kDa fragment [the “C2”-sized fragment (C2-in vitro)] is the predominant product, and IGF-II augments cleavage at this site. Additionally, C2-in vitro corresponds to the 14-kDa IGFBP-2 antigenic fragment in IEC-18 cell lysates, and C1b-in vitro corresponds to the predominant fragment found in conditioned media. Finally, 100X cathepsin L results in total loss of detectable antigen, demonstrating that cathepsin L also has weak hydrolase activity at this pH. Cathepsin B has little or no endopeptidase activity but, at 100X concentration, also acts as a nonspecific protease when IGF-II is present (but not in its absence). Cathepsin D has no detectable IGFBP-2 protease activity in these assays.
findings suggest that there are additional sites of proteolysis that can be utilized without disrupting endocytosis of the COOH-terminus fragment.

**In vitro assays of cathepsin L proteolysis of IGFBP-2.** To determine whether cathepsin L could utilize IGFBP-2 to generate any of the COOH-terminus-sized fragments discovered in vivo, we performed in vitro protease assays using commercially available stocks of human IGFBP-2, with and without IGF-II, using either cathepsin B, D, or L, and then analyzed them by IGFBP-2 Western blots (Figs. 5 and 6). We found that, at low concentrations, cathepsin L predominantly produced a carboxyl fragment identical in size to that of C1b (C1b-in vitro) but also produced a small amount of the fragment identical in size to C2 (C2-in vitro). Production of both fragments was significantly diminished when IGF-II was added in a 2:1 ratio to that of IGFBP-2, indicating that ligand binding reduces the efficiency of proteolysis at both sites when cathepsin L is present in low abundance. In contrast, at a 10-fold higher concentration of cathepsin L, the 14-kDa carboxyl fragment became the predominant fragment, and C1b-in vitro was present only in trace amounts. Also in contrast, the presence of IGF-II significantly enhanced IGFBP-2 degradation at 10×, so ligand binding retards C1b-in vitro cleavage at low cathepsin L concentrations but drives the reaction toward C2-in vitro cleavage when cathepsin L approaches a threshold level. Cathepsin L showed nonspecific protease activity at the 100× concentration in the absence of IGF-II, but, in the presence of IGF-II, a trace amount of novel-sized cleavage product was detected. Interestingly, this 13-kDa band approximated C3b identified in SW 620 cells. Cathepsin B also showed nonspecific proteolysis at the 100× concentration but, in contrast, was only evident when IGF-II was present. Cathepsin D showed no protease activity in any of the conditions.

**DISCUSSION**

We have characterized IGFBP-2 catabolism within IEC-18 cell culture. Our results suggest that cathepsin L is the dominant protease for IGFBP-2 in this model, producing alternate cleavage products at the cell surface vs. the media. IGFBP-2 catabolism is responsive to Dex, which increases cathepsin L transcription, liberates cathepsin L from the cell surface, and significantly reduces IGFBP-2 within the media. However, our proteolysis assays suggest that the sum of these events may be to reduce a subset of IGFBP-2 that does not have IGF-II bound to it because production of the corresponding-sized cathepsin L-derived fragment (C1b-in vitro) is inhibited by the addition of IGF-II. Thus the net effect of Dex may be to selectively attenuate the abundance of empty IGFBP-2 within the extracellular milieu.

We found that C2-positive cells preferentially associate with cathepsin L on their cell surface compared with adjacent cells by immunolocalization and that cathepsin L produces a C2-sized fragment in vitro. These findings suggest that cathepsin L is capable of, and strategically placed for, C2 production. In contrast to C1b, this reaction is enhanced by the addition of IGF-II in vitro, and we note that IGF-II is known to enhance IGFBP-2 heparin binding (2), which we postulate could be the biochemical equivalent of IGFBP-2 proteoglycan binding on the cell surface. This raises the intriguing possibility that cell surface cleavage of IGFBP-2 might provide a mechanism for sustained recruitment of IGF-II to the cell surface (as C2 is actively sequestered coincident with cleavage, thereby removing a potential competitor for IGFBP-2 cell surface binding).

This arrangement appears to be perturbed in at least two colorectal cell lines (Colo 205 and SW 620), where C2 is not sequestered but alternate-sized carboxyl antigens of IGFBP-2 are, suggesting that cathepsin L function is abnormal. In support of this, cathepsin L has been demonstrated to wane with colorectal tumor progression (35, 37). In addition, an alternate isoform of cathepsin L, known as cathepsin L2, which is normally only expressed in thymus and testis, has been found to have widespread expression in colorectal cancers (34). The significance of this finding is unknown, but we speculate that cathepsin L2 might generate alternate carboxyl fragment sizes as found in SW 620.

There are some potential limitations to our study. First, despite several attempts, we have been unable to obtain definitive Edman sequencing on the C2 and C1b fragments and cannot be certain that each fragment size is the result of a single cleavage site. In fact, each attempt has yielded mixed sequences from gel-purified, in vitro-derived samples, suggesting either low fidelity or splintered cleavage sites and/or limited NH2-terminal proteolysis in the resulting fragments. In either case, both cathepsin L cleavage sites are likely to lie within the lysine-glutamate-rich region of the IGFBP-2-variable domain (based on the carboxyl fragment sizes and the fact that a similar cathepsin L site for C1b exists in IGFBP-3) (33, 42). Second, our culture data must still be considered circumstantial in regard to their developmental relevance, because IGFBP-2 has not been demonstrated to affect fetal growth. While our findings do point toward a compelling model for mucosal growth regulation, point mutation analyses in IGFBP-2 will be required to precisely identify the cathepsin L cleavage sites and to confirm their mechanistic significance.
In summary, cathepsin L-mediated proteolysis of IGFBP-2 encompasses two potential mechanisms for regulating IGF-II availability in IEC-18 cells. One of the resulting fragment sizes (C2) is actively sequestered, and we speculate that it represents IGFBP-2 on the cell surface that supplies IGF-II to its receptor. A second cathepsin L-derived fragment size (C1b) is generated from soluble IGFBP-2. This latter cleavage event may represent selective attenuation of excess IGFBP-2, ensuring that the majority of the IGFBP-2 pool has IGF-II bound to it. The presence of dual-cleavage sites for a single endopeptidase is an intriguing finding in IGFBP evolution because each IGFBP family member has endured divergent selection pressure for IGF homeostasis that is contextually driven one way at the cell surface and another in the extracellular milieu. Our findings suggest that mammalian IGFBP-2 appeased these selection pressures by forcing a cleavage “choice” based on whether or not IGF-II ligand is present and whether or not a given cathepsin L protease is soluble or cell surface associated. Finally, our colon cancer survey data suggest that systematic study of lineages with mutations in IGFBP-2 catalobism will further our understanding of IGF-II homeostasis at the single-cell level.

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