Role of pro-IGF-II processing by proprotein convertase 4 in human placental development

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Fetal growth restriction (intrauterine growth restriction, IUGR) is a leading cause of perinatal mortality. However, the causes of aberrant development of the placenta and, thus, of the fetus, are not currently known. Insulin-like growth factor II (IGF-II) has been shown to be an important regulator of fetoplacental growth. This growth factor must undergo posttranslational processing, and, thus, we hypothesized that aberrant processing of pro-IGF-II to IGF-II may be a cause of IUGR. Here, we have found that the proprotein convertase PC4 is expressed in the human placenta and that it cleaves pro-IGF-II to generate the intermediate processed form, IGF-II (1–102) and, subsequently, mature IGF-II (1–67), which are accounted for by the removal of terminal basic residues by carboxypeptidases. This processing confers the ability of IGF-II to activate invasive trophoblast cells through AKT phosphorylation, whereas inhibition of PC4 by a PC4-specific inhibitor blocks pro-IGF-II processing and reduces trophoblast cell migration, which can be partly restored by addition of mature IGF-II. Consistent with the hypothesis that IGF-II processing is implicated in IUGR, sera of patients carrying IUGR fetuses displayed elevated levels of pro-IGF-II. Thus, abnormal processing of IGF-II by PC4 may represent a previously uncharacterized mechanism involved in the pathophysiology of fetoplacental growth restriction, and elevated pro-IGF-II may be a useful clinical marker for risk of IUGR.

insulin-like growth factor II | intrauterine growth restriction

Fetal growth restriction remains a leading cause of perinatal mortality in the developed world and has important consequences for adult health by increasing the risks of hypertension, diabetes, and hyperlipidemias (1, 2). To date, the mechanisms responsible for aberrant fetoplacental growth remain to be fully elucidated. Our deficient knowledge in this area is highlighted by the lack of useful diagnostic and therapeutic interventions for fetuses at risk.

The insulin-like growth factor-II (IGF-II) gene is genomically imprinted and paternally expressed in the fetus and placenta (3). Accumulated evidence suggests that IGF-II plays a key role in regulating fetoplacental growth (3–6) by stimulating extravillous trophoblast migration/invasion (7) and facilitating nutrient supply through the development of placental exchange (8). Disturbances in IGF-II expression and activity are associated with serious complications of human pregnancy, including intrauterine growth restriction (IUGR) (6, 9). Placental-specific IGF-II knockout, without affecting IGF-II level in fetal tissue and circulation, leads to reduced placental, and then fetal, growth, suggesting that it is a major regulator of fetoplacental growth (5).

In addition to dysregulation of IGF-II gene expression, abnormal processing of IGF-II also may alter its biological activity. Mature IGF-II, a 7.5-kDa peptide containing 67 amino acids, is originally synthesized as a biologically inactive pro-IGF-II peptide (156 amino acids), which subsequently undergoes regulated endoproteolytic cleavage to the mature form. In addition to mature IGF-II, two "big" variants, IGF-II (1–87) and IGF-II (1–104), have been identified in human and bovine serum (10–13). They are 11–17 kDa in size, contain 87 or 104 amino acids, respectively, and differ in glycosylation (10–14). It has been suggested that big IGF-II is biologically active but may have different activity in tumor cells when compared with mature IGF-II (15). However, whether posttranslational processing of pro-IGF-II or the balance of mature and big forms are important regulators of placental and fetal growth is not known.

Proprotein convertases (PCs), a family of serine proteases structurally related to the endoproteases bacterial subtilisin and yeast kexin, normally cleave precursors of polypeptides at the carboxyl-terminal end of single or paired basic residues. To date, seven members of mammalian PCs have been identified (16, 17). Of these PCs, furin, PACE4, PC5/PC6, and PC7 exhibit widespread tissue distribution, whereas PC1 and PC2 are expressed only in endocrine and neuroendocrine tissues and cells. Of the proprotein convertases, PC4 is interesting in being expressed solely in reproductive tissues, and the presence of PC4 outside of the gonads (testis and ovary) has never been documented (18–20).

The identification of various IGF-II species and the presence of paired basic residues at the cleavage site of these variants have led to the prediction that pro-IGF-II may be cleaved at Arg-104, Lys-88, and Arg-68 by PCs. However, cotransfection of Furin, PACE4, PC6A, PC6B, or PC7 with pro-IGF-II failed to produce mature IGF-II (1–67) or IGF-II (1–87) (21). To date, the proteolytic pathway leading to mature IGF-II is not understood, and the identity of the specific PC(s) responsible for the physiological maturation of pro-IGF-II, including in placenta, remains unknown.

In a previous study, the proteolysis of a synthetic peptide of IGF-II [IGF-II (63–72), Ac-PA**K**SE**R** ↓ DVST], encompassing the proposed processing site, has been demonstrated *in vitro* with recombinant PC4 (22), suggesting that PC4 might be involved in physiological pro-IGF-II processing. In this study, we investigate the possible placental expression of PC4 and IGF-II and the involvement of PC4 in the processing of pro-IGF-II with its influence on trophoblast migration, an essential step for normal placental and fetal growth. Our current data support the concept that pregnancy is associated with an increased PC4 processing of IGF-II from its pro to active form, possibly promoting fetoplacental growth. Our studies reveal a previously uncharacterized mechanism involved in the control of placental development and provide evidence that dysregulation of this mechanism is important in the pathophysiology of IUGR. In addition, we demonstrate that mothers carrying growth-restricted fetuses have an IGF-II serum profile consistent with aberrant IGF-II processing as an underlying mechanism and suggesting the possibility of a useful screening tool for this important pregnancy complication.

