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Abogado
CARLOS R. OLARTE

ASUNTO

Radicación	08 129 816
Trámite	011
Evento	378
Actuación	519
Oficio	11203
Folios	15

Patente de Invención	✓			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	✓	Capítulo I	✓	Capítulo II	

No. Solicitud Internacional PCT/US2007/070468
Fecha de Presentación Internacional 06/Junio/2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 99 a 170	05/Diciembre/2008
Reivindicaciones:	Folios 171 a 174	05/Diciembre/2008
	Folios 204 a 208	16/Diciembre/2008
Dibujos:	Folios 176 a 195	05/Diciembre/2008
Prioridad:	US 60/812,349	09/Junio/2006
	US 60/862,244	20/Octubre/2006
	US 60/897,187	24/Enero/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Polipéptidos de factor de crecimiento de tipo insulina".

El problema planteado en esta solicitud consiste en la necesidad de estabilizar IGF-1 como un fármaco formando un complejo con una de sus proteínas de unión.

Para resolver este problema técnico la solicitud en estudio proporciona nuevos polipéptidos precursores de IGF-1 y IGF-2 conteniendo substancialmente un péptido

E que ha sido modificado para prevenir, reducir, o evitar la unión típica a proteasa tipica responsable de la liberación del IGF-1 o IGF-2 activo a partir de sus péptidos E.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 38/30, C07K 14/65.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 29 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 30 no es patentable, de acuerdo con el Art 14.

No obstante la reivindicación 31 se encuentra encabezada como un polipéptido, éste se encuentra definido en términos de su uso y por lo tanto no es patentable de acuerdo con el Art 14.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 4, 6 – 12, 14, 17, 21 - 28 se refieren a un polipéptido, definiéndolo por su proceso de obtención con pasos de eliminación, mutación, inserción. Éstas son reivindicaciones de producto, y sólo pueden ser definidas en términos de su proceso de fabricación si: el producto es nuevo e inventivo y no se puede definir por sus caracteristicas estructurales. Se sugiere definir el polipéptido por su secuencia de aminoácidos.

La reivindicación 1 contiene el termino "parir", que según el contexto puede corresponder a "partir" y tratarse de un error de transcripción. Se sugiere realizar las aclaraciones y correcciones necesarias.

Las reivindicaciones 2, 3 y 5 nombran los péptidos Ea, Eb y Ec sin definirlos inequívocamente. Se recuerda al solicitante que un péptido se caracteriza por la secuencia exacta de aminoácidos.

La reivindicación 13 nombra una secuencia de consenso de glicosilación N-enlazada definiéndola como NXS/T, esta manera de definir la secuencia no es adecuada puesto que no permite caracterizarla ni distinguirla del estado de la técnica. Se sugiere al solicitante definirla restringiendo el pliego reivindicatorio según lo que encuentra soporte en la descripción.

Las reivindicaciones 15 - 17 se refieren a un polipéptido que contiene un oligosacárido y a un residuo de la proteína de precursor que se reemplaza por un aminoácido natural, el cual es definido por su grupo químico, o por su origen en el caso de los aminoácidos y no por un compuesto como tal o un aminoácido especifico. Esta manera de reivindicar no es adecuada porque no permite distinguir la solicitud del estado de la técnica. Adicionalmente no se encuentra soporte de la obtención o prueba de algún polipéptido que contenga un oligosacárido. Se sugiere restringir el pliego reivindicatorio según lo que se encuentra debidamente soportado en el capitulo descriptivo.

Se encuentra una incongruencia entre el capitulo reivindicatorio donde en la reivindicación 23 referida a una proteína precursora IGF-1 humana se nombra la modificación de la proteína precursor por eliminación o mutación de P4, S5, E6 y R38, mientras que en el capitulo descriptivo se dice que estas modificaciones se realizan en el precursor hIGF-2 (Pág. 26, líneas 13 – 16, Fl. 124). Se sugiere realizar las aclaraciones y correcciones necesarias.

La reivindicación 25 se refiere al residuo S54 el cual es mutado o eliminado, de lo cual no se encuentra sustento ni soporte en el capitulo descriptivo. Esto puede tratarse de un error en la transcripción por lo cual se sugiere realizar las correcciones y aclaraciones necesarias.

Las reivindicaciones 1, 6 – 11 y 22 - 28 se refieren a eliminaciones o mutaciones de uno o más de los residuos G1, P2, E3, R36, R37, R71, S72 de la proteína de precursor IGF-1 humana, y en las reivindicaciones 23 – 28 se nombran eliminaciones o mutaciones de los residuos P4, S5, E6 y R38. Sin embargo, no existe soporte para la eliminación y mutación de cada uno de estos residuos, ni de que la mutación por cualquier aminoácido produzcan el mismo efecto en cuanto a estabilización de IGF-1 o IGF-2. En el capitulo descriptivo se encuentra soporte para:

Precursor IGF-1	
Sustituciones	**Soporte de los ejemplos presentados**
G1	Eliminación
P2	Eliminación
E3	Eliminación
R36	Eliminación, R36A
R37	Eliminación, R37A
R71	Eliminación
S72	Eliminación

Precursor IGF-2	
Sustituciones	**Soporte de los ejemplos presentados**
P4	Eliminación
S5	Eliminación
E6	Eliminación
R38	R38A
R68	Eliminación
D69	Eliminación

Adicionalmente, en la reivindicación 1 y 23 se encuentra la expresión "uno o más" refiriéndose a los residuos de proteína de precursor, sin embargo no se encuentra soporte de que cualquier modificación inclusive en un solo aminoácido presente algún efecto técnico, por el contrario se encuentra en el capitulo descriptivo que los polipéptidos presentan modificaciones en al menos 4 aminoácidos. Se sugiere restringir la solicitud a una razonable generalización del tipo de polipéptidos que ha sido sintetizado ó probado, es decir, que se encuentran debidamente soportados.

En la reivindicación 32 se define un ácido nucleico por el polipéptido que codifica, lo cual no es aceptable porque no se define al ácido nucleico como tal. Un ácido nucleico se caracteriza por su secuencia de nucleótidos.

En las reivindicaciones 33 y 34 se reclama un vector y una célula transfectada definiéndolas únicamente por el ácido nucleico que contienen que a su vez no se encuentra apropiadamente caracterizado. Se sugiere incluir los vectores y células transfectadas que se desean proteger.

Descripción (Art 28 D 486)

Dado que esta solicitud se refiere a secuencias de nucleótidos y aminoácidos, deben presentarse las listas en forma separada de la descripción, bajo el título: "Listado de secuencias" y anexarlas en formato digital.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el requisito de unidad de invención porque se refiere a 2 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características **esenciales**:

1° Polipéptidos que comprenden una proteína precursora de IGF-1 humana en la que se ha modificado la proteína precursora (Divulgados en D2 y D3).
2° Polipéptidos que comprenden una proteína precursora de IGF-2 humana en la que se ha modificado la proteína precursora.

