Degradation of IGF-binding protein-3 by proteases in cultured FRTL-5 rat thyroid cells

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Abstract

In this study, we have found that IGF-binding protein-3 (IGFBP-3) in calf serum added to tissue culture medium is degraded by cultured FRTL-5 cells and a major 31 kDa fragment of IGFBP-3 is produced. When FRTL-5 rat thyroid cells were cultured in 6H medium (modified F-12M medium containing TSH, insulin, hydrocortisone, somatostatin, transferrin, and glycylyl-histidyl-lysine) containing 5% calf serum, both 44–46 and 31 kDa IGFBPs were found in conditioned medium by ligand blot analysis using 125I-labelled IGF-II. However, predominantly the 44–46 kDa IGFBP was detected in unconditioned 6H medium containing 5% calf serum. When calf serum in the media was replaced by human serum similar results were obtained, and the 44–46 kDa and 31 kDa IGFBPs were recognized using a human IGFBP-3 antibody following Western blot analysis. FRTL-5 cells secreted only small amounts of an endogenous 29 kDa IGFBP, thought to be IGFBP-5.

To separate the 31 kDa fragment of IGFBP-3 from the endogenous IGFBP-5, culture media were fractionated by concanavalin-A–Sepharose chromatography and aliquots of both flow-through and eluate from the column were analyzed by ligand blotting. A 31 kDa IGFBP was found in the eluate fractions from concanavalin-A–Sepharose chromatography following the separation of conditioned 6H medium supplemented with calf serum, suggesting that this species was an N-linked glycoprotein and could be derived from the degradation of serum IGFBP-3 by FRTL-5 cells.

Using a modified zymographic assay, we examined whether the degradation of IGFBP-3 could depend on the cell membrane. Confluent FRTL-5 cells were washed with PBS and overlaid with liquid agarose solution. After the agarose had solidified, unconditioned 6H medium containing 5% calf serum was incubated with the cells at 37°C for 16 h. Both 44–46 and 31 kDa IGFBP species were found in the overlying, conditioned medium by ligand blot. However, the 31 kDa IGFBP was not found in medium in the absence of FRTL-5 cells, and no IGFBP could be found in serum-free conditioned medium from agarose-covered FRTL-5 cells. This suggests that the 44–46 kDa IGFBP-3 in serum was degraded to yield a 31 kDa fragment, while any endogenous IGFBP-5 could not pass out of the agarose. The degradation of 44–46 kDa IGFBP-3 in the modified zymographic assay was inhibited by phenylmethylsulfonyl fluoride, EDTA, and aprotinin, but not by leupeptin. In summary, these results indicated that IGFBP-3 in calf serum added to culture medium could be degraded by FRTL-5 cells and that this may involve calcium-dependent serine proteases.

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Introduction

Insulin-like growth factors (IGFs) in co-operation with thyroid-stimulating hormone (TSH) regulate the growth and function of thyroid cells (Tramontano et al. 1986, Maciel et al. 1988, Roger et al. 1988, Williams et al. 1988, Becks et al. 1992). However, IGFs in serum and other extracellular fluids and in cell culture media are associated with members of a family of six different IGF–binding proteins (IGFBPs) which have been characterized and are designated as IGFBP-1 to -6 (Rechler 1993). The effects of IGFs on thyroid cell growth and function are modulated by IGFBPs (Frauman et al. 1989, Phillips et al. 1994). The degradation of IGFBPs by proteases could allow IGFs to be released from IGFBPs and to act on their target cells (Rechler 1993, Jones & Clemmons 1995, Zapf 1995). Specific proteases which degrade IGFBP-3 have been described in pregnancy sera (Hossenlopp et al. 1990, Davenport et al. 1990, 1992a, Guidice et al. 1990), and have also been shown to be released from cultured cells (Fowlkes & Freemark 1992, Grimes & Hammond 1994, Lalou et al. 1994). Previous studies have shown that calf serum and IGF-I or insulin have similar effects on TSH-stimulated iodide uptake and thymidine incorporation by
FRTL-5 cells (Zakaria & McKenzie 1989). However, most IGFs in serum are bound to IGFBP-3 in association with an acid-labile subunit to form 150 kDa complexes (Kechler 1993). It is not known whether IGFBP-3 in calf serum is degraded by FRTL-5 thyroid cells, thereby releasing IGFs which could, in turn, cooperate with TSH to regulate FRTL-5 cell growth and function. The following studies were conducted to address this question.

