Insulin-like growth factor-2 (IGF-2) is expressed in most embryonic tissues and is required for normal development during gestation. After birth IGF-2 expression is extinguished in most tissues, but the gene is often reactivated during tumorigenesis. Tumors secrete high molecular weight forms of IGF-2 that result from aberrant post-translational processing of pro-IGF-2. As a first step toward understanding how high molecular weight IGF-2 peptides might contribute to tumor progression, we have characterized the biosynthesis of IGF-2 in a human embryonic cell line. We have found that pro-IGF-2 can initially form two disulfide isomers that undergo rearrangement to a single conformation in vivo. The addition of N-acetylgalactosamine to Ser71, Thr72, Thr75, and Thr139 likely occurs in the cis-Golgi apparatus. Sialic acid addition begins in the trans-Golgi apparatus, but IGF-2 peptides must reach the trans-Golgi network for oligosaccharide maturation to be completed. Endoproteolysis occurs concomitant to or slightly after oligosaccharide maturation. Cleavage was observed only at Arg104, resulting in the secretion of IGF-2-(1-104) and free E-peptide. Proteolysis required basic residues in the P1 (Arg104) and P4 (Arg101) positions, was completely blocked by a furin inhibitor, and was enhanced by coexpression with furin, PACE4, PGCA, PC6B, and LPC. These data suggest that members of the subtilisin-related proprotein convertase family mediate processing of pro-IGF-2 at Arg104. We did not detect the IGF-2 peptides that are most abundant in normal serum, mature IGF-2, and IGF-2-(1-87), in this expression system, which indicates that novel endoproteases are responsible for generating these products.

Gene disruption studies in mice indicate that insulin-like growth factor-2 (IGF-2) is required for normal embryonic development. IGF-2 knockout mice are born 40% smaller than wild-type littermates but have normal postnatal growth rates, are fertile, and have average life spans (1). In rodents, IGF-2 gene expression is extinguished in all tissues except the chondral plexus and leptomeninges after birth (2), while hepatic IGF-2 expression persists in adult humans from a liver-specific promoter (3). A number of mechanisms exist to inhibit inappropriate postnatal IGF-2 activity. These include silencing of maternal IGF-2 gene expression through genomic imprinting (4), inhibition of gene expression by tumor suppressor genes (5), and removal of IGF-2 from the circulation and extracellular space by the IGF-2/mannose-6-phosphate receptor (6).

It has been known for some time that many tumors overexpress IGF-2 (7). A recent comparison of the expression profiles of more than 300,000 transcripts derived from about 45,000 genes in normal and tumor tissue from human patients revealed that less than 1% of the genes had altered expression levels in the neoplastic state. Of the genes that were up-regulated in tumors, two alternatively spliced IGF-2 transcripts showed the greatest increase (8). IGF-2 has been shown to be required for tumor progression in a mouse model of oncogene-induced tumorigenesis (9). Overexpression of IGF-2 in tumors has been attributed to loss of imprinting and mutations in tumor suppressor genes (5, 10).

In addition to displaying deregulated IGF-2 gene expression, post-translational processing of the 156-amino acid IGF-2 precursor is abnormal in tumors. IGF-2 was originally isolated from human serum and found to be a 67-amino acid peptide (11), now referred to as mature IGF-2. Recent mass spectrometric analysis of IGF-2 peptides isolated from normal human and bovine serum identified an 87-amino acid peptide that is present in at least 12 forms which vary in the amount of O-linked carbohydrate they contain (Fig. 1) (12, 13). Many investigators have characterized high molecular weight forms of tumor-derived IGF-2 by gel electrophoresis or column chromatography. While the exact composition of these peptides is unknown, it is clear that they differ in size from IGF-2 produced by normal tissues (14–18). In one patient with non-ileal cell tumor hypoglycemia associated with mesothelioma, production of altered forms of IGF-2 was attributed to changes in glycosylation (18).

The question of whether abnormal peptides generated by aberrant processing of pro-IGF-2 in tumor cells are more potent than mature IGF-2 at stimulating tumor progression is unresolved. Before this question can be answered it will first be necessary to define the products generated from post-translational processing of pro-IGF-2. The goal of the present study was to characterize human pro-IGF-2 biosynthesis, including identification of sites of endoproteolysis and glycosylation, in a human embryonic cell line that was previously shown to process pro-IGF-1 to mature IGF-1 (19, 20).
**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The human prepro-IGF-2 cDNA (kindly provided by Dr. Graeme Bell) was amplified by polymerase chain reaction to introduce HindIII and XbaI sites upstream of the initiator methionine and downstream of the chain termination codons, respectively. The polymerase chain reaction product was cloned into the corresponding site of the ReCMV2 expression vector (Invitrogen) to generate ReCMV-IGF-2, and the insert was sequenced to confirm the fidelity of the polymerase chain reaction amplification. Site-directed mutants, pro-IGF-2-FLAG and IGF-2-R104-FLAG were generated using the Quik Change mutagenesis kit (Stratagene). All mutants were verified by DNA sequencing. Expression vectors for pro-IGF-IA, furin, PACE4, PC6A, PC6B, and LPC were described previously (19, 20).