Materials and Methods

Cell Culture. The HTR-8/SVneo cell line was derived from human invasive extravillous trophoblasts and was provided by Charles H. Graham (Queen's University, Kingston, ON, Canada). Cells were cultured in RPMI 1640/10% FBS with antibiotics.

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Abbreviations: dR8, 8-dextro-Arginine; IGF-II, insulin-like growth factor II; IUGR, intrauterine growth restriction; PC, proprotein convertase.

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Sample Collection. Serum and placental tissues were obtained from eight normal pregnant women (birth weight between the 10th and 90th percentile) and seven patients with IUGR (birth weight <10th percentile) in the third trimester immediately after delivery. Fetuses with either chromosomal or congenital anomalies and maternal medical complications were excluded. Patients were matched for gestational age. Placental tissues also were obtained from first trimester pregnancy immediately after therapeutic termination. Serum samples were also obtained from three healthy nonpregnant volunteers.

RT-PCR Analysis. Transcripts of PC4, IGF-II, and human β-actin were determined with RT-PCR.

Western Blot Analysis. Conditioned media were concentrated with Microcon YM-3 columns (Millipore). The proteins in the media were examined with Western blot analysis by using monoclonal IGF-II (1–67) (clone S1F2, Upstate Biotechnology, Lake Placid, NY), polyclonal IGF-II E (89–101) (GroPep, Adelaide, Australia), or monoclonal V5 (Invitrogen) antibody. Whole-cell lysate and placental tissue protein extracts were examined by Western blot analysis using polyclonal PC4 (Belgian, as described in ref. 22), polyclonal phospho-AKT (Cell Signaling Technology, Beverly, MA), and monoclonal GAPDH (loading control, Abcam Ltd., Cambridge, U.K.) antibodies.

***In Situ* Hybridization for Study of PC4 mRNA Expression.** The placental tissue paraffin sections were hybridized with DIG-labeled antisense RNA probe (74-221, GenBank accession no. AY358963, sense RNA probe as negative control).

Immunohistochemistry for Localization of PC4 Protein. The deparaffinized and rehydrated sections of placental tissue were incubated with polyclonal PC4 (606-06) antibody. Primary antibodies were substituted with normal rabbit serum as a negative control.

Enzymatic Deglycosylation. Glycosidase digestion was performed by using the Enzymatic CarboRelease Kit (QA-Bio, San Mateo, CA) under nondenaturing conditions according to manufacturer's instructions.

Plasmid Construction, Site-Directed Mutagenesis, and Transfection. A pEF6/V5-His-TOPO/pro-IGF-II (pEF6/IGF-II) vector was constructed for expression of pro-IGF-II with V5 epitope at C-terminal. pEF6/V5-His-TOPO vector containing LacZ gene (pEF6/LacZ) was used as a control vector. The pEF6/IGF-II(R68A), pEF6/IGF-II(K88A), and pEF6/IGF-II(R104A) mutants were obtained by point mutation by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). A pCIneo vector for expression of rat PC4 carboxyl-terminally tagged with a V5 epitope also was constructed.

Induction of AKT Phosphorylation by IGF-II Variants. HTR8/SVneo cells were starved in serum-free medium overnight and stimulated for 15 min with either recombinant IGF-II variants (pro-IGF-II, big IGF-II (1–104), and mature IGF-II, all provided by GroPep) or pro-IGF-II treated with recombinant PC4 at different time durations. Aliquots of extracted protein from cell lysates (50 μg) were analyzed by Western blot with phospho-AKT antibody and GAPDH as a loading control.

***In Vitro* pro-IGF-II Processing by Recombinant PC4.** The reaction mixture (recombinant pro-IGF-II and recombinant PC4) was split into two aliquots. The IGF-II profiles were analyzed by IGF-II (1–67) immunoblotting from one aliquot, and the ability to induce AKT phosphorylation in HTR8/SVneo cells by pro-IGF-II and its processing product by PC4 were examined with the other aliquot, as described above. Concentrated spent media also were incubated with recombinant PC4 protein to assess the endogenous IGF-II processing by PC4.

PC4 and Furin Inhibitor Assays. The PC4-specific inhibitors used in this study was developed from its own prodomain sequence (YQTLRRRVKR) near the primary activation site. A cell permeable element, 8-dextro-arginine (dR8), was attached through a linker (2 units of epsilon amino hexanoic acid) to rPC4 (75–84). A fluorescent moiety also was linked to the extreme N-terminal end to facilitate the detection of its cellular transport through fluorescence microscopy. The measured inhibition constant (K_i) for our PC4 inhibitor against a small fluorogenic peptide was found to be in the nanomolar range, whereas K_i for other recombinant PCs is in micromolar range, suggesting this inhibitor is highly specific for PC4. Cells were cultured with the PC4 inhibitors (with or without dR8) or furin inhibitor (Decanoyl-RVKR-cmk, Calbiochem) and IGF-II profile in the concentrated media were assessed.