Se sugiere presentar las solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 09/Junio/2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 2004/0116335	Compositions and Methods for Inhibiting the Proliferation and Invasiveness of Malignant Cells Comprising E-Domain Peptides of IGF-1	17/Junio/2004
D2	DUGUAY, S. ET AL, The Journal of Biological Chemistry, Vol. 272, No. 10, Págs. 6663 - 6670	Processing of Wild-type and Mutant Proinsulin-like Growth Factor-IA by Subtilisin-related Proprotein Convertases	07/Marzo/1997
D3	DUGUAY, S. ET AL, The Journal of Biological Chemistry, Vol. 270, No. 29, Págs. 17566-17574	Mutational Analysis of the Insulin-like Growth Factor I Prohormone Processing Site	21/Julio/1995

D1 http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2004116335A1&KC=A1&FT=D&ND=3&date=20040617&DB=EPODOC&locale=en_EP divulga (Abstract) el péptido IGF y sus dominios E, los ácidos nucleicos que las expresan, y la transformación de células con estos ácidos nucleicos.

D2 http://hwmaint.jbc.org/cgi/reprint/272/10/6663 divulga (Pág. 6663, Abstract, Fl. 221) el proceso de maduración de pro-IGF-1A a su forma madura, en el que la Pro-IGF-1A es procesada en Arg-71 para generar IGF-1-(1-70) y en Arg-77 para producir IGF-1-(1-76). La pro-IGF-1A mutante es expresada en células 293 y en células de ovario de hámster chino deficientes de furina.

D3 http://www.jbc.org/content/270/29/17566.full.pdf+html divulga (Pág. 17566, Abstract, Fl. 225) que IGF-1 es sintetizado inicialmente como un precursor pro-hormona que es convertida para madurar a IGF-1 por la remoción del dominio carboxi terminal E y que la regulación de la conversión de pro-IGF-1 a IGF-A madura es un mecanismo por el cual la actividad biológica de este factor de crecimiento puede ser modulado.

9. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

GRUPO INVENTIVO I

La reivindicación 1 consiste en un polipéptido que comprende una proteína de precursor IGF-1 humana, en donde la escisión del péptido E a partir de IGF-1 mediante una proteasa se reduce a través de la modificación de la proteína de precursor, y en donde uno o más de los siguientes residuos de proteína de precursor es eliminado o mutado: G1, P2, E3, R36 y R37.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características **esenciales**	D1	D3
Un polipéptido que comprende:	Péptido:	Péptido:
Una proteína de precursor IGF-1 humana, en donde la escisión del péptido E a partir de IGF-1 mediante una proteasa se reduce a través de la modificación de la proteína de precursor, y en donde uno o más de los siguientes residuos de proteína de precursor es eliminado o mutado: G1, P2, E3, R36 y R37.	IGF-1 y dominios E de IGF-1	Péptido mutante de pro-IGF-1A

Los documentos D1 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1. El documento D1 da a conocer los péptidos IGF-1 y dominios E de IGF-1 (Pág. 1, párrafo 2; Pág. 2, párrafo 10). El documento D3 revela que IGF-1 es sintetizado inicialmente como un precursor pro-hormona que es convertida para madurar a IGF-1 por la remoción del dominio carboxi terminal E (Pág. 17566, Abstract, Fl. 225) y da a conocer péptidos mutantes de pro-IGF-1A (Pág. 17567, Tabla I, Fl. 225; Pág. 17572, Tabla II, Fl. 228).

El documento D1 también divulga los dominios E pro-IGF-1-a, pro-IGF-1-b y pro-IGF-1-c (Pág. 1, párrafo 5), vectores y células transfectadas (Pág. 4 y 5, ejemplos 1 - 3)

La diferencia entre la invención, definida en la reivindicación 1 y el documento D1 es la modificación de la proteína precursor IGF-1 humana.

El efecto que logra el modificar la proteína precursora de IGF-1 es la obtención de polipéptidos biológicamente activos y estables.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el polipéptido conocido en D1 para obtener un polipéptido biológicamente activo y estable".

> Sin embargo, péptidos mutantes de pro-IGF-1A (Pág. 17567, Tabla I, Fl. 225; Pág. 17572, Tabla II, Fl. 228) y el uso de la regulación de la conversión de pro-IGF-1 a IGF-1 madura como mecanismo por el cual la actividad biológica de este factor de crecimiento puede ser modulado, ya se encontraba divulgada en el documento D3 (Pág. 17566, Abstract, Fl. 225).

En consecuencia, la persona del oficio normalmente versada en la materia, realizaría modificaciones en la pro-IGF-1A teniendo en cuenta las enseñanzas divulgadas en el documento D3 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 31 – 34 no mencionan características

inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 35 consiste en un polipéptido que comprende una proteína de precursor de IGF-1 o IGF-2 no humana, en donde la escisión de péptido E de IGF-1 o IGF-2 mediante una proteasa se reduce a través de la modificación de una proteína precursora.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características **esenciales**	**D2**	**D3**
Un Polipéptido que comprende:	Polipéptido que comprende:	Polipéptido que comprende:
Una proteína de precursor de IGF-1 o IGF-2 no humana, en donde la escisión del péptido E de IGF-1 o IGF-2 mediante una proteasa se reduce a través de la modificación de la proteína precursora.	Una proteína mutante de pro-IGF-1.	Una proteína mutante de pro-IGF-1. Las secuencias de proIGF-1A y proIGF-1B son idénticas en los primeros 16 residuos del dominio E, incluyendo el sitio de escisión de la prohormona. Este sitio se ha conservado en mamíferos, aves, anfibios y teleósteos.

Los documentos D2 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 35. El documento D2 enseña el proceso de maduración de pro-IGF-1A a su forma madura, en el que la Pro-IGF-1A es procesada en Arg-71 para generar IGF-1-(1-70) y en Arg-77 para producir IGF-1-(1-76) (Pág. 6663, Abstract, Fl. 221), y mutantes de la proteína pro-IGF-1A (Pág. 6664, Fig. 1, Fl. 221; Pág. 6665, Tabla I, Fl. 222; Pág. 6666, Tabla II, Fl. 222). El documento D3 revela que IGF-1 es sintetizado inicialmente como un precursor pro-hormona que es convertida para madurar a IGF-1 por la remoción del dominio carboxi terminal E y que la regulación de la conversión de pro-IGF-1 a IGF-1 madura es un mecanismo por el cual la actividad biológica de este factor de crecimiento puede ser modulado (Pág. 17566, Abstract, Fl. 225) y da a conocer péptidos mutantes de pro-IGF-1A (Pág. 17567, Tabla I, Fl. 225; Pág. 17572, Tabla II, Fl. 228).

El documento D2 además enseña que los precursores pro-IGF-1A y pro-IGF-1B son idénticos en los dominios B, C, A y D de la IGF-1 madura, así como en los primeros 16 aminoácidos del dominio E de la pro-región (Pág. 6663, Fl. 221), y da a conocer una mutación de la pro-IGF-1 en R71 (Pág. 6664, Fig. 1, Fl. 221).

En el documento D2 también se divulga que los dominios E pro-IGF-1A y pro-IGF-1B en los primeros 16 residuos del dominio E son idénticos (Pág. 17566, Fl. 225).

La diferencia entre la invención, definida en la reivindicación 35 y el polipéptido del documento D2 consiste en que es un precursor de IGF-1 o IGF-2 no humano.

El efecto que logra el utilizar un precursor de IGF-1 o IGF-2 no humano, es la obtención de polipéptidos de IGF-1 o IGF-2 de otras especies diferentes a la humana.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el polipéptido conocido en D2 para obtener un

polipéptido no humano".

> Sin embargo, el documento D3 ya divulgaba que las secuencias de proIGF-1A y proIGF-1B son idénticas en los primeros 16 residuos del dominio E, incluyendo el sitio de escisión de la prohormona y que este sitio se ha conservado en mamíferos, aves, anfibios y teleósteos. (Pág. 17566, Fl. 225).