**Cell Culture and Transfections**—Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum and 100 units/ml penicillin, 100 units/ml penicillin plus 100 μg/ml streptomycin. Cells were transfected in 6-cm dishes with 6 μg of DNA using calcium phosphate (Life Technologies, Inc.). For stably transfected cell lines, 293 cells were co-transfected with an SV40-neomycin plasmid (Promega) and selected for resistance to G418. Resistant colonies were expanded and evaluated for expression of the desired protein by immunoprecipitation from conditioned medium and/or cell lysates, and by immunostaining of cells grown in chamber slides.

**Cell Labeling, Inhibitor Studies, Immunoprecipitation, and Gel Electrophoresis**—36 h after transfection, cells were washed twice with phosphate-buffered saline and incubated for 1 h with cold labeling medium (cysteine-free or methionine-free Dulbecco’s modified Eagle’s medium with 1 mg/ml bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin). The medium was then replaced with fresh cold labeling medium containing 100 μCi/ml [35S]cysteine or 100 μCi/ml [35S]methionine (Amer sham Corp.; 100 Ci/mmol) and the incubation was continued for 24 h unless stated otherwise. For studies with the inhibitor decanoyl-RVKR-chloromethylketone (dec-RVKR-CMK, a kind gift from Dr. Wolfgang Garten), cells were incubated with cold labeling medium without cysteine in the presence of 50 μM dec-RVKR-CMK. After 30 min, [35S]cysteine was added to a final concentration of 100 μCi/ml, and incubation was continued for 4 h.

After the labeling period, conditioned medium was collected and centrifuged to remove cell debris. One-tenth volume of 10% immunoprecipitation buffer (250 mM Tris-Cl, pH 7.4, 10% Triton X-100, 10 mM CaCl2) was added to the medium (typically 500 μl of medium were used). Cells were washed twice with phosphate-buffered saline and lysed in 500 μl of 1% immunoprecipitation buffer containing 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, freeze-thawed, and centrifuged to remove debris. Immunoprecipitation was performed with 3 μg of monoclonal antibody to rat IGF-2 (Upstate Biotechnology Inc.) or the FLAG peptide (Kodak/IBI) at 4 °C overnight. Antibody-antigen complexes were precipitated with protein G-Sepharose (Amer sham Pharmacia Biotech), and pellets were washed twice in buffer containing 25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 mM CaCl2, 1% Triton X-100, and then once in buffer consisting of 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 1 mM CaCl2.

Immunoprecipitates were solubilized in SDS-sample buffer, without reducing agents unless stated otherwise, by heating at 95 °C for 5 min. Samples were electrophoresed on 15% glycine-buffered polyacrylamide gels with a 5% stack. Gels were fixed for 30 min (10% acetic acid, 25% isopropanol) and then soaked for 20 min in a fluorographic solution (Amplify, Amersham), and dried. Dried gels were typically exposed to x-ray film (Eastman Kodak Co.) with an intensifying screen for 4 h to 2 days.

**Glycosidase Digests**—For digestion with glycosidases, immunoprecipitated pellets were resuspended in 25 μl of digestion buffer (20 mM sodium cacodylate, 10 mM calcium acetate, pH 6.5) and 1 milliunit of neuraminidase or 1 milliunit of O-glycosidase (Boehringer Mannheim) was added. Samples were incubated overnight at 37 °C. For the double digest, samples were first incubated with neuraminidase for 6 h, then O-glycosidase was added and incubation continued overnight. After digestion was complete, 25 μl of 2X SDS-sample buffer were added, and samples were electrophoresed as described above.

**Pulse-Chase Experiments**—A 293 cell line that stably expressed human pro-IGF-2 was used for all pulse-chase experiments. Cells were incubated for 30 min in cysteine-free cold labeling medium and then pulsed for 10 min with cold labeling medium containing 1 μCi/ml [35S]cysteine. Cells were then washed with phosphate-buffered saline and incubated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and excess cold cysteine. Conditioned medium and cell lysates were collected at various time points, immunoprecipitated, and analyzed by SDS-PAGE as described above. Some pulse-chase experiments were performed in the presence of 2 μg/ml brefeldin A or 2 μg/ml monensin (Calbiochem).

