Insulin-like growth factor I (IGF-I) is required for normal embryonic development and postnatal growth. Like most hormones and growth factors, IGF-I is synthesized as a proprotein that is converted to the mature form by endoproteolysis. Processing of pro-IGF-I to mature IGF-I occurs by cleavage within the unique basic processing motif Lys-X-X-Lys-X-Arg\textsuperscript{71}-X-Arg-X-Arg\textsuperscript{77}. We have previously shown that human embryonic kidney 293 cells process pro-IGF-IA at Arg\textsuperscript{71} to generate IGF-I-(1–70) and at Arg\textsuperscript{77} to produce IGF-I-(1–76). Cleavage at each of these sites requires upstream basic residues, indicating that subtilisin-related proprotein convertases (SPCs) may be involved. In order to investigate the identity of the endogenous enzymes involved in maturation of pro-IGF-IA, we have expressed wild-type and mutant pro-IGF-IA in 293 cells and in the furin-deficient Chinese hamster ovary cell line, RPE.40. We have also co-expressed these constructs with SPCs that are thought to play a role in processing precursor proteins in the constitutive pathway: furin, PACE4, PC6A, PC6B, and LPC. The results show that furin is most active at cleaving wild-type and mutant pro-IGF-IA and can cleave these precursors at multiple sites within the pro-region of the pro-region (1).

In our previous study on pro-IGF-I processing, we found that expression of pro-IGF-IA in human embryonic kidney 293 cells resulted in secretion of N-glycosylated pro-IGF-IA, pro-IGF-IA, IGF-I-(1–76), and IGF-I-(1–70). Using site-directed mutagenesis, we demonstrated that IGF-I-(1–76) is produced by cleavage at Arg\textsuperscript{77} and that IGF-I-(1–70) is generated by cleavage at Arg\textsuperscript{71}. Cleavage at both of these positions required a P4 basic residue (see Fig. 1). Furthermore, cleavage at Arg\textsuperscript{71} occurred in the furin-deficient LoVo and RPE.40 cell lines, indicating that another processing enzyme must be involved in maturation of pro-IGF-IA (13). In an attempt to identify the enzyme(s) involved in maturation of pro-IGF-IA, we have expressed wild-type and mutant pro-IGF-IA in 293 and RPE.40 cells. We have also co-expressed these constructs with candidate pro-IGF-I-converting enzymes, furin, PACE4, PC6A, PC6B, and LPC.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The expression vector pCMVigf1-FLAG has been described previously (13). The human furin cDNA was excised from the Human Embryonic Kidney 293 (HEK 293) cell line and subcloned into the pCMV expression vector. The constructs were then transfected into 293 cells using the lipofection method. The cells were harvested 48 hours after transfection and the supernatants were collected and analyzed for the presence of IGF-I by immunoblotting.

**Results**

We have previously shown that human embryonic kidney 293 cells process pro-IGF-IA at Arg\textsuperscript{71} to generate IGF-I-(1–70) and at Arg\textsuperscript{77} to produce IGF-I-(1–76). Cleavage at each of these sites requires upstream basic residues, indicating that subtilisin-related proprotein convertases (SPCs) may be involved. In order to investigate the identity of the endogenous enzymes involved in maturation of pro-IGF-IA, we have expressed wild-type and mutant pro-IGF-IA in 293 cells and in the furin-deficient Chinese hamster ovary cell line, RPE.40. We have also co-expressed these constructs with SPCs that are thought to play a role in processing precursor proteins in the constitutive pathway: furin, PACE4, PC6A, PC6B, and LPC. The results show that furin is most active at cleaving wild-type and mutant pro-IGF-IA and can cleave these precursors at multiple sites within the pro-region of the pro-region (1).