Migration Assay. Migration of trophoblast cells was determined by their ability to cross the 8-μm pores of migration chambers as described in ref. 23 with modification.

Determination of IGF-II(s) Content in the Serum. Aliquots of serum sample (1 μl) were applied to Western blot analysis under nonreducing condition by using the IGF-II (1–67) monoclonal antibody.

Statistical Analysis. Cell migration data are expressed as the mean ± SEM for three independent experiments and analyzed by using one-way ANOVA and subsequently with Newman–Keual multiple comparison tests and the two-tailed Student *t* test.

Supporting Information. A detailed description of all materials and methods is provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

Results

PC4 Is Expressed in Human Trophoblast Cells and Placental Tissue. The PC4-specific PCR product, a 678-bp band on agarose gel electrophoresis, was amplified by RT-PCR in HTR8/SVneo cells and human placental tissues (first and third trimesters) (Fig. 1*a*). Western blot analysis of HTR8/Svneo cell lysate and human placental tissue extracts revealed bands for 72-kDa pro-PC4 and mature PC4, a 54-kDa protein (Fig. 1*b Left*). A strong 72-kDa band and a weak 54-kDa band in cells transfected with PC4-V5 vector showed immunoreactivity when probed with the V5 antibody (Fig. 1*b Right*), confirming the specificity of the bands detected by the PC4 antibody. *In situ* hybridization revealed that PC4 mRNA was localized predominantly in the cytotrophoblast layer during the first trimester and in syncytiotrophoblast and stroma cells and, to a lesser extent, in cytotrophoblast during the third trimester (Fig. 1*c*). PC4 immunosignals were detected in villous cytotrophoblasts and syncytiotrophoblasts in both first and third trimester placental tissues (Fig. 1*d*).

ProIGF-II, Big IGF-II (1–102), and Mature IGF-II (1–67) Are Secreted by Human Trophoblast Cells. In addition to the expected 7.5-kDa band for mature IGF-II (1–67), two bands at 22 and 24 kDa belonging to pro-IGF-II, along with at least three bands of big-IGF-II between 10 and 17 kDa, also were detected with monoclonal antibody against IGF-II (1–67) in the conditioned medium (Fig. 2*a*). Pro-IGF-II was also detected in the placental tissue. To distinguish whether big forms of IGF-II is IGF-II (87) or IGF-II (1–104), the membrane was also probed with a polyclonal antibody against IGF-II (89–101) in which pro-IGF-II and big IGF-II bands were also recognized. After enzymatic deglycosylation, the 26-kDa band was reduced in size to 24 kDa (Fig. 2*b*), suggesting that these bands represent pro-IGF-II glycosylated and nonglycosylated forms, respectively. IGF-II immunoblotting also revealed that the multiple

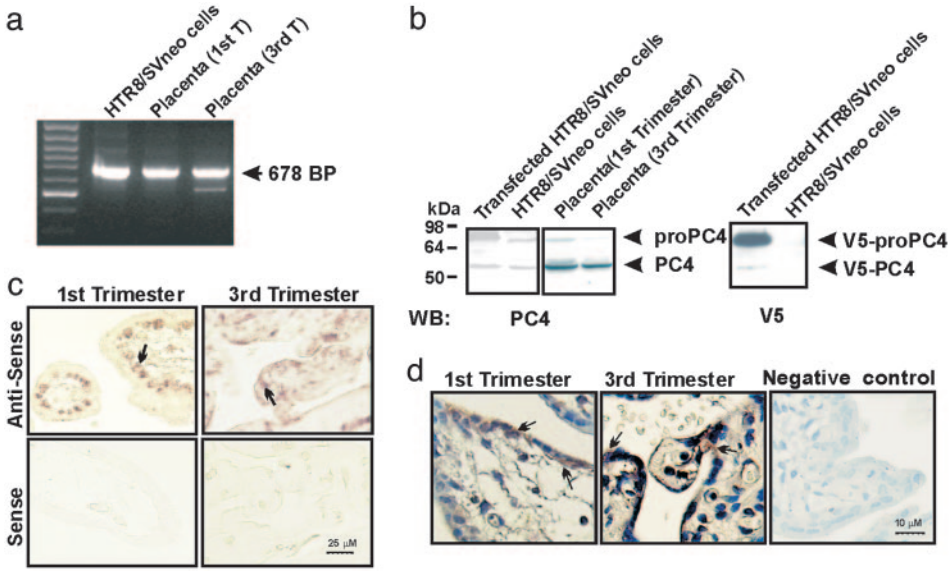

Fig. 1. Expression and localization of PC4 in human trophoblasts (HTR8/Svneo cells) and placental tissues. (*a*) Presence of PC4 mRNA in trophoblast cells and placental tissues from first and third trimesters of pregnancy was demonstrated by RT-PCR. (*b*) Detection of PC4 protein in trophoblast cells/placental tissues (*Left*) and PC4-V5 protein in trophoblasts transfected with PC4-V5 cDNA (*Right*) by PC4 and V5 immunoblotting, respectively. (*c*) Localization of PC4 mRNA in placental tissues by *in situ* hybridization. (*d*) Localization of PC4 protein in placental tissues by immunohistochemistry. PC4 immunosignals are indicated by arrows.

bands between 11 and 17 kDa were transformed to a single 11-kDa band after treatment with glycosidase, suggesting that all these protein bands represent one cleaved form of pro-IGF-II with different sugar moieties (Fig. 2*b*).