En consecuencia, la persona del oficio normalmente versada en la materia, según las enseñanzas del documento D3 en cuanto a la similitud de las pro-IGF en diferentes especies realizaría modificaciones en la proteína precursora de IGF-1 o IGF-2 no humana para llegar así al objeto de la reivindicación 35 de la solicitud. Por lo cual se considera obvia.

Conclusión:
La reivindicación 35 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 2004/0116335	1, 31 – 34	Nivel Inventivo
D2	DUGUAY, S. ET AL, The Journal of Biological Chemistry, Vol. 272, No. 10, Págs. 6663 - 6670	35	Nivel Inventivo
D3	DUGUAY, S. ET AL, The Journal of Biological Chemistry, Vol. 270, No. 29, Págs. 17566-17574	1, 31 – 34	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del titulo primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

27 JUN. 2010

Elaborado por: Jeny Paola Villate Becerra P.Villate
Revisado por: Consuelo Leguizamón

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Processing of Wild-type and Mutant Proinsulin-like Growth Factor-IA by Subtilisin-related Proprotein Convertases*

(Received for publication, October 11, 1996, and in revised form, December 31, 1996)

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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 pentabasic processing motif Lys-*X*-*X*-Lys-*X*-*X*-Arg^{71}-*X*-*X*-Arg-*X*-*X*-Arg^{77}. We have previously shown that human embryonic kidney 293 cells process pro-IGF-IA at Arg^{71} to generate IGF-I-(1–70) and at Arg^{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 pentabasic motif. PC6A and LPC are less active than furin but cleave only at Arg^{71}. PACE4 and PC6B have very little activity on pro-IGF-IA precursors. Wild-type pro-IGF-IA was correctly processed to mature IGF-I in 10 of 10 cell lines that were tested. Since furin, PC6A, and LPC are known to have a broad pattern of tissue distribution and we have demonstrated expression of LPC in RPE.40 cells, our results suggest that these SPCs may be responsible for the endogenous pro-IGF-IA processing activity observed in a wide variety of cell lines.

Insulin-like growth factor I (IGF-I)[1] is expressed ubiquitously throughout embryonic development and postnatal growth of mammalian organisms (1). IGF-I is an important regulator of growth; mice that are homozygous for a disrupted IGF-I gene display a number of developmental abnormalities and most die shortly after birth (2, 3). Overproduction of IGF-I has been associated with many cancers, in which IGF-I may act as an autocrine or paracrine growth factor (4) or an inhibitor of apoptosis (5). Regulation of the biological activity of IGF-I is therefore an important physiological process.

Most hormones and growth factors are initially synthesized as inactive propeptide precursors that are post-translationally processed to the biologically active mature peptide. The subtilisin-like proprotein convertases (SPCs) are a family of enzymes that have been shown to play an important role in the maturation of prohormones and growth factors as well as a number of other protein precursors. PC2 and PC3 (also known as PC1) are restricted in expression to endocrine and neuroendocrine cells and process substrates such as proinsulin and proopiomelanocortin. PC4 is expressed in the testis, and substrates for this processing enzyme have not yet been identified. In contrast, furin, PACE4, PC6A, PC6B, and LPC (also known as PC7, SPC7, and PC8) are expressed in many tissues and may have an important function in proteolytic processing of precursors in the constitutive secretory pathway (6–12).

The mature, 70-amino acid IGF-I molecule can be generated from two different prohormone precursors, pro-IGF-IA or pro-IGF-IB. Pro-IGF-IA and pro-IGF-IB are derived from a single gene by alternative splicing of 3′-exons. The prohormone sequences are identical through the B, C, A, and D domains of mature IGF-I as well as the first 16 amino acids of the E domain or pro-region. Pro-IGF-IA and pro-IGF-IB, therefore, contain the same prohormone processing site at the junction of the D and E domains but diverge in sequence at the carboxyl terminus 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^{77} and that IGF-I-(1–70) is generated by cleavage at Arg^{71}. Cleavage at both of these positions required a P4 basic residue (see Fig. 1). Furthermore, cleavage at Arg^{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

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[1] The abbreviations used are: IGF-I, insulin-like growth factor I; SPC, subtilisin-related proprotein convertase; CMV, cytomegalovirus; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type.

from pBluescript with *Eco*RI and *Sal*I and cloned into *Eco*RI/*Sal*I-digested pCMV6c to generate pCMV-furin. The human PACE4 cDNA was removed from pBluescript by digestion with *Xho*I and *Sal*I. The purified PACE4 fragment was end-filled and blunt-end ligated to pCMV6c that had been digested with *Sma*I to generate pCMV-PACE4. Human pBSK-LPC was digested with *Sac*I 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 digestion and DNA sequencing. cDNAs coding for mouse PC6A (14) and PC6B (15) were subcloned into pRc-CMV (Invitrogen).

Site-directed Mutagenesis—The following pCMVigf1-FLAG mutants have been described previously (13): K71A, 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′-TTGCGCAGGCTCCGGCCTGCCAAGTCAG; K68R, 5′-TCAAGCCTGCCCGGTCAGCTCGCTCTG; K65R/K68S, 5′-TTGCGCACCCTCCGGCCTGCCCGGTCAGCTCGCTCTG; K68R/A70K, 5′-CCCTCAAGCCTGCCCGGTCAAAGCGCTCTGTCCGTGCC.

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 and 100 μg/ml streptomycin. 293 cells were transfected by the calcium phosphate method (17). RPE.40 cells were grown in Ham's F12 medium with 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. RPE.40 cells were transfected using LipofectAMINE™ following the manufacturer's protocol (Life Technologies, Inc.). For co-transfection experiments, 5 μg of prohormone cDNA was co-precipitated with 5 μg of SPC cDNA.

Cell Labeling, Immunoprecipitation, and Gel Electrophoresis—24 h after transfection cells were washed two times in PBS and incubated for 2 h in cysteine-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 cysteine-free Dulbecco's modified Eagle's medium containing 100 μCi/ml [^{35}S]cysteine (Amersham Corp.; 1000 Ci/mmol), 1 mg/ml bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin. After labeling for 24 h, conditioned medium was collected and centrifuged to remove cellular debris. Conditioned medium was concentrated on a Sep-Pak (Waters), eluted and lyophilized, as described previously (13). Lyophilized proteins were reconstituted in 500 μl of immunoprecipitation buffer (25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). Immunoprecipitation was performed with 3 μg of M1 anti-FLAG monoclonal antibody (Kodak/IBI) and protein G-Sepharose (Pharmacia Biotech Inc.). The immunoprecipitates were washed two times in buffer containing 25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$ and then once in buffer consisting of 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$.

Immunoprecipitates were solubilized in SDS-sample buffer containing 2-mercaptoethanol and denatured by heating at 95 °C for 5 min. Samples were electrophoresed on 16% Tricine buffered polyacrylamide gels with a 4% stack at 4 °C (18). Gels were fixed in 10% acetic acid, 25% isopropyl alcohol for 30 min and then soaked in a fluorographic solution (Amplify, Amersham) for 15 min. Dried gels were exposed to x-ray film (Kodak) with an intensifying screen for 4 h to 2 days unless stated otherwise. Quantitation of individual bands was done using a PhosphorImager (Molecular Dynamics).