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from pBluescript with EcoRI and SalI and cloned into EcoRI/SalI-digested pCMV6c to generate pCMV-furin. The human PACE4 cDNA was removed from pBluescript by digestion with XhoI and SalI. The purified PACE4 fragment was end-filled and blunt-end-ligated to pCMV6c that had been digested with SmaI to generate pCMV-PACE4. Human pBSK-LPC was digested with ScaI and 3′-overhangs were removed with T4 DNA polymerase under conditions favoring exonuclease activity. The purified fragment was blunt-end-ligated to pCMV6c to create pCMV-LPC. pCMV-furin, pCMV-PACE4 and pCMV-LPC were analyzed by restriction site and DNA sequencing. cDNAs coding for mouse PC6A (14) and PC6B (15) were subcloned into pC800.

Site-directed Mutagenesis—The following pCMVigf1-FLAG mutants have been described previously (13): R71A, R74A/R77A, and R77X. Additional mutants of pCMVigf1-FLAG were made using the restriction site elimination method (16) with the following mutagenic oligonucleotides: K65R, 5′-TGTCGAGCTCCTGGCATTCGCAAGTCA; K65R/E68R, 5′-TGTCGACCCCTCGCCATTCGGTCATCTGTCTG; K65R/E68R/A70K, 5′-CGCCCTCAAGGCGCCTGGTCATTCGGTCATCTGTCTG; K65R/A70K, 5′-CCCCTCAAGGCGCCTGGTCATTCGGTCATCTGTCTG.

Cell Culture and Transfections—Human embryonic kidney 293 cells were grown in Dublecco’s modified Eagle’s medium (Life Technologies, Inc) with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was then replaced with cysteine-free Dulbecco’s modified Eagle’s medium with 1% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Processing of Pro-IGF-I by SPCs

RESULTS

Processing of Wild-type and Mutant Pro-IGF-I by SPCs in 293 Cells—Our previous study on pro-IGF-I processing utilized the pCMVigf1-FLAG expression vector. This vector directs expression of human prepro-IGF-I containing the eight-amino acid FLAG epitope between Ala1 and Gly3 of the B domain. We have shown that the FLAG peptide does not affect co-translational or post-translational processing of prepro-IGF-I. Human embryonic kidney 293 cells transfected with pCMVigf1-FLAG secrete N-glycosylated pro-IGF-I, pro-IGF-I, IGF-I-(1–76), and IGF-I-(1–70) (13). In the present work, we sought to determine 1) if any of the known SPCs could process pro-IGF-I and 2) if Arg for Lys substitutions at the P4 and/or the P7 positions would affect processing by endogenous or exogenous enzymes. The primary sequences of the cleavage sites of wild-type and mutant pro-IGF-I-FLAG constructs used in this study are indicated in Fig. 1. The results of expression of these constructs in 293 cells and their processing by endogenous enzymes or SPCs are shown in Fig. 2 and Table I.

When wild-type pro-IGF-I and pCMV6c (as a negative control) are co-transfected in 293 cells, two major bands corresponding to IGF-I-(1–70) and IGF-I-(1–76) are detected in conditioned medium (Fig. 2A). The diffuse band that migrates at approximately 20 kDa corresponds to N-glycosylated pro-IGF-I (see marker on Fig. 2E). Cells are continuously labeled for approximately 24 h after transfection, and under these conditions approximately 45% of the IGF-I recovered from conditioned medium is IGF-I-(1–70) and 45% is IGF-I-(1–76). When wild-type pro-IGF-I is co-transfected with pCMV-furin, 89% of the precursor is processed to IGF-I-(1–70). An additional band is visible that migrates slightly faster than IGF-I-(1–70) and has a molecular mass of approximately 7 kDa. The 7-kDa band is not present in the absence of pCMV-furin. Furin has been reported to cleave at lysine residues (19), and this band may result from processing at Lys65 or Lys68. The K65R mutant by enzymes endogenous to 293 cells is qualitatively identical to processing of wild-type pro-IGF-I. The K65R mutant is co-transfected with pCMV6c in 293 cells, IGF-I-(1–70) and 45% is IGF-I-(1–76). The K65R mutant is co-transfected with pCMV6c in 293 cells, IGF-I-(1–70) and 45% is IGF-I-(1–76). In the presence of furin the endocytic product is again IGF-I-(1–70). A 7-kDa band is also present and is more intense than the minor cleavage product produced by co-expression of mutant pro-IGF-I with furin. This band may result from cleavage at Arg36 or Lys48. The K65R mutant shifts the Lys48-X-Arg71 processing motif of pro-IGF-I upstream by three amino acids to Lys65-X-Arg68 and creates a new minimal furin site at Arg68-X-Arg71 (see Fig. 1). When the K68R mutant is co-transfected with pCMV6c in 293 cells, IGF-I-(1–70) is the major product, and IGF-I-(1–76) is not detected (Fig. 2A). A faint band is visible below IGF-I-(1–70) in Fig. 2A.