Pro-IGF-II Is Proteolytically Processed at Arg-104 and Arg-68, but Not at Lys-88. To identify the cleavage sites of pro-IGF-II that generate the different IGF-II isoforms, we constructed a pro-IGF-II vector and three mutants [IGF-II(R68A), IGF-II(K88A), and IGF-II(R104A)] by replacing Arg-68, Lys-88, and Arg-104 residues with Ala, respectively, by using site-directed mutagenesis. HTR8/SVneo cells were transfected with either the control vector, wild-type pro-IGF-II, or one of the three pro-IGF-II mutants [IGF-II(R68A), IGF-II(K88A), and IGF-II(R104A)]. The concentrated spent media from the experimental groups were analyzed by immunoblotting by using either IGF-II (1–67) (for detection of N-terminal fragment of pro-IGF-II) or V5 (for detection of C-terminal fragment of pro-IGF-II) antibody. After transfection with IGF-II(R104A) mutant, neither big IGF-II nor the corresponding cleavage fragment IGF-II(CT)-V5 were generated (Fig. 3*a*). There was an accumulation of pro-IGF-II, suggesting that Arg-104 is an important residue for pro-IGF-II processing (Fig. 3*a*). Expression of IGF-II(R68A) vector decreased mature IGF-II (1–67) content compared with the control and led to an accumulation of big IGF-II (Fig. 3*a*), suggesting that mature IGF-II is produced by cleavage of big IGF-II at Arg-68. Moreover, after IGF-II(R104A) transfection, mature IGF-II (1–67) did not increase compared with the content of endogenous product in the transfection with control vector. These data suggest that processing of pro-IGF-II at Arg-104 is necessary for the generation of big IGF-II and the subsequent mature IGF-II. However, expression of IGF-II(K88A) did not influence the generation of big IGF-II and IGF-II(CT)-V5 or the level of mature IGF-II compared with wild-type IGF-II expression (Fig. 3), suggesting that Lys-88 is not a site of pro-IGF-II processing in this cell line.

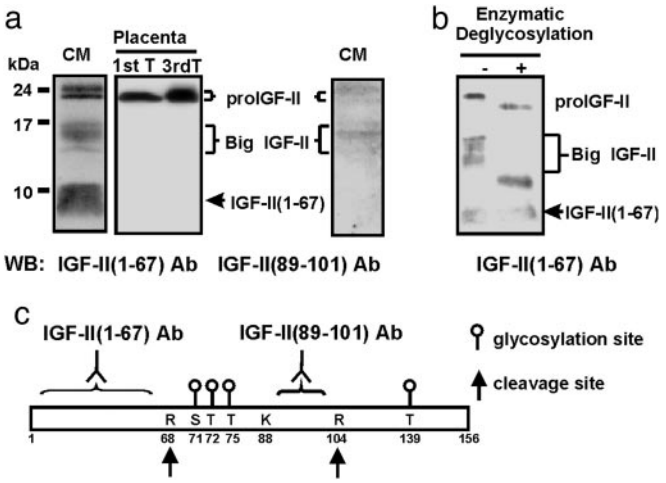

Fig. 2. HTR8/SVneo cells secrete different isoforms of IGF-II. (*a*) IGF-II variants in the concentrated conditioned medium (CM) of HTR8/SVneo cells and human placental tissues were assessed by Western blot with antibodies against IGF-II peptide 1–67 and 89–101, respectively. (*b*) Proteins from concentrated spent media with or without glycosidase digestion were analyzed by IGF-II (1–67) immunoblotting. (*c*) Schematic representation of pro-IGF-II structure. The cleavage sites are indicated by arrows. The various epitopes for the IGF-II antibodies and the sites of glycosylation are also indicated.

PC4 Activates pro-IGF-II by Proteolytic Action to Generate Big and Mature IGF-II. We next examined the ability of inducing AKT phosphorylation by different IGF-II isoforms, thus assessing their biological activity. Mature and big IGF-II increased phosphorylated AKT protein content at a concentration as low as 0.1 nM (Fig. 4*a*). However, induction of AKT phosphorylation by pro-IGF-II was only found at the concentration of 10 nM. We also examined the role of PC4 in proteolytic processing of pro-IGF-II and the influence of pro-IGF-II processing by PC4 on the ability to induce AKT phosphorylation in HTR8/SVneo cells. After treatment with recombinant PC4, pro-IGF-II was converted to big IGF-II, and subsequently to mature IGF-II as the time of incubation is increased (Fig. 4*b*). Phospho-AKT content of HTR8/SVneo cells increased after treatment with pro-IGF-II preincubated with PC4 (Fig. 4*c*). To assess the role of PC4 in the processing of endogeneous pro-IGF-II and big IGF-II, concentrated spent medium from