Cloning and Expression of Hamster LPC—Total RNA was prepared from RPE.40 cells and transcribed to cDNA with Superscript (Life Technologies, Inc.). Hamster LPC was amplified using degenerate PCR primers LPC-3 and LPC-4 that were designed to anneal to unique sequences coding for the P domain. The LPC-3 primer (5′-TT(T/C)TC(T/C)CCNAG(T/C)GGNATGATG) corresponds to the amino acid sequence [illegible]FCPSGMM[illegible], and LPC-4 (5′-ATG(A/G)TA(A/G)TANCC(A/G)TC(T/C)TC(T/C)TC) encodes [illegible]EEDGYTI[illegible]. The 342-bp PCR product was end filled, blunt end-ligated to pBluescript (Stratagene), and sequenced.

For Northern blot analysis, 4 μg of poly(A)$^+$ RNA from RPE.40 cells was electrophoresed through 1% agarose, blotted to a HyBond membrane (Amersham), and probed with a ^{32}P-labeled 342 base pair PCR product encoding the P domain of hamster LPC. Standard molecular biology protocols were followed (17).

RESULTS

Processing of Wild-type and Mutant Pro-IGF-IA by SPCs in 293 Cells—Our previous study on pro-IGF-IA processing utilized the pCMVigf1-FLAG expression vector. This vector directs expression of human prepro-IGF-IA containing the eight-amino acid FLAG epitope between Ala[-1] of the signal peptide and Gly[1] of the B domain. We have shown that the FLAG peptide does not affect co-translational or post-translational processing of prepro-IGF-IA. Human embryonic kidney 293 cells transfected with pCMVigf1-FLAG secrete *N*-glycosylated pro-IGF-IA, pro-IGF-IA, 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-IA 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-IA-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-IA 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-IA (see marker on Fig. 2E). Cells are continuously labeled for approximately 24 h after transfection, and under these conditions approximately 45% of the IGF recovered from conditioned medium is IGF-I-(1–70) and 45% is IGF-I-(1–76). When wild-type pro-IGF-IA 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 Lys^{65}-*X*-*X*-Lys^{68} to generate IGF-I-(1–67). The pattern observed for processing of the K65R mutant by enzymes endogenous to 293 cells is qualitatively and quantitatively identical to processing of wild-type pro-IGF-IA. In the presence of furin the main processing product is again IGF-I-(1–70). A 7-kDa band is also present and is more intense than the the minor cleavage product produced by co-expression of wild-type pro-IGF-IA with furin. This band may result from cleavage at Arg^{65} or Lys^{68}. The K68R mutant shifts the Lys^{68}-*X*-*X*-Arg^{71} processing motif of pro-IGF-IA upstream by three amino acids to Lys^{65}-*X*-*X*-Arg^{68} and creates a new minimal furin site at Arg^{68}-*X*-*X*-Arg^{71} (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

residues			65 (P7)			68 (P4)			71 (P1)			74			77		
WT	P	L	K	P	A	K	T	S	R	S	V	R	A	Q	R	H	T
K65R			R			K			-			R			R		
K68R			K			R			-			R			R		
K65R/K68R			R			R			R			R			R		
K68R/A70K			K			R		K	-			R			R		
R74A/R77A			K			K			-			A			A		
R71A			K			K			A			R			R		
R77X			K			K			R			R			X		

FIG. 1. **Wild-type and mutant IGF-I prohormone-processing sites.** The amino acid sequence of the wild-type pro-IGF-I processing site is shown in single letter code. Basic residues are *numbered*, and substitutions generated by site-directed mutagenesis are indicated by *boldface type*. *X*, stop codon. The Lys^{68}-*X*-*X*-Arg^{71} and Arg^{74}-*X*-*X*-Arg^{77} processing motifs utilized in wild-type pro-IGF-I are in *brackets*, and the putative scissile bonds are indicated by *vertical arrows*. The P1, P4, and P7 positions relative to the Lys^{68}-*X*-*X*-Arg^{71} processing motif are indicated at the *top*.

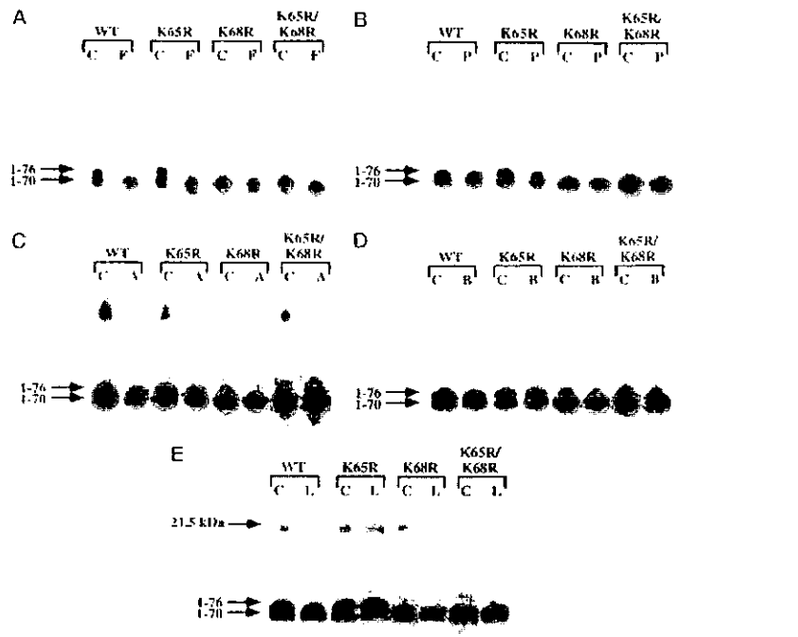

FIG. 2. Processing of wild-type and mutant pro-IGF-IA by SPCs in 293 cells. 293 cells were co-transfected with wild-type or mutant pro-IGF-IA-FLAG, as indicated at the *top*, and with expression vectors for each of the SPCs. *Panel A*, pCMV-furin (*F*); *panel B*, pCMV PACE4 (*P*); *panel C*, pCMVmPC6A (*A*); *panel D*, pCMVmPC6B (*B*); *panel E*, pCMV-LPC (*L*). *C*, pCMV6c expression vector alone. Cells were metabolically labeled with [^{35}S]cysteine and peptides were immunoprecipitated from conditioned medium with the M1 antibody. Immunoprecipitates were resolved on 16% Tricine-buffered gels, as described under "Experimental Procedures." *Arrows* on the *left* indicate the positions of IGF-I-(1–76) and IGF-I-(1–70) markers. The position of the 21.5 kDa molecular mass marker is shown in *panel E*.

TABLE I

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 Arg^{70}, Arg^{71}, and Lys/Arg^{65} was calculated.

Constructs	Lys/Arg^{65a}	Arg^{71}	Arg^{77}	Pro
		%		
WT	0	45	45	10
WT + furin	3	89	0	10
WT + PACE4	0	55	43	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	1
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

[a] Assignment of this cleavage site is tentative

E). 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 Lys^{65}-*X*-*X*-Arg^{68} 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. 2*A*). The upper band represents 65% of the precursor and corresponds to IGF-I-(1–70). The 7-kDa band represents 26% of the precursor and may result from cleavage at Arg^{65} or Arg^{68}. Furin processes 89% of the K65R/K68R precursor to the 7-kDa form, 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. 2*B*). 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. 2*C*). 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.