**Immunostaining**—Cells were grown in 4-well chamber slides (Fisher) that had been pretreated with polylysine. Cells were fixed in 4% formaldehyde, permeabilized with 0.075% Triton X-100, and blocked in 1× TBS (50 mM Tris-Cl, pH 7.4, 150 mM NaCl), containing 2 mg/ml bovine serum albumin. Mouse monoclonal antibodies to rat IGF-2 (10 μg/ml, Upstate Biotechnology Inc.), or GRP78/Bip (5 μg/ml, StressGen) were incubated with cells overnight at 4°C. Fluorescence isothiocyanate-conjugated anti-mouse IgG (Sigma) diluted 1:500 was used as a secondary antibody. Cells were also stained with rhodamine-wheat germ agglutinin (Vector Laboratories) diluted 1:8000. Images were acquired using an Olympus BH-2 microscope with a 60X objective that was equipped with a Sony 3CCD color video camera, personal computer, and Image-Pro Plus software (Media Cybernetics).

**RESULTS**

**Multiple IGF-2 Peptides Are Generated by O-Glycosylation and Endoproteolysis**—We chose to study the post-translational processing of human pro-IGF-2 in the human embryonic kidney 293 cell line. This cell line has been previously shown to efficiently convert human pro-IGF-IA to mature IGF-I (19, 20). When transfected with the ReCMV-IGF-2 expression vector, 293 cells secrete six immunoreactive IGF-2 peptides into conditioned medium (Fig. 2A). The three most abundant bands range in size from 14 to 18 kDa, and three faint bands are visible in the 23–26-kDa size range. The anti-rat IGF-2 monoclonal antibody (antibody no. 166) does not recognize any peptides in conditioned medium from cells transfected with the...
Multiple IGF-2 peptides are generated by O-glycosylation and endoproteolysis. A, 293 cells were transiently transfected with RcCMV (CMV), RcCMV-IGF-2 (igf2), or the Arg<sup>66</sup> stop codon mutant (R68X). IGF-2 peptides were immunoprecipitated from conditioned medium with an anti-IGF-2 monoclonal antibody and analyzed by SDS-PAGE as described under “Experimental Procedures.” B, 293 cells were transiently transfected with the pro-IGF-2-FLAG expression vector (shown below), and peptides were immunoprecipitated from conditioned medium with monoclonal antibodies to IGF-2 (166), N-terminal FLAG (M1), or FLAG (M2) and analyzed by SDS-PAGE. C, IGF-2 peptides were immunoprecipitated from conditioned medium of a 293 cell line that stably expressed pro-IGF-2. Peptides were incubated with digestion buffer alone (- - -), neuraminidase (N), O-glycosidase (O), or both (N+O), as described under “Experimental Procedures.” After digestion peptides were analyzed by SDS-PAGE.

In order to identify peptides containing the full-length 156-amino acid IGF-2 precursor, we modified the RcCMV-IGF-2 vector to include the FLAG epitope on the carboxyl terminus. When pro-IGF-2-FLAG was transfected into 293 cells and conditioned medium immunoprecipitated with the anti-IGF-2 antibody, the same six bands that were generated from the RcCMV-IGF-2 vector were observed, indicating that the FLAG peptide did not interfere with pro-IGF-2 processing (Fig. 2B). The M1 antibody, which will recognize only the FLAG peptide if the amino terminus is accessible, served as a negative control and did not recognize any peptides from conditioned medium. The three high molecular mass bands (23–26 kDa) were immunoprecipitated by the M2 anti-FLAG antibody, indicating that all of these peptides contained the full-length IGF-2 precursor.