Materials and Methods

Materials

FRTL-5 cells (F2 sub-clone) were kindly provided by Dr Leonard D Kohn, Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD, USA. Materials were purchased from the following sources: transferrin, bovine insulin, bovine TSH, hydrocortisone, glycy1-histidyl-lysine, somatostatin, fatty acid-free BSA, Dulbecco’s phosphate buffer (PBS) powder, Tween-20, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, Coon’s modified F-12 medium powder, non-essential amino acids, and ExtrAvidin peroxidase staining kit from Sigma Chemical Co., St Louis, MO, USA; recombinant human IGF-I and IGF-II from Bachem Inc., Torrance, CA, USA; Na[I25I] (IMS30) from Amersham International, Mississauga, Ontario, Canada; pre-stained molecular weight standards for SDS-PAGE, and nitrocellulose membranes (0.2 µm) from Bio-Rad Laboratories (Canada) Ltd, Mississauga, Ontario, Canada; and Centricon microconcentrators (10 kDa cut-off) from Amicon Corporation, Beverly, MA, USA. Calf serum, trypsin, and collagenase were purchased from Gibco BRL, Burlington, Ontario, Canada. Concanavalin-A-Sepharose was purchased from Pharmacia Fine Chemical Corp, Ronkonkoma, NY, USA. Polyclonal antiserum against purified recombinant human (h) IGFBP-3 was purchased from Upstate Biotechnology Incorporation (UBI), Lake Placid, NY, USA. Pooled human serum was prepared from two normal healthy adult male volunteers.

Cell culture

FRTL-5 cells were cultured and passaged as described by Ambesi-Impomiato & Perrild (1989). Cells were cultured in 6H medium (F-12 medium containing TSH (0.1 IU/ml), hydrocortisone (0.4 µg/ml), transferrin (5 µg/ml), somatostatin (10 ng/ml), insulin (10 µg/ml), and glycy1-histidyl-lysine (10 ng/ml) and also containing 5% calf serum). The medium was changed every 3 days. The conditioned media from near-confluent cells were collected and stored at −20 °C until analysis by ligand blot. Cells between four and ten passages were used in these studies.

Iodination of IGFs

Recombinant hIGF-I or hIGF-II were iodinated with Na[I125I] to specific activities of 120–215 µCi/µg, using the chloramine T procedure as previously described (Hill et al. 1989).

Ligand blot

Conditioned media were analyzed by ligand blot as previously described (Wang et al. 1990). In brief, 60 µl culture medium were mixed with 20 µl of 4 × Laemmli sample buffer and separated on 10% SDS–polyacrylamide gels under non-reduced conditions. The proteins in the gel were transferred to nitrocellulose membranes by electroblotting, which were further incubated with 125I-labelled IGF-I or 125I-labelled IGF-II. The nitrocellulose membrane was washed with Tris buffer and then exposed to X-ray film for 24–72 h at −80 °C.

Western blot

6H media containing 5% calf serum, or 5% human serum, were analyzed by Western blot as previously described (Wang et al. 1990). In brief, 60 µl culture medium were mixed with 20 µl of 4 × Laemmli sample buffer and separated by 10% SDS-PAGE before proteins in the gel were transferred to nitrocellulose membranes as described for ligand–blot analysis. The nitrocellulose membrane was incubated with 1:1000 dilution of a rabbit polyclonal antibody against recombinant hIGFBP-3 for 16 h at 4 °C, and, after washing with PBS, was further incubated with biotinylated goat anti-rabbit IgG (1:1000) for 2 h at 23 °C. The complexes of IGFBP-3 and its antibody were visualized using freshly prepared 3,3’-diaminobenzidine tetrahydrochloride (1:89 mm containing 0.03% (v/v) of hydrogen peroxide). The reaction was quenched by washing in excess 50 mm Tris buffer.