However, this product is not produced consistently from K68R by the endogenous processing enzymes (see Fig. 2, B, C, D, and...
In the presence of furin, 54% of the K68R precursor is processed to IGF-I-(1–70), and 34% is processed to a 7-kDa product that likely represents cleavage at the Lys65-X-X-Arg68 site. The K65R/K68R mutant contains arginine residues at positions 65, 68, 71, 74, and 77. Processing enzymes endogenous to 293 cells generate two products from this precursor (Fig. 2A). The upper band represents 65% of the precursor and corresponds to IGF-I-(1–76), and IGF-I-(1–70) is not detected. When PACE4 is co-expressed with wild-type pro-IGF-IA or the K65R mutant, IGF-I-(1–70) and IGF-I-(1–76) are the only cleavage products, lower molecular weight forms are not produced (Fig. 2B). PhosphorImager analysis indicates a slight increase in the production of IGF-I-(1–70) from the wild type and the K65R precursor in the presence of pCMV-PACE4. The K68R mutant is processed only to IGF-I-(1–70), and the amount of product produced is not affected by PACE4. As seen previously, the K65R/K68R mutant is processed primarily to IGF-I-(1–70) by endogenous enzymes, with a smaller peptide composing 27% of the final product. Processing of this mutant is unaffected by PACE4.

Co-expression of PC6A with either the wild-type pro-IGF-IA or the K65R mutant enhances production of IGF-I-(1–70), and lower molecular weight forms are not generated (Fig. 2C). PC6A processes 59% of the wild-type precursor and 71% of the K65R mutant to IGF-I-(1–70). PC6A does not qualitatively or quantitatively alter the processing of the K68R or K65R/K68R mutants.

| Constructs         | Lys/Arg68 | Arg71 | Arg77 | Pro  |
|--------------------|-----------|-------|-------|------|
| WT                 | 0         | 45    | 45    | 10   |
| WT + furin         | 1         | 89    | 0     | 10   |
| WT + PACE4         | 0         | 55    | 13    | 2    |
| WT + PC6A          | 0         | 59    | 34    | 7    |
| WT + PC6B          | 0         | 54    | 38    | 8    |
| WT + LPC           | 0         | 73    | 18    | 9    |
| K65R               | 0         | 45    | 45    | 10   |
| K65R + furin       | 31        | 60    | 0     | 9    |
| K65R + PACE4       | 0         | 61    | 36    | 4    |
| K65R + PC6A        | 0         | 71    | 23    | 6    |
| K65R + PC6B        | 0         | 59    | 32    | 9    |
| K65R + LPC         | 0         | 67    | 23    | 10   |
| K68R               | 0         | 87    | 0     | 13   |
| K68R + furin       | 34        | 54    | 0     | 12   |
| K65R/K68R          | 26        | 65    | 0     | 9    |
| K65R/K68R + furin  | 89        | 0     | 0     | 11   |

* Assignment of this cleavage site is tentative.

Table 1

Processing of wild-type and mutant pro-IGF-IA in 293 cells

The radioactivity in gels used for autoradiography in Fig. 2 was measured using a PhosphorImager. The percentage of pro-IGF-I and IGF-I processed at Arg68, Arg71, and Lys/Arg68 was calculated.
Fifty-four percent of the wild-type precursor and 59% of the K65R mutant are processed to IGF-I-(1–70) in the presence of PC6B, compared with 45 and 48% IGF-I-(1–70) product by endogenous enzymes (Fig. 2D). PC6B does not qualitatively or quantitatively alter the processing of the K68R or K65R/K68R mutants.