One likely explanation for the molecular heterogeneity of the pro-IGF-2 peptide would be the presence of carbohydrate. Pro-IGF-2 does not contain any sites for N-linked glycosylation, but there are eight serine and threonine residues in the E domain that could be used for O-linked glycosylation. The existence of carbohydrate was assessed by immunoprecipitating IGF-2 peptides from medium conditioned by a 293 cell line that stably expressed pro-IGF-2 and subjecting the immunoprecipitates to digestion with neuraminidase and/or O-glycosidase. Upon digestion with neuraminidase, the six peptides were converted to smaller molecular weight species (Fig. 2C). The electrophoretic mobility of the six peptides was unchanged after digestion with O-glycosidase. Since O-glycosidase cannot remove O-linked carbohydrates that contain sialic acid, this result confirms that all IGF-2 peptides observed in this expression system contained sialic acid. When peptides were digested with both neuraminidase and O-glycosidase, only two bands were observed. Digestion of pro-IGF-2-FLAG peptides that were immunoreactive to the M2 antibody confirmed that the upper band (approximately 20 kDa in size) was full-length pro-IGF-2 (data not shown). The lower band likely represents pro-IGF-2 that has been subjected to endoproteolysis in the E domain (see below). The slight difference in molecular weight between peptides digested with neuraminidase alone compared with those digested with neuraminidase and O-glycosidase could represent the loss of N-acetylgalactosamine that is attached to serine and threonine side chains to initiate the process of O-glycosylation.

**Identification of Four Sites of O-Linked Carbohydrate Attachment**—We employed site-directed mutagenesis to determine which of the eighteen serine and threonine residues in the pro-IGF-2 E domain were used as sites of carbohydrate attachment. Each potential glycosylation site was mutated to an alanine residue, and the effect of individual mutations was assessed. When Ser<sup>71</sup>, Thr<sup>72</sup>, or Thr<sup>75</sup> was mutated, the 18-kDa band (the upper most of the abundant triplet, indicated by the arrowhead in Fig. 3A) disappeared, which would be consistent with the loss of one O-glycosylation site in each case. Overexpression of this gel revealed that the Thr<sup>139</sup> mutation resulted in the loss of the highest molecular mass band that migrates at approximately 26 kDa (data not shown).

When all permutations of double mutations at Ser<sup>71</sup>, Thr<sup>72</sup>, and Thr<sup>75</sup> were expressed, the upper two bands of the abundant triplet (17 and 18 kDa, labeled with arrowheads in Fig. 3B) disappeared. This result is consistent with the loss of two O-glycosylation sites for each peptide. The 12-kDa band that is generated from the T72A/T75A mutant does not contain carbohydrate (data not shown), indicating that only these two sites are glycosylated on a subset of IGF-2 peptides. The S71A/T72A/T75A triple mutant migrated as a major band at approximately 12 kDa and a faint band at about 22 kDa. The 12-kDa peptide was resistant to glycosidase digestion, confirming that all carbohydrate attachment sites had been removed from this protein. Glycosidase digestion revealed a slight shift in the migration of the 22-kDa band, which likely resulted from removal of carbohydrate at Thr<sup>139</sup> (data not shown). Therefore, in addition to identifying Ser<sup>71</sup>, Thr<sup>72</sup>, Thr<sup>75</sup>, and Thr<sup>139</sup> as sites of carbohydrate attachment, these data also suggest that endoproteolysis occurs between Thr<sup>75</sup> and Thr<sup>139</sup> to convert full-length glycosylated pro-IGF-2 peptides to lower molecular weight gly-
Experiments with phenyl-N-acetyl-L-galactosamine, which inhibits all O-linked glycosylation, demonstrated that addition of carbohydrate to pro-IGF-2 is not a prerequisite for endoproteolysis and secretion (data not shown).

**Endoproteolysis Occurs Only at Arg<sup>104</sup> and Requires Basic Residues in Both the P1 and P4 Positions**—A site-directed mutagenesis strategy was also used to identify sites of endoproteolysis in the pro-IGF-2 E domain. Since hormone and growth factor precursors are often cleaved at basic amino acids, each of the 10 lysine and arginine residues were mutated to alanine. The mutation of Arg<sup>104</sup> to alanine resulted in the accumulation of high molecular weight pro-IGF-2 peptides in the medium, and little or no lower molecular weight forms were detected (Fig. 4A). Arginine residues were also present upstream of Arg<sup>104</sup>, in the P2 and P4 positions. Failure of the R101A mutant to be processed indicates that both the P1 and P4 residues are of critical importance for pro-IGF-2 processing (Fig. 4B).