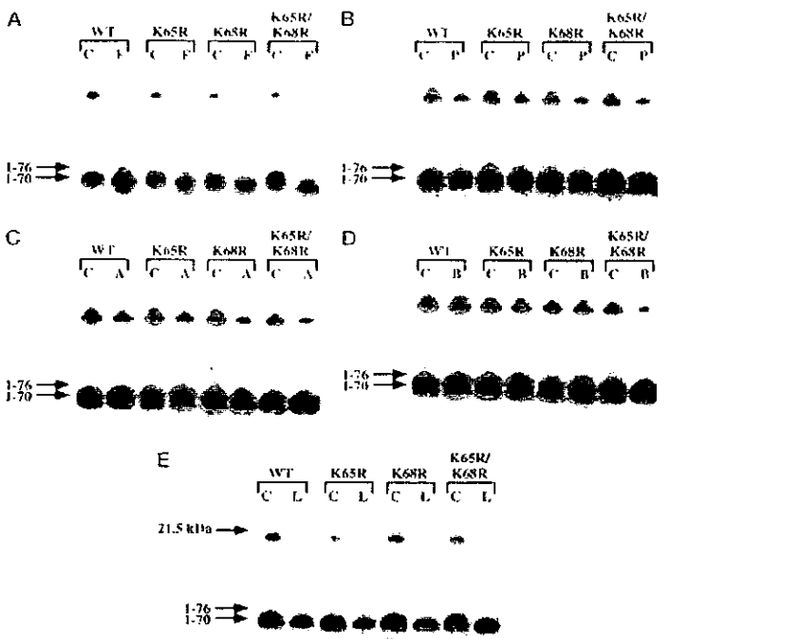

FIG. 3. Processing of wild-type and mutant pro-IGF-IA by SPCs in RPE.40 cells. RPE.40 cells were co-transfected with wild type or mutant pro-IGF IA FLAG, as indicated at the *top*, and with expression vectors for each of the SPCs. *Panel A*, pCMV-furin (*F*); *panel B*, pCMV PACE4 (*P*); *panel C*, pCMVmPC6A (*A*); *panel D*, pCMVmPC6B (*B*); *panel E*, pCMV LPC (*L*). *C*, pCMV6c expression vector alone. Cells were metabolically labeled with [^{35}S]cysteine and IGF I peptides were analyzed as described under "Experimental Procedures." *Arrows* on the *left* indicate the positions of IGF-I-(1–76) and IGF-I-(1–70) markers. The position of the 21.5-kDa molecular mass marker is shown in *panel E*.

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. 2*D*). 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. 2*E*). 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

TABLE II

Processing of wild-type and mutant pro-IGF-IA in RPE.40 cells

The radioactivity in gels used for autoradiography in Fig. 3 was measured using a PhosphorImager. The percentage of pro-IGF I and IGF-I processed at Arg^{70}, Arg^{71}, and Lys/Arg^{65} was calculated.

Constructs	Lys/Arg^{65a}	Arg^{71}	Arg^{77}	Pro
		%		
WT	0	81	0	19
WT + furin	13	85	0	2
WT + PACE4	0	82	0	18
WT + PC6A	0	83	0	17
WT + PC6B	0	75	0	25
WT + LPC	0	85	0	15
K65R	0	81	0	19
K65R + furin	26	72	0	2
K65R + LPC	0	85	0	15
K68R	0	82	0	18
K68R + furin	23	75	0	2
K68R + LPC	0	91	0	9
K65R/K68R	0	85	0	15
K65R/K68R + furin	98	0	0	2
K65R/K68R + LPC	0	93	0	7

[a] Assignment of this cleavage site is tentative

is processed to IGF-I-(1–70), while 20% is secreted as *N*-glycosylated pro-IGF-IA (Fig. 3*A*). 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-

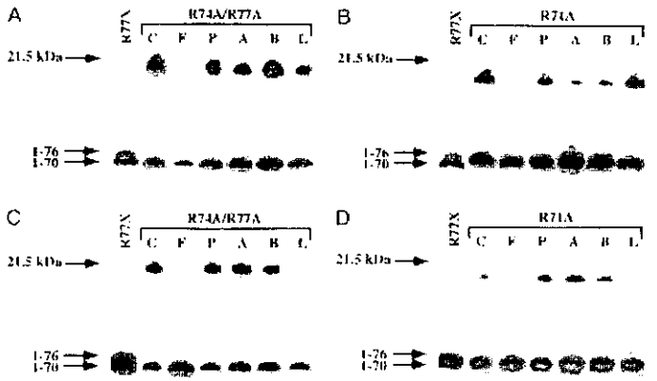

FIG. 4. Cleavage of pro-IGF-IA at Arg^{71} or Arg^{77}. 293 cells (*panels A* and *B*) or RPE.40 cells (*panels C* and *D*) were co-transfected with R74A/R77A (*panels A* and *C*) or R71A (*panels B* and *D*), and each of the SPCs, as indicated. *C*, pCMVGc expression vector alone, *F*, pCMV furin; *P*, pCMV-PACE4; *A*, pCMVmPC6A; *B*, pCMVmPC6B; *L*, pCMV-LPC. Cells were labeled, and immunoprecipitates were analyzed as described under "Experimental Procedures." IGF-I-(1–76) and IGF-I-(1–70) markers are shown on the *left* and indicated by *arrows*. The position of the 21.5 kDa molecular mass marker is also indicated.

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. 3*B*), PC6A (Fig. 3*C*), or PC6B (Fig. 3*D*) 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 figure 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-IA. Eighty-five to 93% of each precursor is processed to IGF-I-(1–70) when co-expressed with LPC, and smaller peptides are not produced (Fig. 3*E*).

Cleavage of Pro-IGF-IA at Arg^{71} or Arg^{77} by SPCs—In order to determine if SPCs discriminate between the Lys^{68}-*X*-*X*-Arg^{71} and Arg^{74}-*X*-*X*-Arg^{77} sites of pro-IGF-IA we co-expressed these enzymes with the R74A/R77A and R71A mutants. Each of these mutants contains only one of the two potential processing sites (Fig. 1). When the R74A/R77A mutant is expressed in 293 cells, 48% of the precursor is processed at Arg^{71} (Fig. 4*A*). In the presence of furin, nearly 100% of the precursor is cleaved at Arg^{71}. PACE4, PC6A, PC6B, and LPC cleave at Arg^{71} to generate 59, 66, 60, and 58% IGF-I-(1–70), respectively. Seventy-seven percent of the R71A precursor is cleaved by endogenous enzymes in 293 cells (Fig. 4*B*). Processing at Arg^{77} increased to 96, 81, 91, 88, and 70% of the precursor in the presence of furin, PACE4, PC6A, PC6B, and LPC, respectively.

When expressed in RPE.40 cells, 47% of the R74A/R77A precursor is processed at Arg^{71}. Furin processed this precursor very efficiently, but PACE4, PC6A, and PC6B had no apparent affect. In contrast, LPC had a moderate affect, processing 57% of the precursor (Fig. 4*C*). Sixty-eight percent of the R71A mutant is cleaved at Arg^{77} in RPE.40 cells. In the presence of furin, cleavage at this site increases to 93% of precursor. PACE4, PC6A, PC6B, and LPC do not affect processing of this mutant in the RPE.40 cells (Fig. 4*D*). We often observe some additional processing or degradation of the R71A precursor in RPE.40 cells, resulting in faint bands below the IGF-I-(1–76) product.