In the presence of LPC, 73% of wild-type pro-IGF-IA was converted to IGF-I-(1–70), as compared with 45% conversion by endogenous enzymes (Fig. 2E). LPC was less active on the K65R, K68R, and K65R/K68R mutants. LPC did not generate peptides smaller than IGF-I-(1–70) from any of the precursors.

Processing of Wild-type and Mutant pro-IGF-IA by SPCs in RPE.40 Cells—The co-expression experiments in 293 cells indicated that furin will efficiently process wild-type and mutant pro-IGF-IA to IGF-I-(1–70) and will also cleave the mutant precursors at other sites. Processing enzymes endogenous to 293 cells process all precursors to IGF-I-(1–70) and cleave only the K65R/K68R mutant at an alternate site. By co-expressing pro-IGF-IA constructs with SPCs in the furin-deficient RPE.40 cell line (20), we sought to determine 1) if furin was responsible for alternative cleavage of K65R/K68R and 2) the activity of PACE4, PC6A, PC6B and LPC for pro-IGF-IA in the absence of furin. The results of these experiments are shown in Fig. 3 and Table II.

As we have shown previously (13), when pro-IGF-IA is expressed in RPE.40 cells, IGF-I-(1–70), but not IGF-I-(1–76), is generated. In RPE.40 cells, approximately 80% of the precursor is processed to IGF-I-(1–70), while 20% is secreted as N-glycosylated pro-IGF-IA (Fig. 3A). When co-expressed with furin, nearly 100% of the pro-IGF-IA is converted to IGF-I-(1–70).

Approximately 80% of the K65R and K68R precursors ap-
peared as IGF-I-(1–70). In the presence of furin IGF-I-(1–70) is still the major product, but a smaller peptide is also generated from both mutants. The processing enzymes endogenous to RPE.40 cells generate only IGF-I-(1–70) from the K65R/K68R mutant. However, when co-expressed with furin, nearly 100% of the precursor is processed at an alternative site to generate the smaller 7-kDa form.

When wild-type or mutant pro-IGF-IA is co-expressed with PACE4 (Fig. 3B), PC6A (Fig. 3C), or PC6B (Fig. 3D) in RPE.40 cells, IGF-I-(1–70) is the only cleavage product. Approximately 80% of each precursor is converted to mature IGF-I, and this effect is not affected by the presence of exogenous enzymes.

In the background of RPE.40 cells, LPC is nearly as efficient as furin at processing pro-IGF-I-A. Eighty-five to 93% of each precursor is converted to mature IGF-I, and this effect is not affected by the presence of exogenous enzymes.

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sequence identity to human LPC and 95% sequence identity to rat PC7 (data not shown). When this cDNA was used to probe a Northern blot of RPE-40 poly(A)⁺ RNA, a single transcript of 4.4 kilobase pairs was detected (Fig. 7), indicating that LPC is expressed in RPE-40 cells.

Processing of Human Pro-IGF-IA in Various Cell Lines—Since IGF-I is synthesized by all tissues of the body, we were interested in determining if pro-IGF-IA-processing enzymes are also ubiquitous. We expressed pro-IGF-IA-FLAG in cell lines derived from several mammalian sources as well as one invertebrate cell line. Secreted IGF-I peptides were immunoprecipitated from conditioned medium and analyzed by gel electrophoresis, as described under “Experimental Procedures.” The results have been summarized in Table III.

The proteolytic processing of pro-IGF-IA is complex, and the enzymes responsible for producing the various IGF-I-(1–76) fragments remain to be identified. Our data suggest that a furin-related enzyme is involved in the production of IGF-I-(1–70) from pro-IGF-IA. This conclusion is based on the following observations:

1. Production of IGF-I-(1–70) was observed in all cell lines examined, including the furin-deficient RPE-40 cell line. This suggests that another enzyme, such as a furin-related enzyme, is involved in the production of IGF-I-(1–70).
2. Production of IGF-I-(1–70) was not observed in the furin-deficient cell line, indicating that furin is not responsible for this cleavage.
3. The production of IGF-I-(1–70) was not affected by the overexpression of furin, suggesting that furin is not the primary enzyme responsible for this cleavage.