Concanavalin-A–Sepharose chromatography

Since IGFBP-3 is an N-glycosylated protein whereas IGFBP-5 is an O-glycosylated protein, concanavalin-A–Sepharose chromatography was used as previously described to separate these two IGFBPs in the culture medium of FRTL-5 cells (Wang et al. 1990). In brief, a concanavalin-A–Sepharose column (bed volume 6 ml) was equilibrated with TBS buffer (Tris 50 mm, NaCl 1 mm, and CaCl2 1 mm, pH 7.4) and 2 ml fractions were collected. Two millilitres of conditioned or unconditioned 6H medium containing 5% calf serum were loaded on the column. After incubation at 25 °C for 30 min, non-N-glycosylated proteins were washed out from the column with 40 ml TBS buffer and referred to as ‘flow-through’. TBS buffer (50 ml) containing 0.5 µM α-methylmannoside was added to the column to elute N-glycosylated proteins.
Aliquots of both flow-through and eluate were examined by competitive charcoal-binding assay to determine the presence of \(^{125}\text{I}\)-labelled IGF-II-binding activity (Wang et al. 1990). Fractions containing binding activity for \(^{125}\text{I}\)-labelled IGF-II were separately pooled and concentrated on Centricon microconcentrators (molecular weight cut-off 10 kDa). Aliquots from each pool were analyzed by ligand blot using either \(^{125}\text{I}\)-labelled IGF-I or \(^{125}\text{I}\)-labelled IGF-II.

**Detection of IGFBP-3 proteolytic activity in conditioned medium of FRTL-5 cells**

To examine whether serum-derived IGFBP-3 could be degraded by proteases in the conditioned medium from FRTL-5 cells, bovine serum (3 µl) was incubated with either 60 µl serum-free 6H conditioned medium or 60 µl 50 × concentrated serum-free 6H conditioned medium at 37 °C for 16 h. The incubation was mixed with 20 µl Laemmli sample buffer and analyzed by ligand blot as described above. We also used \(^{125}\text{I}\)-labelled recombinant human IGFBP-3 as the substrate to examine the activity of IGFBP-3 proteases in the culture medium. \(^{125}\text{I}\)-Labelled recombinant human IGFBP-3 was incubated with serum-free conditioned 6H medium. After the incubation was separated by SDS-PAGE, the gel was dried and exposed to X-ray film to visualize the degradation products.

**Modified zymographic assay**

To examine whether the calf serum-derived IGFBP-3 in culture medium of FRTL-5 cells was degraded by membrane-associated proteases, a modified zymographic assay was used (Granelli-Piperno & Reich 1978). Near-confluent FRTL-5 cells were washed three times with PBS. A 1-25% agarose solution in water was boiled before use and maintained at 42 °C in a water bath. Warmed agarose solution was overlaid on the cell layer of a 24-well tissue culture plate (0.15 ml/well), or in 10 cm tissue culture plates (3 ml). After the agarose solidified, 6H medium (0.15 ml/well for 24-well tissue culture plates or 3 ml/10 cm tissue culture plate), with or without 5% calf serum, was added to the agarose-covered FRTL-5 cells and incubated at 37 °C for 16 h. Aliquots of 60 µl were collected from the incubation medium and mixed with 20 µl 4 × Laemmli sample buffer before analysis by ligand blot. To characterize the proteases which could degrade serum IGFBP-3, agarose-covered FRTL-5 cells in a 10 cm tissue culture plate were incubated with 3 ml 6H medium containing 5% calf serum for 16 h at 37 °C. Aliquots of 60 µl were collected from the incubation after 1, 3, 6, and 9 h, mixed with 20 µl 4 × Laemmli buffer to stop the reaction, and stored at −80 °C until analyzed by ligand blot. 6H medium containing 5% calf serum was incubated in an Eppendorf tube at 37 °C for 16 h as the control. To examine the action of specific proteases, protease inhibitors PMSF (4 mM), aprotinin (7.4 U/ml), EDTA (100 mM), and leupeptin (1 mg/ml) were also included in the incubations of agarose-covered cells with culture media.