Processing of IGF-I-(1–76) to IGF-I-(1–70). The previous experiments show that PACE4, PC6A, and PC6B can cleave wild-type and mutant pro-IGF-IA when co-expressed in 293 cells, but these enzymes do not appear to process pro-IGF precursors in RPE.40 cells. One possible explanation is that furin first cleaves pro-IGF-IA at Arg^{77} to generate IGF-I-(1–76), which then becomes a substrate for these other SPCs. To test this hypothesis we co-expressed the R77*X* truncation mutant with the SPCs. In 293 cells, 61% of the IGF-I-(1–76) is processed to IGF-I-(1–70). Furin and PC6A convert 90% of this precursor to IGF-I-(1–70). PACE4, PC6B, and LPC have no effect on processing efficiency (Fig. 5*A*). In RPE.40 cells, 56% of the IGF-I-(1–76) is processed to IGF-I-(1–70). Furin very efficiently processes the precursor to IGF-I-(1–70), but PACE4, PC6A, PC6B, and LPC have no effect (Fig. 5*B*).

Processing of the K68R/A70K Mutant by SPCs in RPE.40 Cells—In the previous experiments we have not observed processing of IGF-IA precursors by PACE4, PC6A, or PC6B in RPE.40 cells. In order to determine if these enzymes are active in RPE.40 cells, we co-expressed them with the K68R/A70K mutant. This mutant contains an Arg-*X*-Lys-Arg^{71} cleavage site (Fig. 1), which is also found in other precursors that are processed by PACE4, PC6A, and PC6B (21–24). The K68R/A70K mutant was processed very efficiently by the endogenous RPE.40 processing enzymes, and it was therefore necessary to overexpose the gel to detect pro-IGF-IA. Overexposure of the gel revealed that processing is enhanced by furin, PACE4, PC6A, and PC6B (Fig. 6). This effect was observed in three independent experiments.

Cloning and Expression of Hamster LPC—The data on co-expression of pro-IGF-IA with SPCs in RPE.40 cells indicates that furin and LPC are most active in this cell line. The fact that both alleles of furin are known to be inactive in RPE.40 cells due to mutation (20) suggests that LPC may be responsible for the observed pro-IGF-IA processing. In order to determine if RPE.40 cells express LPC, we obtained a partial clone by PCR of RPE.40 cDNA. The 342-base pair cDNA encodes a 113-amino acid segment of the P domain that shares 92% 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.

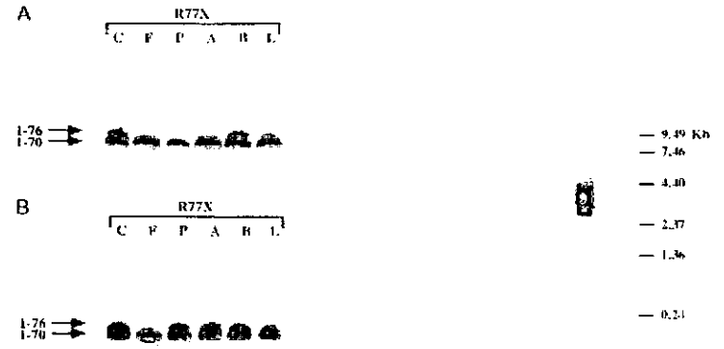

FIG. 5. Processing of IGF-I-(1–76) to IGF-I-(1–70). 293 cells (*panel A*) or RPE.40 cells (*panel B*) were co-transfected with R77*X* and each of the SPCs, as indicated. *C*, pCMVGc expression vector alone, *F*, pCMV furin; *P*, pCMV-PACE4; *A*, pCMVmPC6A; *B*, pCMVmPC6B; *L*, pCMV-LPC. Cells were labeled, and immunoprecipitates were analyzed as described under "Experimental Procedures." IGF-I-(1–76) and IGF-I-(1–70) markers are shown on the *left* and indicated by *arrows*.

FIG. 6. Processing of the K68R/A70K mutant by SPCs in RPE.40 cells. RPE.40 cells were co-transfected with K68R/A70K and each of the SPCs, as indicated. *C*, pCMVGc expression vector alone, *F*, pCMV furin, *P*, pCMV-PACE4, *A*, pCMVmPC6A, *B*, pCMVmPC6B. Cells were labeled, and immunoprecipitates were analyzed as described under "Experimental Procedures." The gel was exposed to film for 3 days.

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. All cell lines examined processed pro-IGF-IA to IGF-I-(1–70). IGF-I-(1–76) was also generated by all cells except the furin-deficient LoVo and RPE.40 cells and the *Drosophila* cell line. Pro-IGF-IA was also processed very efficiently. Endogenous enzymes were able to convert at least 50% of overexpressed precursor to final product in all cell lines (data not shown).

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 Lys^{68}-*X*-*X*-Arg^{71} to yield IGF-I-(1–70) and at Arg^{74}-*X*-*X*-Arg^{77} to produce IGF-I-(1–76) (Fig. 2). The K68R mutant is processed at Arg^{65}-*X*-*X*-Arg^{71} 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 Arg^{65}-*X*-*X*-Arg^{71} over Arg^{71}-*X*-*X*-Arg^{77}. 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 Lys^{68} 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 major product corresponds to IGF-I-(1–70) generated by cleavage at Arg^{65}-*X*-*X*-Arg^{71}. The smaller peptide likely corresponds to IGF-I-(1–67) resulting from cleavage at the newly created furin site, Arg^{65}-*X*-*X*-Arg^{68}. 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*D*).

When furin is co-expressed with wild-type pro-IGF-IA in 293 cells, IGF-I-(1–70), but not IGF-I-(1–76), is produced (Fig. 2*A*).

FIG. 7. Expression of LPC in RPE.40 cells. Four micrograms of poly(A)$^+$ RNA from RPE.40 cells was fractionated on a 1% agarose gel and blotted to a nylon membrane. The blot was hybridized with a P^{32}-labeled nick-translated probe to hamster LPC and exposed to film for 3 days with an intensifying screen. Molecular size markers are indicated on the *right*. Kb, kilobase pairs.

TABLE III
Processing of human pro-IGF-IA in various cell lines

Cells were transfected with pCMVigfI FLAG, and the secreted IGF-I peptides were analyzed by gel electrophoresis, as described "Experimental Procedures." IGF-I-(1–70) was produced by all cell lines examined, as indicated by the plus sign. The presence (+) or absence (−) of IGF-I-(1–76) is indicated in the column at the far right.

Cell line	Origin	IGF-I-(1–70)	IGF-I-(1–76)
293	Human, embryonic kidney	+	+
GM03652C	Human, fibroblast	+	+
LoVo	Human, colon carcinoma	+	−
SaOs 2	Human, osteosarcoma	+	+
Rat-1	Rat, fibroblast	+	+
CHO-K1	Chinese hamster, ovary	+	+
RPE.40	CHO-K1 derivative	+	−
CV-1	Monkey, kidney	+	+
COS 7	CV-1 derivative	+	+
S2	*Drosophila*	+	−

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^{68}-X-X-Arg^{71} over Arg^{74}-X-X-Arg^{77} 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 (Fig. 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^{65}-X-X-Arg^{68} 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^{68}-X-X-Arg^{71} or Arg^{74}-X-X-Arg^{77} (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 convertase may prefer the Lys-X-X-Arg^{71} site to the Arg-X-X-Arg^{77} 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 *Pseudomonas* exotoxin A (29). RPE.40 cells fail to process several proprotein precursors due to mutations in both furin alleles (20). When expressed in RPE.40 cells, wild-type pro-IGF-IA is processed exclusively to IGF-I-(1–70) by endogenous enzymes (Fig. 3). Similar results were obtained previously in LoVo cells (13). It therefore appears that furin is required to generate IGF-I-(1–76), although co-expression of wild-type pro-IGF-IA and furin in 293 or RPE.40 cells results in processing to IGF-I-(1–70), not IGF-I-(1–76) (Figs. 2A and 3A). This may be due to sequential processing of pro-IGF-IA by furin, as discussed above.