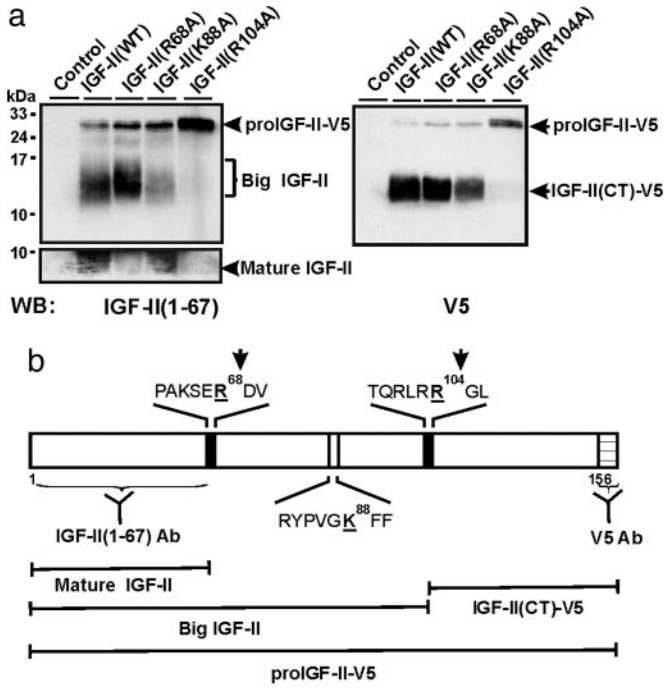

Fig. 3. ProIGF-II is proteolytically processed at Arg-104 and Arg-68 but not at Lys-88. (*a*) ProIGF-II-V5 and processed fragments in the concentrated spent media were analyzed by immunoblotting with IGF-II (1–67) (*Left*) or V5 antibody (*Right*). HTR8/SVneo cells were transfected 48 h with pFE6/lacZ (control vector), wild-type IGF-II [IGF-II(WT)-V5], or one of the three mutants [IGF-II (R68A), IGF-II (K88A), and IGF-II (R104A)]. Blots in *Upper Left* (short exposure time) and *Lower Left* (long exposure time) were from the same gel to show extremely high exogenous pro- and big IGF-II and a relative low content of mature IGF-II, respectively. (*b*) Schematic representation of pro-IGF-II-V5 structure. The sequences of predicted cleavage sites are indicated by arrows. Amino acids underlined are mutated to alanine in three mutant vectors. The regions recognized by antibodies and products generated by cleavage are also indicated.

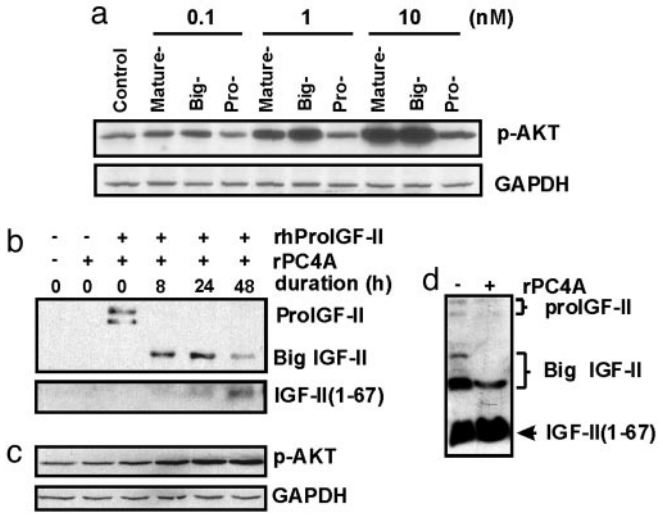

Fig. 4. PC4-mediated pro-IGF-II processing increases AKT phosphorylation in HTR8/SVneo cells by generating big and mature IGF-II. (*a*) Mature and big IGF-II are more potent in inducing AKT phosphorylation than pro-IGF-II. HTR8/SVneo cells starved overnight in serum-free medium were stimulated for 15 min with recombinant mature, big and pro-IGF-II at different concentrations (0.1, 1, and 10 nM). The protein content of *p*-AKT and GAPDH were analyzed by Western blot. (*b*) Pro-IGF-II is proteolytically processed by recombinant PC4 to generate big and mature IGF-II. Recombinant pro-IGF-II was incubated with recombinant PC4 for different time durations (0, 8, 24, and 48 h). The protein contents of IGF-II variants in the reaction mixture were analyzed by IGF-II immunoblotting. (*c*) The products of PC4-mediated pro-IGF-II processing are more potent in inducing AKT phosphorylation in HTR8/SVneo cells than pro-IGF-II without processing. HTR8/Svneo cells were treated for 15 min with pro-IGF-II preincubated with recombinant PC4 as described above. Phospho-AKT and GAPDH content were analyzed by Western blot. (*d*) Endogenous pro-IGF-II and big IGF-II are cleaved by recombinant PC4. Concentrated spent medium of HTR8/SVneo cells were treated for 16 h with or without recombinant PC4 protein. The protein content of IGF-II variants were assessed by Western blot.