We constructed a third expression vector to determine if the fragment of the E domain released by endoproteolysis at Arg<sup>104</sup> was subsequently subjected to further processing. The FLAG epitope was inserted immediately after Arg<sup>104</sup> to generate IGF-2-R104-FLAG (Fig. 4C). This peptide can be labeled on either the amino-terminal side of the FLAG epitope with [³⁵S]cysteine, or on the carboxyl-terminal side with [³⁵S]methionine. Specific peptides, including the E domain fragment released by cleavage after Arg<sup>104</sup> and beginning with the exposed amino terminus of the FLAG, can be immunoprecipitated with antibodies to IGF-2 or FLAG. When the amino terminus of the precursor was labeled with [³⁵S]cysteine, full-length pro-IGF-2 and IGF-2(1–104) glycopeptides were detected with the anti-IGF-2 antibody. The full-length pro-IGF-2 peptides, but not the E domain fragment, were visualized by immunoprecipitation with the M2 antibody. Because the precursor was labeled with [³⁵S]cysteine, no peptides were visualized with the M1 antibody, since this monoclonal antibody will recognize the FLAG only after it has been released by cleavage at Arg<sup>104</sup>, and the corresponding E domain fragment does not contain any cysteine residues. When the precursor was labeled with [³⁵S]methionine, only full-length pro-IGF-2 peptides were detected with the anti-IGF-2 antibody. A low molecular weight peptide corresponding to the epitope-tagged E domain was detected with the M1 antibody, and both the E domain fragment and full-length pro-IGF-2 peptides could be visualized with M2. Since the methionine residue is located in position 151 of pro-IGF-2, it is unlikely that endoproteolysis occurs between this site and Arg<sup>104</sup>. The only basic residues downstream of Met<sup>151</sup> are Arg<sup>155</sup> and Lys<sup>156</sup>. It is possible that these amino acids could be removed by exoproteolysis.

**Subtilisin-like Proprotein Convertases Process Pro-IGF-2 at Arg<sup>104</sup>**—The requirement for basic residues in both the P1 and P4 positions of the pro-IGF-2 processing site suggested that members of the subtilisin-related proprotein convertase (SPC) family might be involved in cleaving this precursor. Furin, PACE4, PC6A, PC6B, and LPC are expressed in a variety of cell types, making them candidate pro-IGF-2 processing enzymes. A potent inhibitor of furin, dec-RVKR-CMK, has been developed (21). It is likely that this inhibitor will also be effective on other members of the SPC family. In the presence of 50 μM dec-RVKR-CMK, a 293 cell line that stably expresses pro-IGF-2 secreted only uncleaved peptides (Fig. 5A). In co-expression experiments the amount of unprocessed pro-IGF-2 secreted from 293 cells (either transiently or stably transfected) was greatly diminished by PACE4 and LPC, and completely eliminated by furin, PC6A, and PC6B (Fig. 5B). No low molecular weight IGF-2 peptides were generated by co-expression with PACE4, PC6A, PC6B, or LPC. In the presence of furin, a peptide that was smaller than mature IGF-2, was produced and is likely an artifact resulting from cleavage at an inappropriate site by the overexpressed enzyme. We have shown previously that the endogenous processing enzyme activity in 293 cells can convert more than 90% of the overexpressed pro-IGF-IA to mature IGF-I (19). The experiment described here shows that the enzymes that process pro-IGF-IA fail to process pro-IGF-2 to mature IGF-2.

**Acidic Residues in the P2 and P1' Positions Interfere with Cleavage of Pro-IGFs**—We had previously shown that processing of pro-IGF-IA to IGF-I-(1–70) required an arginine in the P1 position and a lysine in the P4 position (19). Since the cleavage site that is likely to be used to generate mature IGF-2 also contains arginine and lysine in the P1 and P4 sites, respectively (Fig. 1), it was unexpected to find that this product was not generated by the endogenous processing enzymes in 293 cells or in co-expression experiments with the SPCs. Examination of alignments of pro-IGF-1 and pro-IGF-2 sequences from various organisms revealed that the P1 Arg of pro-IGF-2 was usually flanked by acidic residues in the P2 and P1' positions, while acidic side chains were never present in the vicinity of the P1 of pro-IGF-I (22). In order to test the hypothesis that the acidic residues were interfering with cleavage of pro-IGF-2, we constructed a series of site-directed mutants in which we converted the pro-IGF-2 processing site to a pro-IGF-IA site by substituting nonacidic residues for acidic ones.
Conversely, we introduced acidic residues into the pro-IGF-IA site to create a pro-IGF-2 processing motif (Fig. 6).

When the E67A mutant of pro-IGF-2, which converts the P2 glutamic acid to an alanine, was expressed in 293 cells a small amount of low molecular weight IGF-2 corresponding in size to mature IGF-2 was generated (Fig. 6A). Low molecular weight IGF-2 was also detectable when the D69S mutant, which converts the P1 aspartic acid to serine, was expressed. The E67A/
D69S double mutant converts both the P2 and P1' residues to the nonacidic counterparts found in pro-IGF-IA, thus creating a pro-IGF-IA cleavage site within pro-IGF-2. Mature IGF-2 was readily detectable when the double mutant was expressed in 293 cells.