**Results**

A 31 kDa IGFBP was found in conditioned medium of cultured FRTL-5 cells

When unconditioned 6H medium containing 5% calf serum was analyzed by ligand blot, a doublet of 44–46 kDa IGFBP was found (Fig. 1, lane B). In contrast, in addition to this 44–46 kDa species, an abundant 31 kDa IGFBP was found in 6H medium containing 5% calf serum conditioned by FRTL-5 cells, together with minor species of 34 kDa and 21–23 kDa, while the abundance of the 46 IGFBP was decreased (Fig. 1, lane C). A barely visible 31 kDa IGFBP, previously identified as IGFBP-5 (Backejauw et al. 1993), was found in conditioned serum-free 6H medium (Fig. 1, lane A). Abundant IGFBP-5 could only be detected by ligand blot when the serum-free medium was concentrated 25–50 times (results not shown). These results indicate that the calf serum-derived 44–46 kDa IGFBP was partly degraded and a major 31 kDa IGFBP was produced following culture with FRTL-5 cells. A species with molecular weight greater than 112 kDa, possibly representing soluble IGF-II receptor (Westlund et al. 1991), was also found by ligand blot (Fig. 1, lanes B and C).

**The 46 kDa and 31 kDa IGFBPs in conditioned 6H medium supplemented with calf serum represent IGFBP-3**

When calf serum in 6H medium was replaced by human serum, similar results were obtained by ligand blot (Fig. 2). Only the major 44–46 kDa IGFBP was seen in non-cell-conditioned medium containing 5% human serum (Fig. 2, lanes A and B) or 5% calf serum (Fig. 2, lane C) following ligand blot. Following incubation with FRTL-5 cells, however, 31 kDa, 34 kDa, and 21 kDa IGFBPs were also seen in the conditioned medium supplemented with human serum (Fig. 2, lanes D and E) or calf serum (Fig. 2, lane F). Both 44–46 kDa and 31 kDa IGFBPs derived from human serum were also recognized by a polyclonal antibody against recombinant hIGFBP-3 following Western blot (Fig. 3). The 46 and 31 kDa IGFBPs in calf serum were visualized less well than were the hIGFBP species (Fig. 3, lane A vs lanes B and C), since the hIGFBP-3 antibody has a very weak cross-activity with IGFBP-3 in calf serum. Other proteins with different molecular sizes were also bound by the hIGFBP-3 antibody, but were not capable of binding \(^{125}\text{I}\)-labelled IGF-I or IGF-II following ligand blot (Fig. 2).
Figure 1 Ligand blot analysis of conditioned media from FRTL-5 cells. Near-confluent FRTL-5 cells were cultured in 6H medium with (lane C) or without (lane A) 5% calf serum for 72 h. The conditioned media and unconditioned 6H medium containing 5% calf serum (lane B) were analyzed by ligand blot using 125I-labelled IGF-II. Molecular weight markers are shown to the left. Results are representative of three similar experiments.

The 31 kDa IGFBP-3 in the cultured medium from FRTL-5 cells can be separated from endogenous IGFBP-5 by concanavalin-A–Sepharose chromatography

The competitive charcoal assay showed that fractions of both flow-through and eluate from concanavalin-A–Sepharose columns contained IGFBPs, suggesting that both non-N-glycosylated and N-glycosylated IGFBPs were present in conditioned medium of FRG-5 cells (Fig. 4). Although the same amount of unconditioned and conditioned medium was separately loaded on concanavalin-A–Sepharose columns, the binding activity of IGFBPs in fractions of both flow-through and eluate was increased in comparison with IGFBPs in unconditioned medium analyzed in the same experiment. This could be due to the secretion of endogenous IGFBP-5 by FRTL-5 cells and to the fact that more binding sites were exposed after IGFs were released from IGFBPs. As shown in the ligand blot performed with the resulting fractions, the abundance of the 31 kDa IGFBP in flow-through fractions for conditioned 6H medium supplemented with 5% calf serum was increased in comparison with that from the serum-containing unconditioned 6H medium, suggesting that this species could contain the endogenous IGFBP-5 (Fig. 5, lanes C, D, E, and F in (b) vs lanes C, D, and E in (a)). However, a 31 kDa IGFBP was also found in fractions of eluate from the concanavalin-A–Sepharose column following the separation of conditioned 6H medium supplemented with 5% calf serum. (Fig. 5, lanes G, H, I, and J in (b) vs F, G, H, and I in (a)). This species, therefore, was probably an N-glycosylated protein and could be a fragment derived from the degradation of the 46 kDa