HTR8/SVneo cells were incubated with recombinant PC4 and the IGF-II profiles were examined by immunoblotting. After incubation with PC4, both pro-IGF-II (1–156) and big IGF-II contents decreased, whereas the protein content of mature IGF-II (1–67) increased compared with those without PC4 treatment (Fig. 4*d*).

Intracellular Pro-IGF-II Processing Is Blocked by the PC4-Specific Inhibitor. If PC4 is indeed involved in the maturation of IGF-II, it is expected that inhibition of PC4 activity should reduce the production of mature IGF-II (1–67) and result in the accumulation of pro-IGF-II (1–156) and big IGF-II. To test this hypothesis, recently developed PC4-specific inhibitors with or without a cell-permeable element (dR8) were used. PC4 activity assay in the presence of inhibitors showed that both inhibitors have similar ability to inhibit recombinant PC4 activity (Fig. 8, which is published as supporting information on the PNAS web site). Only the PC4 inhibitor with dR8 element was able to penetrate HTR8/SVneo cells, as indicated by green fluorescence after 24-h incubation with the inhibitors (25 μM, Fig. 9, which is published as supporting information on the PNAS web site). Upon treatment with the cell-permeable inhibitor, there was an accumulation of both big IGF-II and pro-IGF-II, with mature IGF-II (1–67) content in the spent media being reduced extensively at 50 μM. The PC4 inhibitor without dR8 had no effect on the IGF-II profile (Fig. 5). Semiquantitative RT-PCR revealed that the PC4 inhibitors had no influence on the abundance of IGF-II mRNA (Fig. 10, which is published as supporting information on the PNAS web site). To confirm the specificity of this inhibitor, the protein content of IGF-IR, a furin substrate (24), was examined in HTR8/SVneo cells treated with either the PC4 inhibitor or a commercially available furin inhibitor (Decanoyl-RVKR-cmk). As expected, the furin inhibitor increased the 200-kDa pro-IGF-IR content and decreased the protein content of α and β subunits. However, the PC4 inhibitor had no effect on IGF-1R protein content (Fig. 11, which is published as supporting information on the PNAS web site). In contrast, the furin inhibitor had no influence on mature IGF-II content, although a concentration-dependent accumulation of pro-IGF-II content was observed (Fig. 12, which is published as supporting information on the PNAS web site).

PC4 Inhibitor Reduces Trophoblast Cell Migration. Consistent with previous studies (25), we have demonstrated that recombinant

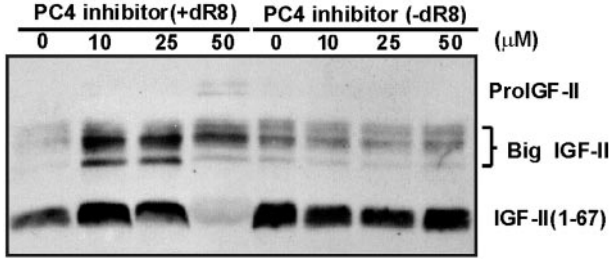

Fig. 5. Intracellular pro-IGF-II processing is inhibited by the PC4-specific inhibitor with dR8. HTR8/SVneo cells were incubated for 48 h with PC4 inhibitors (± dR8 at 0, 10, 25, and 50 μM) in serum-free medium. The protein content of IGF-II isoforms was examined by Western blot.

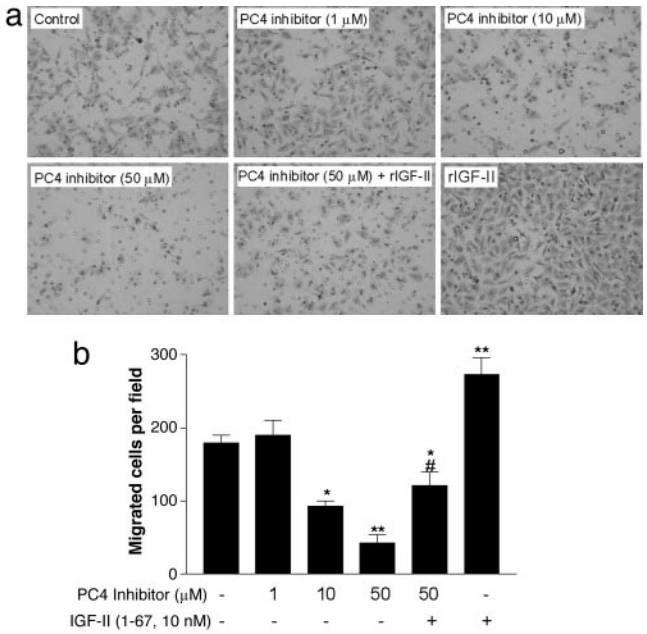

Fig. 6. PC4 inhibitor reduces trophoblast cell migration. (*a*) Representative images of migrated HTR8/SVneo cells. The migration assay was conducted in the presence of different concentrations of the PC4 inhibitor (0, 1, 10, and 50 μM) or IGF-II (50 nM) with or without PC4 inhibitor (50 μM). (*b*) Graphical presentation of cell migration ability. The data are expressed as the mean ± SE of three measurements. *, $P < 0.05$, and **, $P < 0.01$, compared with control; #, $P < 0.05$ compared with 50 μM of PC4 inhibitor alone.

human mature IGF-II (1–67) (10 nM) stimulates trophoblast migration, as indicated by increases in the number of cells found on the lower surface of the migration chambers ($P < 0.01$, Fig. 6*a Bottom Right*). Treatment with the cell-permeable PC4 inhibitor reduced HTR8/SVneo cell migration in a concentration-dependent manner (Fig. 6*b*) without influencing cell viability (as assessed by MTT assays; data not shown). The addition of mature IGF-II (10 nM) attenuated the effect of the PC4 inhibitor and partly restored cell migration (Fig. 6).