An unexpected result from the experiments with RPE.40 cells was that PACE4, PC6A, and PC6B do not process pro-IGF-IA precursors in this cell line (Fig. 3 and Table II). This could be due to a requirement for furin to activate PACE4, PC6A, and PC6B. However, others have shown that these enzymes cleave prosomatostatin and neurotropin precursors in LoVo cells (21, 22, 30). Furthermore, we have found that PACE4, PC6A, and PC6B will cleave the K68R/A70K mutant in RPE.40 cells. This mutant contains a basic residue in the P2 position and may therefore be a better substrate for these enzymes (Fig. 6). It is also possible that 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 by endogenous and transfected enzymes in RPE.40 cells is summarized in Fig. 8B.

Our previous work clearly identified the processing site for final maturation of pro-IGF-IA as Lys^{68}-X-X-Arg^{71} (13). This dibasic motif has been shown to be cleaved by enzymes of the SPC family (8). The data presented here indicate that furin,

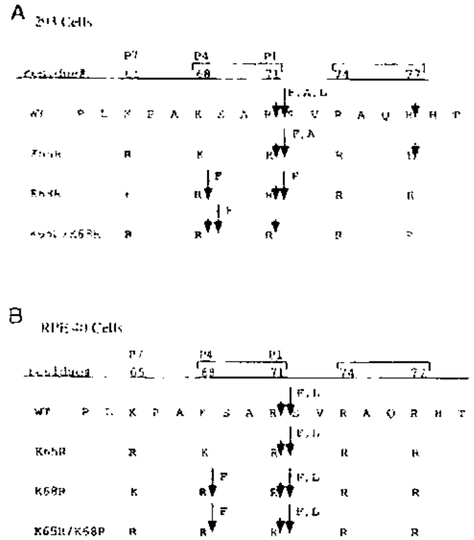

FIG. 8. **Summary of processing of wild-type and mutant pro-IGF-I by endogenous enzymes and SPCs.** The amino acid sequence of the wild-type pro-IGF-I processing site is shown in single-letter code. Basic residues are numbered, and substitutions generated by site-directed mutagenesis are indicated by *boldface type*. The Lys^{68}-X-X-Arg^{71} and Arg^{74}-X-X-Arg^{77} processing motifs utilized in wild type pro-IGF-I are in *brackets*. The P1, P4, and P7 positions relative to the Lys^{68}-X-X-Arg^{71} processing motif are indicated at the *top*. The processing sites utilized by enzymes endogenous to 293 cells (*A*) and RPE.40 cells (*B*) are indicated by *short arrows*. Processing sites utilized by transfected furin (*F*), PC6A (*A*), and LPC (*L*) are indicated by *long arrows*.

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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Mutational Analysis of the Insulin-like Growth Factor I Prohormone Processing Site*

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Insulin-like growth factor I (IGF-I) is a mitogenic peptide that is produced in most tissues and cell lines and plays an important role in embryonic development and postnatal growth. IGF-I is initially synthesized as a prohormone precursor that is converted to mature IGF-I by endoproteolytic removal of the carboxyl-terminal E-domain. Regulation of the conversion of proIGF-I to mature IGF-I is a potential mechanism by which the biological activity of this growth factor might be modulated. Endoproteolysis of the IGF-I prohormone occurs at the unique pentabasic motif Lys-*X*-*X*-Lys-*X*-*X*-Arg^{71}-*X*-*X*-Arg-*X*-*X*-Arg. Recently, a family of enzymes which cleave prohormone precursors at sites containing multiple basic residues has been discovered. The goals of this study were 1) to determine which basic residues in the pentabasic proIGF-I processing site were necessary for proper cleavage and 2) to examine the role that subtilisin-related proprotein convertase 1 (SPC1/furin) might play in proIGF-I processing. We have shown that an expression vector coding for an epitope-tagged proIGF-I directs synthesis and secretion of mature IGF-I-(1–70), extended IGF-I-(1–76), proIGF-I, and *N*-glycosylated proIGF-I in human embryonic kidney 293 cells. Extended IGF-I-(1–76) is produced by cleavage at Arg^{77} and requires both Arg^{74} (P4) and Arg^{77} (P1). Cleavage at Arg^{77} does not occur in the SPC1-deficient cell lines RPE.40 and LoVo, suggesting that processing at this site is mediated by SPC1. Mature IGF-I-(1–70) is produced by cleavage at Arg^{71} and requires both Lys^{68} (P4) and Arg^{71} (P1). Lys^{65} in the P7 position is important for efficient cleavage. SPC1 is not required for processing at Arg^{71} since this cleavage occurs in RPE.40 and LoVo cells. These data suggest the existence of a processing enzyme which is specific for the Lys-*X*-*X*-Arg motif of proIGF-I.

Insulin-like growth factor I (IGF-I)[1] circulates in human serum as a 70-amino-acid peptide consisting of four domains: B, C, A, and D. The physiological importance of IGF-I for normal development and growth is underscored by the severity of the phenotype of mice lacking a functional IGF-I gene. Mice carrying a disrupted IGF-I gene display growth deficiencies, delayed bone development, infertility, and a high mortality rate (1, 2). Liver is the main site of production of circulating IGF-I, although this growth factor is also synthesized and secreted by most tissues. Mature IGF-I can be derived from either of two IGF-I prohormones by removal of the E-domain (see Fig. 2). Alternative splicing of IGF-I mRNA is responsible for generating the two IGF-I prohormones, and the physiological significance of the different forms is unknown (3). ProIGF-IA contains a 35-amino-acid E-domain, while the E-domain of proIGF-IB is 77 amino acids in length. The sequences of human proIGF-IA and proIGF-IB are identical through the first 16 residues of the E-domain (4), including the unique pentabasic prohormone cleavage motif Lys-*X*-*X*-Lys-*X*-*X*-Arg^{71}-*X*-*X*-Arg-*X*-*X*-Arg (Fig. 1). This motif has been conserved in mammals, birds, amphibians, and teleosts.

It is now well established that most peptide hormones and growth factors are initially synthesized as biologically inactive precursors that are converted to active forms by endoproteolysis at specific sites. Proinsulin has served as a model for studies of prohormone processing. From these studies it has been determined that the C-peptide of proinsulin is excised by proteolytic cleavage at paired dibasic residues to produce insulin (5, 6). Cleavage is mediated by two subtilisin-related proprotein converting (SPC) enzymes, SPC2 (PC2) and SPC3 (PC1/3) (7, 8). These enzymes are serine proteases and have been shown to process several prohormones to mature hormones, including proglucagon (9) and the proopiomelanocortin precursor (10–12). Expression of SPC2 and SPC3 is limited to endocrine and neuroendocrine tissues, and the preferred cleavage site is carboxyl-terminal to Arg-Arg and Lys-Arg doublets (6). Other members of the mammalian subtilisin-related proprotein convertase family include SPC1 (furin), SPC4 (PACE4), SPC5 (PC4), and SPC6 (PC5/6). SPC1 and SPC4 are widely distributed and cleave proprotein precursors at tri- and tetrabasic sites (13). The preferred cleavage site for SPC1 appears to be Arg-*X*-Lys/Arg-Arg (14), and the Arg-*X*-*X*-Arg sequence may serve as a minimal processing site (15). SPC4 has been reported to have a more strict requirement for a basic residue in the P2 position than does SPC1 (16, 17).