Figure 2 Ligand blot analysis of conditioned media from FRTL-5 cells. Near-confluent FRTL-5 cells were cultured in 6H medium containing 5% human serum (lanes D and E) or calf serum (lane F) for 72 h. The unconditioned medium containing 5% human serum (lanes A and B) or 5% calf serum (lane C), and the conditioned media (lanes D, E, and F) were analyzed by ligand blot using 125I-labelled IGF-II. Molecular weight markers are shown to the left. Results are representative of three similar experiments.
IGFBP-3 from calf serum in the presence of FRTL-5 cells. Since fractions of the flow-through and eluate from concanavalin-A-Sepharose column were pooled and concentrated by Centricon filtration, protein loading in these fractions was considerably higher than in Figs 1 and 2. The appearance of serum-derived IGFBPs in non-cell-conditioned medium showed the existence of minor species with molecular weights of 34, 31, 28, and 21 kDa (Fig. 5, lane A in (a) and (b)).

Degradation of the IGFBP-3 in serum depends on co-incubation with FRTL-5 cells

Ligand blot analysis showed that the relative abundance of the 31 kDa IGFBP was not increased if serum-free 6H medium was conditioned by FRTL-5 cells, removed, and subsequently incubated with calf serum in the absence of cells (Fig. 6, lane D). This suggests that degradation of IGFBP-3 in serum was unlikely to be due primarily to the release of soluble protease by FRTL-5 cells. Very little IGFBP was seen in serum-free conditioned medium (Fig. 6, lane C). However, in the same experiment a relative increase in the 31 kDa IGFBP and decrease in 44-46 kDa
Figure 5 Ligand blot of fractions from concanavalin-A-Sepharose chromatography for culture medium from FRTL-5 cells as described in Fig. 4. (a) Fractions 1–2 (lane C), 3–5 (lane D), 6–8 (lane E), 19–20 (lane F), 21–23 (lane G), 24–26 (lane H), and 27–29 (lane I) from the column profiles for unconditioned medium and (b) fractions 1–3 (lane C), 4–6 (lane D), 7–9 (lane E), 10–13 (lane F), 22–24 (lane G), 25–27 (lane H), 28–30 (lane I), and 31–33 (lane J) for conditioned medium were separately pooled and concentrated. Aliquots from each pool were analyzed by ligand blot using IGF-II. Conditioned (lane B) and unconditioned (lane A) 6H media containing 5% calf serum were used as control. The molecular weight markers are shown to the left. Results are representative of three similar experiments.

IGFBP occurred when serum-containing medium was in contact with FRTL-5 cells (Fig. 6, lane B vs lane A). Conditioned 6H medium from FRTL-5 cells was also incubated with \(^{125}\)I-labelled recombinant hIGFBP-3, and analyzed by SDS electrophoresis to examine the degradation of the radiolabelled IGFBP-3. However, \(^{125}\)I-labelled IGFBP-3 itself separated on SDS gel with an apparent molecular weight of approximately 31 kDa, making it unsuitable as an experimental tool to examine whether 31 kDa IGFBP in conditioned medium of FRTL-5 cells was derived from the degradation of 43 kDa IGFBP-3 (data not shown).

Degradation of IGFBP-3 in calf serum could be demonstrated by a modified zymographic assay

The relative abundance of the 31 kDa IGFBP was increased in the medium following the incubation of agarose-covered FRTL-5 cells with 6H medium containing 5% calf serum (Fig. 7, lane C vs lane A). In contrast, no IGFBP could be found following the incubation of agarose-covered FRTL-5 cells with serum-free 6H medium (Fig. 7, lane D), suggesting that the endogenous IGFBP-5 produced by the cells did not penetrate the agarose into the culture medium. This may be due to the
strong affinity of IGFBP-5 for cell membrane-associated molecules such as glycosaminoglycans. As a control, both unconditioned and conditioned 6H media containing 5% calf serum from FRTL-5 cells without agarose were analyzed within the same ligand blot. As expected, the abundance of 31 kDa IGFBP was substantially increased in the conditioned 6H medium supplemented with 5% calf serum compared with that in unconditioned 6H medium containing 5% calf serum (Fig. 7, lane B vs lane A). Ligand blot analysis also showed that the 31 kDa IGFBP species was not a major species when 6H medium containing serum was simply incubated in the absence of cells at 37°C for 16 h (Fig. 8, lane A) but was increased following the incubation of agarose-covered FRTL-5 cells with 6H medium containing calf serum at 37°C for as little as 1 h (Fig. 8, lane B). These data suggest that the 31 kDa IGFBP species is primarily derived from the degradation of serum IGFBP-3 by FRTL-5 cells.