Pro-IGF-II Is Present in Human Serum and Its Level Increased in Pregnancy with IUGR. The presence of IGF-II variants in human serum was assessed by immunoblotting. In addition to the detection of a 7.5-kDa protein (mature IGF-II) and 10- to 14-kDa proteins corresponding to high molecular mass IGF-IIs, a 26-kDa protein was also detected (Fig. 13, which is published as supporting information on the PNAS web site). The size of this protein suggests that it may be pro-IGF-II; however, the presence of pro-IGF-II in the circulation has not been documented previously (11). To confirm the identity of this peptide, the same serum samples were assessed by immunoblotting with two different antibodies (monoclonal and polyclonal IGF-II) in the absence or presence of recombinant IGF-II protein (10 μg/ml) as a blocking peptide. This 26-kDa protein was detected by both antibodies and the appearance of the 26-kDa band, along with mature IGF-II and the higher molecular mass IGF-II signals, were abolished when incubated with IGF-II antibody preabsorbed with recombinant human mature IGF-II(1–67) (Fig. 13), thereby confirming that the 26-kDa protein is indeed pro-IGF-II. Comparing IGF-II serum profiles from pregnant and nonpregnant individuals revealed increased levels of mature IGF-II and decreased levels of pro-IGF-II to be associated with pregnancy (Fig. 14, which is published as supporting information on the PNAS web site), suggesting increased processing from establishment of the fetoplacental unit. We then compared IGF-II profiles of sera from eight normal pregnant women with those of seven women with

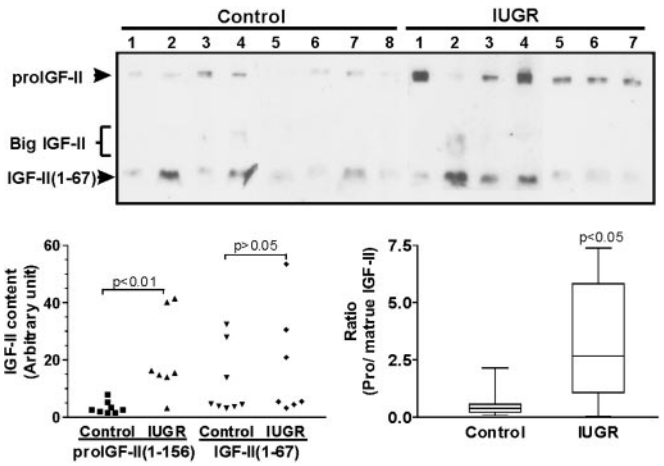

Fig. 7. The level of Pro-IGF-II in human serum and the ratio of pro-IGF-II (1–156) to mature IGF-II (1–67) are higher in the IUGR patient. (*Upper*) Western blot depicting changes in density of serum pro-IGF-II, big IGF-II, and mature IGF-II in women with normal pregnancy and pregnancy with IUGR. (*Lower Left*) Relative protein content of pro-IGF-II and mature IGF-II with densitometric value. (*Lower Right*) The serum ratio of pro-IGF-II to mature IGF-II in pregnancies complicated with IUGR and control group.

IUGR all in the third trimester. Pro-IGF-II content and the ratio of pro-IGF-II to mature IGF-II were significantly higher in the IUGR patients compared with those with normal pregnancies ($P < 0.01$ for pro-IGF-II and $P < 0.05$ for the ratio, Fig. 7), whereas no difference in mature IGF-II was seen between the two groups.

Discussion

We have demonstrated that PC4 is expressed in human placenta and in extravillous invasive trophoblasts and that PC4 is able to completely process pro-IGF-II at Arg-104 and Arg-68 to generate big IGF-II and mature IGF-II (1–67). We also showed that IGF-II is biologically activated by PC4 through posttranslational modification. This activation is reflected in the promotion of trophoblast migration, a step essential to adequate growth of the placenta and the fetus and which is recognized as severely impaired in pregnancies complicated by fetal growth restriction. In addition, IGF-II serum profiles of women carrying fetuses with growth restriction display greater levels of pro-IGF-II when compared with controls, thus reflecting aberrant processing and supporting a role for IGF-II processing in clinical IUGR. Taken together, these data provide a mechanism to explain the posttranslational regulation of IGF-II in placental development and how aberrant processing of this essential growth factor by PC4 plays a role in inadequate trophoblast migration and, thus, fetal growth restriction. Finally, we also suggest the possibility that maternal IGF-II serum profiles may represent a marker for fetuses at risk of growth restriction.