ProIGF-IA is normally processed at Arg71 to generate IGF-I-(1–70) and Arg77 to produce IGF-I-(1–76) when expressed in 293 cells (19). Cleavage at these sites is very efficient, with more than 90% of the precursor processed to these two products (Fig. 6B). When the P2 alanine was mutated to glutamic acid (A70E), processing at Arg71 was drastically reduced. When the P1' serine residue was mutated to aspartic acid (S72D), processing at both Arg71 and Arg77 was diminished. The S72D mutation was more detrimental to processing at the Arg77 site than at the Arg71 site. This is not surprising since Ser72 is in the P6 position, relative to Arg77, and P6 has been shown to be important for substrate recognition by the SPCs. The A70E/S72D mutant converts the pro-IGF-IA site to a pro-IGF-2 site by flanking Arg71 with acidic residues. About 20% of the precursor is converted to IGF-I-(1–70) or IGF-I-(1–76), and both pro-IGF-IA and N-glycosylated pro-IGF-IA accumulate in the medium.

Pulse-Chase Analysis of Pro-IGF-2 Processing—Pulse-chase experiments revealed that pro-IGF-2 appeared as a doublet in cell lysates immediately after the 10-min pulse period (Fig. 7A). By 120 min the lower band of the doublet had chased into the upper band. Glycosylated pro-IGF-2 and IGF-2-(1–104) appeared in the lysates at the 40-min time point, and these peptides were visible in conditioned medium 1 h after the pulse. We initially believed that the upper band of the doublet represented pro-IGF-2 with N-acetylglalactosamine attached to the serine and threonine side chains. However, we found that the doublet was present after only a 2-min pulse (data not shown), which would require very fast passage through the endoplasmic reticulum into the cis-Golgi compartment where the addition of N-acetylgalactosamine is known to occur (28).

Furthermore, the doublet was resistant to digestion with O-glycosidase (Fig. 7B). Upon reduction with dithiothreitol, the doublet migrated as a single band on SDS-PAGE (Fig. 7C), indicating that pro-IGF-2 can initially form two disulfide isomers that eventually undergo rearrangement to a single form.

The doublet underwent disulfide isomerization to a single band at the same rate in the presence of brefeldin A (BfA) (Fig. 7D). O-Linked carbohydrate was also added when cells were treated with BfA but complete oligosaccharide maturation to the high molecular weight forms seen previously did not occur. Endoproteolysis and secretion were also inhibited by BfA. Disulfide isomerization was unaffected by monensin (Fig. 7E). However, oligosaccharide maturation, endoproteolysis, and secretion were inhibited by this drug. It is likely that N-acetylglalactosamine addition would occur in the presence of monensin, since transport from the endoplasmic reticulum to the cis- and medial Golgi apparatus is not impeded by this treatment.

We have digested pro-IGF-2 peptides immunoprecipitated from lysates of monensin-treated cells with O-glycosidase, but the resulting shifts in molecular weight were too small and inconsistent to allow us to draw any conclusions regarding the presence of N-acetylglalactosamine (data not shown).

Subcellular Localization of IGF-2 by Immunofluorescence—293 cells that stably express pro-IGF-2 were immunostained with primary antibodies to the endoplasmic reticulum-resident chaperone BiP or IGF-2 (Fig. 8). Rhodamine-conjugated wheat germ agglutinin, which binds N-acetylglucosamine, was used with fluorescein isothiocyanate-conjugated secondary antibodies in order to simultaneously visualize the Golgi compartment. IGF-2 immunoreactivity was localized to a distinct subcellular compartment.
compartment that overlapped with staining by wheat germ agglutinin, indicating that IGF-2 accumulates in the Golgi compartment.

**DISCUSSION**

The goal of this work has been to characterize the post-translational modifications that normally take place on the human IGF-2 precursor and thereby define the processing products generated from this prohormone. A model for the biosynthesis of human pro-IGF-2 can now be constructed from these experiments (Fig. 9). In pulse-chase experiments pro-IGF-2 always first appeared as a doublet, even if the pulse was shortened to only 2 min (data not shown). The doublet was resistant to digestion with O-glycosidase but was converted to a single band in the presence of dithiothreitol followed by iodoacetamide (C), and analyzed by SDS-PAGE.