The cell-mediated degradation of IGFBP-3 is inhibited by protease inhibitors

When the protease inhibitors, PMSF, EDTA, and aprotinin were included in incubations of agarose-covered FRTL-5 cells with 6H medium containing 5% calf serum, ligand blot analysis showed that the 31 kDa species was relatively decreased in abundance compared with incubation without protease inhibitors (Fig. 9, lanes E, F, and G vs lane C). Cell morphology and attachment remained as in control cultures. The abundance of the 31 kDa IGFBP was not obviously changed when leupeptin was included in the incubation (Fig. 9, lane H vs lane C). However, no IGFBP was found in medium from agarose-covered FRTL-5 cells with serum-free 6H medium (Fig. 9, lane D). As a control, unconditioned and conditioned 6H media supplemented with 5% calf serum were also analyzed within the same ligand blot. Abundant 31 kDa IGFBP was found in conditioned 6H medium supplemented with 5% calf serum but was less abundant in...
unconditioned medium (Fig. 9, lane B vs lane A). These results indicate that the degradation of IGFBP-3 by cultured FRTL-5 cells could be inhibited by PMSF, EDTA, and aprotinin, but not by leupeptin.

Discussion

In this study, we found that the 44-46 kDa IGFBP-3 present in calf serum was degraded and a 31 kDa fragment produced following culture with FRTL-5 cells. The 44-46 kDa and 31 kDa IGFBPs in calf serum likely represent IGFBP-3 since they are identical to the reported size of hIGFBP-3 and the 44-46 kDa doublet following SDS-PAGE is also characteristic of IGFBP-3 (Rechler 1993).

It is possible that the 31 kDa species of IGFBP generated by incubation with FRTL cells could consist, in part, of endogenous IGFBP-5 released by the cells. Backejaw et al. (1993) reported that FRTL-5 cells secreted IGFBP-5 with a molecular weight around 30 kDa, but the release of IGFBP-5 was very low since abundant endogenous IGFBP-5 could only be detected by ligand blot when the conditioned medium was concentrated 25-50 times. They also reported that only IGFBP-5 mRNA, but not that of any other IGFBP, could be found in FRTL-5 cells, suggesting that IGFBP-1, -2, -3, -4, and -6 were not synthesized. It is possible that endogenous IGFBP-5 and the 31 kDa fragment derived from the degradation of serum IGFBP-3 in cultured FRTL-5 cells could both be components of the abundant 31 kDa species of IGFBPs detected by ligand blot in our studies. However, we found that endogenous IGFBP-5 could be separated from 31 kDa IGFBP-3 using concanavalin-A-Sepharose chromatography, since IGFBP-3 is an N-glycosylated protein and IGFBP-5 is an O-glycosylated protein (Rechler 1993, Jones & Clemmons 1995).

Fragments of IGFBP-3 with an approximate molecular weight of 30 kDa have been found in serum. For example, a 29 kDa N-linked glycosylated IGFBP was found in the 150 kDa IGFB complex in rat serum, suggesting that this species could be derived from IGFBP-3 (Yang et al. 1989). A 31 kDa fragment of IGFBP-3 was found during the purification of IGFBPs from human and rat serum (Shimonaka et al. 1989, Zapf et al. 1990). A 30 kDa fragment of IGFBP-3 was produced when IGFBP-3 was degraded in human and rat pregnancy serum, and in the serum of patients after elective surgery (Davenport et al. 1990, 1992a, 1992b, Giudice et al. 1990, Hossenlopp et al. 1990). The degradation of IGFBP-3 in pregnancy serum was inhibited by protease inhibitors, PMSF, EDTA, and aprotinin (Davenport et al. 1992b, Hossenlopp et al. 1990). Furthermore, a 30 kDa fragment of IGFBP-3 was also produced when bovine serum was incubated with human and rat pregnancy serum or with uterine homogenates (Davenport et al. 1992a). These observations suggest that the 31 kDa IGFBP-3 fragment is derived from the degradation of intact IGFBP-3. IGFBP-4 is also an N-glycosylated protein and has a molecular weight around 30 kDa. However, concentrations of IGFBP-4 and other species of IGFBPs including IGFBP-2, -5, and -6 are very low in serum (Rechler 1993, Jones & Clemmons 1995).