The results from Table III suggest that a furin-related enzyme is involved in the production of IGF-I-(1–70) from pro-IGF-IA. The exact nature of this enzyme and its role in the processing of pro-IGF-IA remains to be determined. Further studies are needed to identify the specific enzymes responsible for the production of the various IGF-I-(1–76) fragments.

DISCUSSION

We have expressed wild-type and mutant pro-IGF-IA with furin, PACE4, PC6A, PC6B, and LPC in order to determine which of these enzymes might play a role in pro-IGF processing. In 293 cells, endogenous enzymes process wild-type pro-IGF-IA and the K65R mutant at Lys⁶⁸-X-X-Arg⁷¹ to yield IGF-I-(1–70) and at Arg⁷⁴-X-X-Arg⁷⁷ to produce IGF-I-(1–76) (Fig. 6). The K68R mutant is processed at Arg⁶⁸-X-X-Arg⁷¹ to generate IGF-I-(1–70), and IGF-I-(1–76) is apparently not produced from this precursor by 293 enzymes. The lack of IGF-I-(1–76) could be due to a preference for Arg⁶⁸-X-X-Arg⁷¹ over Arg⁷⁴-X-X-Arg⁷⁷. It is also possible that pro-IGF-IA is processed sequentially from IGF-I-(1–76) to IGF-I-(1–70) and that the Arg for Lys⁶⁸ substitution creates a more efficient cleavage site or a site favorable to additional processing enzymes, resulting in complete processing to the final product. Pulse-chase analysis will be necessary to determine if the K68R precursor is sequentially cleaved. Endogenous processing enzymes in 293 cells produce two products from the K65R/K68R precursor (Fig. 2). The main product corresponds to IGF-I-(1–70) generated by cleavage at Arg⁶⁸-X-X-Arg⁷¹. The smaller peptide likely corresponds to IGF-I-(1–67) resulting from cleavage at the newly created furin site, Arg⁶⁸-X-X-Arg⁶⁸. This suggestion is supported by the fact that the smaller peptide is not generated from K65R/K68R in the furin-deficient RPE-40 cell line (Fig. 3).

We have confirmed the presence of wild-type pro-IGF-IA in 293 cells, IGF-I-(1–70), but not IGF-I-(1–76), is produced (Fig. 2A).
A faint band migrating slightly faster than IGF-I-(1–70) is also produced and could represent cleavage at an upstream lysine. The fact that furin generates IGF-I-(1–70), but not IGF-I-(1–76), from pro-IGF-IA could be due to a preference for Lys<sup>68</sup>-X-X-Arg<sup>71</sup> over Arg<sup>74</sup>-X-X-Arg<sup>77</sup> or the result of sequential processing, with IGF-I-(1–70) being the major final product. Since each site is cleaved with high efficiency when the R74A/R77A and R71A mutants are co-expressed with furin in 293 or RPE.40 cells (Figs. 4, A and B), we suggest that the latter hypothesis is more likely. Furin processes the K65R and K68R mutants to IGF-I-(1–70) and a smaller band that may result from cleavage at one of the two upstream basic residues (Fig. 2A). Only one product, which migrates slightly ahead of IGF-I-(1–70), is produced from the K65R/K68R mutant. This 7-kDa band likely represents IGF-I-(1–67) produced by cleavage at the Arg<sup>65</sup>-X-X-Arg<sup>68</sup> site. Again, cleavage may be sequential or reflect processing site specificity.