suggesting that pro-IGF-2 can initially assume two conformations that differ in the arrangement of the disulfide bonds. In **vitro** refolding experiments with both mature IGF-I and pro-IGF-IA have shown that these peptides can form two thermodynamically stable products (23–25). Approximately 60% of the peptide reformed the native disulfide arrangement (Cys18–Cys61, Cys6–Cys48, and Cys47–Cys52) and 40% assumed the “mismatched” or “IGF-I swap” conformation (Cys18–Cys61, Cys6–Cys47, and Cys48–Cys52). Our data suggests that pro-IGF-2 can form two disulfide isomers **in vivo** and also demonstrate that disulfide rearrangement to single form occurs (Fig. 7, A, D, and E). Since the pro-IGF-2 doublet chased to a single band in the presence of monensin (Fig. 7E), which blocks transport from the medial to the trans-Golgi apparatus (26, 27),

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**Fig. 7.** Pulse-chase analysis of pro-IGF-2 processing. A, D, and E, 293 cells that stably express pro-IGF-2 were pulsed for 10 min with [35S]cysteine and chased in medium containing excess unlabelled cysteine, as described under “Experimental Procedures.” Media and lysate samples were collected at various time points after the pulse, as labeled at the top of the gel, and analyzed for IGF-2 peptides as described previously. In panels D and E, cells were treated with brefeldin A or monensin, respectively. B and C, after 10 min of chase, cell lysates were collected and IGF-2 peptides were immunoprecipitated. Peptides were then digested with O-glycosidase (B) or treated with dithiothreitol followed by iodoacetamide (C) and analyzed by SDS-PAGE.

**Fig. 8.** Subcellular localization of IGF-2 by immunofluorescence. 293 cells that stably express pro-IGF-2 were grown on chamber slides and immunostained as described under “Experimental Procedures.” The upper panels show immunostaining for the endoplasmic reticulum-resident molecular chaperone BiP (anti-GRP78) and staining for sialic acid with wheat germ agglutinin (WGA). The lower panels show immunostaining for IGF-2 (anti-IGF-2) co-localized with staining for sialic acid by wheat germ agglutinin.
disulfide isomerization must occur very early in the secretory pathway. The endoplasmic reticulum, which contains protein disulfide isomerase, is the most likely site for this rearrangement to occur. The mechanism of disulfide rearrangement and the involvement of trans-acting factors such as protein disulfide isomerase or IGF-binding proteins remains to be investigated.

The process of O-glycosylation begins with the linkage of N-acetylglalactosamine (GalNAc) to serine and threonine side chains in a compartment between the transitional elements of the endoplasmic reticulum and the cis-Golgi stack (28). A variety of hexoses can be added to construct the O-linked core unit, and carbohydrate maturation is often completed by the addition of galactose, fucose, or sialic acid in various Golgi compartments (29). Since most of these groups have a molecular mass of less than 200 Da, it is sometimes difficult to evaluate their presence by SDS-PAGE analysis. Digestion with neuraminidase indicated that IGF-2 peptides secreted by 293 cells contained a significant amount of sialic acid (Fig. 2C). Large band shifts associated with desialylation of high molecular weight IGF-2 peptides isolated from human and bovine serum have also been observed (13, 30, 31). In pulse-chase experiments some sialic acid was added in the presence of BFA, while monensin prevented the sialic acid addition altogether (Fig. 7, D and E), indicating that carbohydrate maturation of pro-IGF-2 begins in the trans-Golgi apparatus but is not completed until peptides reach the trans-Golgi network.

In deglycosylation experiments of IGF-2 peptides isolated from lysates of monensin-treated cells during pulse-chase analysis, we were unable to observe reproducible band shifts that should be associated with the loss of GalNAc (data not shown). However, it is likely that the small shift in molecular weight between secreted IGF-2 peptides digested with neuraminidase versus peptides digested with neuraminidase and O-glycosidase represents the removal of GalNAc (Fig. 2C). Similar band shifts were observed when IGF-2 peptides isolated from human and bovine serum were desialylated and digested with O-glycosidase (13, 30). Mass spectrometry analysis of IGF-2-(1–87) peptides isolated from human and bovine serum suggested that addition of various amounts of hexose and sialic acid at one, two, or three locations could account for the observed carbohydrate mass (12, 13).