IGFBPs in the cellular environment are degraded by different proteases, which could be regulated by a complex mechanism. In this study, we found that calf serum-derived IGFBP-3 was not degraded following incubation with serum-free conditioned medium from FRTL-5 cells, suggesting that physical contact with the cells could be required for degradation. Using a modified zymographic assay and ligand blot analysis, we found that cell membrane could be involved in the degradation of IGFBP-3. The zymographic assay was used previously to analyze plasminogen activators (Granelli-Piperno & Reich 1978) and matrix metalloproteinase (Sato et al. 1994), and was also used to examine the degradation of IGFBP-3 in conditioned medium from dermal fibroblasts (Fowlkes et al. 1994). We found that a 31 kDa IGFBP was still generated following the incubation of agarose-covered FRTL-5 cells with medium containing 5% calf serum. It is unlikely that the 31 kDa IGFBP was derived from cell membrane-associated IGFBPs, since no IGFBP could be found in the
Degradation of IGFBP-3 by proteases · J F WANG and others

conditioned medium from agarose-covered FRTL-5 cells incubated with serum-free medium. The cell-dependent degradation of IGFBP-3 was inhibited by EDTA and PMSF, suggesting that calcium-dependent serine proteases could be responsible. Since FRTL-5 cells were overlaid with agarose before incubation with calf serum in culture medium, IGFBP-3 could be degraded by the membrane-associated proteases or by proteases which are activated by a membrane-associated apparatus. The proteases which degrade IGFBP-3 in human and rat pregnancy serum, and in the serum of patients after elective surgery, were also shown to be calcium-dependent serine proteases (Giudice et al. 1990, Hossenlopp et al. 1992a, b). Fowlkes et al. (1994) further found that the degradation of IGFBP-3 in rat pregnancy serum and in cultures of dermal thyroid cells was inhibited by the specific tissue inhibitor for metalloproteinase-1 (TIMP-1), suggesting that metalloproteinases were involved in the degradation of IGFBP-3. Matrix metalloproteinase-2 has also been detected by immunohistochemistry in human neoplastic thyroid tissues (Campos et al. 1992) and mRNA of inhibitor of TIMP-2 has been found in transformed rat thyroid cells (PCC13 epithelia cells) (Santoro et al. 1994). However, metalloproteinases are secreted as inactivatedzymogen and the activation of metalloproteinases in both physiological and pathophysiological conditions is not completely known (Senior & Shapiro 1992). Whether serum-derived IGFBP-3 is degraded by metalloproteinases in cultured FRTL-5 cells is under further investigation by us.

IGFBPs could also be degraded by other proteases. For example, IGFBP-3 was degraded by plasminogen activator/Plasminogen activator in cultured porcine ovarian granulosa cells and in cultured human osteosarcoma cells (Grimes & Hammond 1994, Lalou et al. 1994). Both granulosa cells (Liu et al. 1987) and osteosarcoma cells (Fawthrop et al. 1992) secrete plasminogen activators, which could trigger the plasmin cascade. Plasminogen activators were found in cultured sheep and porcine thyroid cells (Mak et al. 1984, Degryse et al. 1993). However, Cassano et al. (1989) reported that fully differentiated FRTL-5 cells did not produce detectable amounts of plasminogen activator or plasminogen activator inhibitors. Therefore, it is unlikely that the serum IGFBP-3 was degraded by FRTL-5 cell-produced plasminogen activator and plasmin in the present experiments. It has also been reported that lysosomal protease cathespin D could degrade IGFBPs (Brulke et al. 1995). Whether IGFBP-3 could be degraded by lysosomal proteases derived from FRTL-5 cells is under investigation.

In summary, the results in these studies indicate that IGFBP-3 in calf serum added to medium is degraded by calcium-dependent serine proteases in cultured rat FRTL-5 cells, and a 31 kDa fragment of IGFBP-3 is produced. Since IGFs are bound to IGFBPs in serum and in the extracellular interstitial environment, the degradation of IGFBPs could modulate the bioavailability of IGFs and thereby contribute to the regulation of thyroid growth and function by IGFs. Further identification and characterization of these proteases and their regulation should help to elucidate endocrine, paracrine, and autocrine actions of IGFs in the thyroid gland.

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