In contrast to furin, co-expression of PACE4, PC6A, PC6B, or LPC with pro-IGF precursors in 293 cells does not qualitatively change the pattern of processing products generated by the endogenous enzymes (Fig. 2, B, C, D, and E). However, some quantitative changes are apparent. PC6A and LPC are most efficient at generating IGF-I-(1–70) from pro-IGF-IA, followed by PC6B and then PACE4 (Table I). These enzymes appear to have a more strict substrate specificity than furin, since they produce only IGF-I-(1–70) from pro-IGF precursors. However, PACE4, PC6A, and PC6B are capable of cleaving at Lys<sup>68</sup>-X-X-Arg<sup>71</sup> or Arg<sup>74</sup>-X-X-Arg<sup>77</sup> (Fig. 4, A and B). Other studies comparing specificity and activity of SPCs have also found that furin has the widest substrate specificity and is more active than PACE4, PC6A, and PC6B. Furin will efficiently cleave both Arg-X-(Lys/Arg)-Arg and Arg-X-X-Arg motifs (25, 26), while PACE4 and PC6A prefer substrates with a basic residue in the P2 position (14, 19, 27, 28). Neurotropin precursors and human immunodeficiency virus gp160 contain Arg-X-(Lys/Arg)-Arg motifs that are cleaved more efficiently by furin than PACE4 or PC6B, and not at all by PC6A (21, 22, 24). However, PC6A is more active than PACE4 at cleaving the Arg-X-(Lys/Arg)-Arg site of receptor protein-tyrosine phosphatase μ (23), indicating that structural features are also important for processing activity. Although we have not extensively examined the substrate specificity of LPC, our data suggest that this conver- tase may prefer the Lys-X-X-Arg<sup>71</sup> site to the Arg-X-X-Arg<sup>77</sup> site (Fig. 4). Processing of pro-IGF by endogenous and transfected enzymes in 293 cells is summarized in Fig. 8A.

From our experiments in 293 cells it is clear that the endogenous processing enzymes cleave pro-IGF-IA very efficiently and that furin is more active on pro-IGF precursors than PACE4, PC6A, PC6B, or LPC. We therefore thought it would be informative to examine processing of pro-IGF-IA in a furin-deficient cell line. We have used RPE.40 cells for this purpose. These cells were derived from CHO-K1 cells by exposure to the mutagen ethyl methane sulfonate and selection for resistance to neomycin. The expression levels did not strictly correlate with processing activity, however. For example, PACE4 had the highest mRNA levels but the lowest activity in both cell lines. Processing of pro-IGF-IA by endogenous and transfected enzymes in RPE.40 cells is summarized in Fig. 8B.

Of the pro-IGF-IA precursors that were expressed, PACE4, PC6A, and PC6B act primarily on IGF-I-(1–76) that would be generated by furin but is not produced in RPE.40 cells. This possibility was eliminated by showing that PACE4, PC6A, and PC6B process IGF-I-(1–76) to IGF-I-(1–70) in 293 cells but not in RPE.40 cells (Fig. 5). A third possibility is that expression levels of exogenous enzymes may vary in 293 and RPE.40 cells. Using ribonuclease protection assays, we determined that mRNA levels for PACE4, PC6A, and PC6B were 1.5–2.3-fold higher in 293 cells than RPE.40 cells (data not shown), suggesting that lower expression may account for their lack of activity in RPE.40 cells. Expression levels did not strictly correlate with processing activity, however. For example, PACE4 had the highest mRNA levels but the lowest activity in both cell lines. Processing of pro-IGF-IA by endogenous and transfected enzymes in RPE.40 cells is summarized in Fig. 8F.

Our previous work clearly identified the processing site for final maturation of pro-IGF-IA as Lys<sup>68</sup>-X-X-Arg<sup>71</sup> (13). This dibasic motif has been shown to be cleaved by enzymes of the SPC family (8). The data presented here indicate that furin,
PC6A, and LPC are capable of efficiently processing pro-IGF-IA to mature IGF-I. We have also found that processing activity can be influenced by enzyme expression levels and that all cells examined contain endogenous enzymes that efficiently and correctly process pro-IGF-IA. Furin, PC6A, and LPC are expressed in many tissues (6–12), suggesting that these and perhaps other members of the SPC family that have not yet been described may account for our observation that pro-IGF-I processing is a function common to all cells.

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