Glycosylated forms of pro-IGF-2 appeared in lysates approximately 40 min after a pulse with [35S]cysteine (Fig. 7A). Mutational analysis has identified Ser71, Thr72, Thr75, and Thr139 as sites of O-linked carbohydrate attachment (Fig. 3). Biochemical analysis of a 15-kDa IGF-2 peptide isolated from Cohn fraction IV revealed the presence of O-linked carbohydrate containing sialic acid on Thr75 (30). Addition of carbohydrate was eliminated in a pro-IGF-2 mutant encoding a triple substitution at Pro71, Pro74, and Thr75 that was expressed in NIH 3T3 cells (32). From these data it would appear that Thr75 is a major site of pro-IGF-2 glycosylation, and other side chains might be glycosylated in a tissue-specific manner.

Expression experiments with pro-IGF-2 mutants containing substitutions at potential cleavage sites revealed that pro-IGF-2 is processed at Arg130 in 293 cells (Fig. 4A). Specific labeling of the amino and carboxyl termini of the IGF-2-R104-FLAG precursor, followed by immunoprecipitation of the precursor, IGF-2-(1–104) or the E domain fragment, demonstrated that endoproteolysis occurs only at Arg130 (Fig. 4C). Experiments with additional mutants revealed that both Arg130 and Arg131 were required for endoproteolysis (Fig. 4B). The requirement for basic residues in the P1 and P4 positions is characteristic of the substrate specificity of some members of the SPC family (33). A furin inhibitor completely blocked conversion of pro-IGF-2 to IGF-2-(1–104), and coexpression of pro-IGF-2 with furin, PACE4, PC6A, PC6B, or LPC resulted in enhanced or complete processing of the precursor (Fig. 5). Pulse-chase data indicated that pro-IGF-2 was cleaved concomitantly or slightly after oligosaccharide maturation (Fig. 7A). Lack of endoproteolysis in the presence of BFA and monensin (Fig. 7, D and E) is consistent with the requirement for furin to reach the trans-Golgi network in order to be activated (34, 35). The accumulation of pro-IGF-2 in the Golgi apparatus (Fig. 8) was not unexpected since furin (36) and many of the enzymes involved in glycosylation are found in this compartment.

The forms of IGF-2 that are most abundant in normal serum, mature IGF-2 and IGF-2-(1–87), were not produced by 293 cells (this work) (37). Mutations at Lys88 (Fig. 4), as well as Gly87, Phe89, and Phe90 (data not shown), had no effect on processing in our expression system. Furthermore, we did not observe IGF-2-(1–87) when the precursor was co-expressed with SPCs (Fig. 5), suggesting that a novel enzyme mediates cleavage at Lys88.

Given the similarity of the Lys-Xaa-Xaa-Arg site utilized for final maturation of pro-IGF-I to the site that is likely used for pro-IGF-2 processing, it was surprising to learn that mature IGF-2 was not generated by endogenous 293 processing enzymes or during co-expression with SPCs. Manipulation of the
pro-IGF-1 and pro-IGF-2 processing sites revealed that the acidic residues flanking Arg26 of the IGF-2 precursor interfered with cleavage (Fig. 6). Since conversion of the P2 and P1' residues to nonacidic amino acids did not result in efficient processing, it is likely that other elements in the pro-IGF-2 E domain also inhibit interaction with processing enzymes in 293 cells. We have found that HepG2 cells will convert a small amount of precursor (<1%) to mature IGF-2 (data not shown). It is possible that a novel member of the SPC family expressed in the liver may be responsible for final maturation of pro-IGF-2.

Other studies on pro-IGF-2 biosynthesis indicate that there are both species- and tissue-specific differences in post-translational processing. Endogenous rat pro-IGF-2 synthesized in the liver-derived BRL-3A cell line appeared as a 20-kDa precursor (38). Higher molecular mass forms that would be associated with glycosylation were not observed during pulse-chase analysis. Instead the precursor was converted to 19-, 10-, 8-, and 7-kDa proteins that were detectable intracellularly and secreted 40 min to 1 h after pulse-labeling. It is likely that the 7-kDa peptide corresponded to mature IGF-2. Since a fragment of the rat pro-IGF-2 E-domain with the amino terminus beginning at Met117 was isolated from medium conditioned by BRL-3A cells (39), the 19-kDa peptide could be IGF-2-(1–116).

Explants from rat neonatal brain and adult pituitary secreted the 19-kDa peptide could be IGF-2-(1–116). The 17-kDa peptide corresponds to IGF-2-(1–104). These data suggested that IGF-2 to high molecular weight IGF-2 peptides in assays for translational processing. Endogenous rat pro-IGF-2 synthesized in 293 cells. We have found that HepG2 cells will convert a small amount of precursor (<1%) to mature IGF-2 (data not shown). It is possible that a novel member of the SPC family expressed in the liver may be responsible for final maturation of pro-IGF-2.

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