We have demonstrated that pro-IGF-II is processed at two separate sites: **RLRR^{104}**↓ and **KSER^{68}**↓ to generate IGF-II (1–104) and IGF-II (1–68), respectively. Exposed basic residues at the carboxyl terminal are further cleaved by carboxypeptidases E and D (26), which are widely distributed in the placenta (27). Therefore, Arg-68 is further cleaved from IGF-II (1–68) to generate a peptide containing 67 amino acids, the mature IGF-II (1–67) form. By the same mechanism, exposed carboxyl-terminal Arg-104 and Arg-103 of IGF-II (1–104) might be removed sequentially to generate IGF-II (1–102) terminated as Leu-102. More precisely, the high molecular mass IGF-II detected in the conditioned medium of trophoblasts may represent this form of IGF-II (1–102). In this study, we have also shown that IGF-II (1–104) or IGF-II (1–102) is further processed at Arg-68 to generate mature IGF-II (1–67), an N-terminal cleavage fragment. In another system,

a 34-amino acid peptide corresponding to Asp-69 to Leu-102 of IGF-II was identified in cultured murine βTC6-F7 beta cells (28), further supporting the finding that pro-IGF-II is proteolytically processed *in vivo* at position Arg-104 and Arg-68. We did not find pro-IGF-II proteolytically processed at Lys-88 in the trophoblasts. However, the existence of big IGF-II (1–87) in human serum (12) suggests that a previously uncharacterized enzyme-mediated cleavage at Lys-88 may exist in other cell systems.

There is little information available regarding the subcellular localization of PC4, and it is not known whether active PC4 is able to be secreted extracellularly. We have demonstrated that all IGF-II isoforms are present in the medium. However, because of their very rapid secretion, big IGF-II and mature IGF-II cannot be detected in placental tissue itself (Fig. 2*a*) and are only present in very limited amounts within the cell (data not shown). To examine the location of pro-IGF-II processing, PC4 inhibitors with or without permeable element were developed. The PC4 inhibitor with dR8 blocked pro-IGF-II processing, whereas that without dR8 had no effect on processing, suggesting that PC4-mediated pro-IGF-II processing takes place intracellularly. It has been shown that polyarginines alone serve as potent furin inhibitors (29). However, in our study, we showed that the PC4 inhibitor containing dR8 had no effect on the processing of pro-IGF1R (a furin substrate, Fig. 11), suggesting that the cell-permeable element did not inhibit furin and confirming the specificity of the PC4 inhibitor. On the other hand, the furin inhibitor, Decanoyl-RVKR-cmk had no influence on mature IGF-II (1–67) and big IGF-II protein content, which is consistent with the previous reports that furin is not involved in the final maturation of IGF-II (21).

Whether different IGF-II forms (including its pro form) possess different biological activities is not known. IGF-II activates the phosphatidylinositol 3-kinase pathway by IGF-1R ligation. A downstream effect of this signaling cascade is AKT phosphorylation, which has important roles in glucose metabolism, cell proliferation, survival, and cell migration (30). Previously we have demonstrated that AKT phosphorylation is essential for extravillous trophoblast migration (23). In this study, we observed that induction of AKT phosphorylation by big and mature IGF-II is more potent than that by pro-IGF-II (Fig. 4*a*). Pro-IGF-II upon processing by recombinant PC4 increased its ability to stimulate AKT phosphorylation (Fig. 4*c*). All of these roles suggest that pro-IGF-II processing is physiologically significant.

Shallow trophoblast invasion is an important etiological factor in fetoplacental growth restriction. IGF-II stimulates extravillous trophoblast invasiveness primarily by promoting migration (7, 25). Consistent with this finding, we have found that mature IGF-II induces HTR8/Svneo cell migration. The PC4-specific inhibitor reduced haptotaxis of trophoblasts in a concentration-dependent manner, and this inhibitory effect was attenuated by the addition of mature IGF-II. MTT assay indicated PC4 inhibitor had no effect on cell viability (data not shown). These data suggest that inhibition of HTR8/SVneo migration by a PC4-specific inhibitor can be explained, at least, in part, by the reduction of active mature IGF-II (1–67) resulting from decreased PC4-mediated processing of pro-IGF-II.

Although IGF-II is involved in the regulation of fetal growth, the correlation of IGF-II levels in the maternal circulation with fetal growth is not consistent (31, 32). However, serum IGF-II levels in these reported studies were determined by the immunoassays that could not distinguish between mature IGF-II, big IGF-II, and pro-IGF-II. In this study, Western blot analysis was used to separate different forms of IGF-II, and we thus were able to show that women with IUGR had higher circulating levels of pro-IGF-II compared with women with normal pregnancies, suggesting that IUGR is associated with abnormal posttranslational processing of IGF-II. Although there is no difference in the serum level of mature IGF-II in women with IUGR compared with women with normal pregnancy, whether higher pro-IGF-II level in the former group may influence the function of mature IGF-II by interfering with receptor ligation requires further investigation. In addition, IGF-binding proteins (IGFBPs) are known to modify the bioactivity of IGF-IIs (33), and it is possible that abnormal processing products of pro-IGF-II may have a different ability to form complexes with IGFBPs.

In conclusion, our data support the role of PC4-mediated IGF-II processing in fetoplacental development and in the pathology of fetal growth restriction.

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