The paradigm established for processing of proinsulin suggests that proIGF-I would be converted to mature IGF-I by cleavage at the carboxyl terminus of Arg^{71}, followed by removal of the basic residue by a carboxypeptidase. SPC1 is a candidate proIGF-I convertase since, like IGF-I, it is expressed ubiquitously and it does not have a strict requirement for a basic residue in the P2 position. We have used site-directed mutagenesis to determine the importance of each of the basic residues in the proIGF-I cleavage site for recognition by the processing enzyme. We have also examined the role of SPC1 in proIGF-I processing through the use of cell lines deficient in SPC1 activity.

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[1] The abbreviations used are: IGF-I, insulin-like growth factor I; SPC, subtilisin-related proprotein convertase; PCR, polymerase chain reaction; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

EXPERIMENTAL PROCEDURES

Expression Constructs—PreproIGF-IA was amplified by PCR from cDNA obtained from the human fibroblast cell line GM 03652C (ATCC). Primers specific for the Met^{-48} region of the signal peptide (hIGF1-5) and the translation termination codon region of the E-domain (hIGF1-6) were used: 5′-GGGAATTCTTGAAGGTGAAGATGCACAC and 5′-GGGGATCCCCTACATCCTGTAGTTCTTGT. The PCR product was cloned into the *Eco*RI and *Bam*HI sites of the pCMV6c expression vector, which contains the CMV promotor and the SV40 poly(A) tail, to generate pCMVigf1. pCMVigf1 was then used as a template for recombinant PCR (18) to generate pCMVigf1-FLAG. The signal peptide of IGF-I was amplified using primers hIGF1-5 and Flg-2 (5′-CTTGTTCATCGTCGTCCTTGTAGTCAGCCGTGGCAGAGCTGGT). Flg-2 codes for Thr^{-5} to Ala^{-1} of the signal peptide and contains the 24-nucleotide antisense FLAG sequence on the 5′ end. The coding region of proIGF-I was amplified using primers hIGF1-6 and Flg-1 (5′-GACTACAAGGACGACGATGACAAGGGACCGGAGACGCTCTGC). Flg-1 codes for Gly^{1} to Cys^{6} of the B-domain and contains the 24-nucleotide sense FLAG sequence on the 5′ end. The PCR products were purified by polyacrylamide gel electrophoresis, and 50 ng of each was mixed, annealed, and extended under PCR conditions and amplified by 12 cycles of PCR with hIGF1-5 and hIGF1-6. The recombinant PCR product was cloned into the *Eco*RI and *Bam*HI sites of pCMV6c to generate pCMVigf1-FLAG. The fidelity of PCR amplification was verified by DNA sequencing.

Site-directed Mutagenesis—Site-directed mutants were generated using the Altered Sites II *in vitro* Mutagenesis System (Promega) and double-stranded proIGF-I-FLAG plasmid DNA as template. Oligonucleotide primer sequences and corresponding templates are shown in Table I. Mutants were subcloned from pAlter to pCMV6c and verified by DNA sequencing.

Cell Culture and Transfections—Human embryonic kidney 293 cells were grown in DMEM (Life Technologies, Inc) with 10% fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin. 293 cells were transfected by the calcium phosphate method (19). CHO-K1 and RPE.40 cells (provided by Dr. Thomas Moehring, University of Vermont) were grown in Ham's F-12 medium with 5% fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin. CHO-K1 and RPE.40 cells were transfected using LipofectAMINE™ following the manufacturer's protocol (Life Technologies, Inc).

Cell Labeling, Immunoprecipitation, and Gel Electrophoresis—24 h after transfection cells were washed twice in phosphate-buffered saline and incubated for 2 h in cysteine-free (or histidine-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 cysteine-free (or histidine-free) Dulbecco's modified Eagle's medium containing 100 μCi/ml [^{35}S]cysteine (Amersham, 1000 Ci/mmol) or [^{3}H]histidine (DuPont NEN, 385 Ci/mmol), 1 mg/ml bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin. After labeling for 24 h, conditioned medium was collected and centrifuged to remove cellular debris. Conditioned medium was stored at −20 °C. Cells were washed twice with phosphate-buffered saline and removed from the plate in immunoprecipitation (IP) buffer (25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100), sonicated, and stored at −20 °C.

Conditioned medium (1 to 5 ml) was acidified with trifluoroacetic acid (0.1% final concentration) and loaded onto a Sep-Pak C-18 cartridge (Millipore) that had previously been equilibrated with 20% acetonitrile, 0.1% trifluoroacetic acid. After washing the column in 20% acetonitrile, 0.1% trifluoroacetic acid, peptides were eluted in 80% acetonitrile, 0.1% trifluoroacetic acid, lyophilized, and reconstituted in 500 μl of IP buffer. Immunoprecipitation was performed with 3 μg of M1 or M2 anti-FLAG monoclonal antibody (Kodak/IBI) or 5 μl of UB3-189 anti-human somatomedin-C polyclonal antisera (National Hormone Pituitary Program, NIDDK) overnight at 4 °C. M1 and M2 immune complexes were precipitated with protein G-Sepharose (Pharmacia Biotech Inc.), and UB3-189 immune complexes were precipitated with protein A-Sepharose. The immunoprecipitates were washed twice in buffer containing 25 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 mM $CaCl_2$, and then once in buffer consisting of 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$. Cell lysates were thawed and centrifuged to remove cell debris. 500 μl of supernatant was preabsorbed with protein G-Sepharose and immunoprecipitated with M1 antibody as described above.

Immunoprecipitates were solubilized in SDS-sample buffer containing 2-mercaptoethanol and denatured by heating at 95 °C for 5 min. Samples were electrophoresed on Tricine-buffered gels with a 4% stack

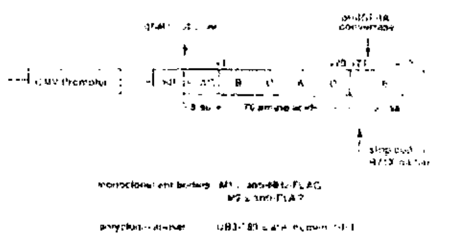
```
                          ↓
            P7 P6 P5 P4 P3 P2 P1  P1' P2' P3' P4' P5' P6'
residue #   65       68       71          74          77
 P  L       K  P  A  K  S  A  R   S   V   R   A   Q   R   H  T
```

FIG. 1. **The IGF-I prohormone processing site.** The amino acid sequence of the proIGF-I processing site is shown in *single-letter code*. Basic residues are indicated by *boldface type* and *numbered*. The scissile bond connecting the P1 Arg^{71} and the P1′ Ser^{72} is indicated by the *vertical arrow*.

TABLE I
ProIGF-I expression mutants

Amino acid substitutions and corresponding nucleotide changes are indicated by boldface type. Residues mutated to stop codons are indicated by *X*. Nonbasic residues in mutant sequences are represented by dots.

Construct	Sequence	Template	Oligonucleotides
	65 68 71 74 77		
Wild-type (WT)	KPAKSARSVRAQR		
K65A	A..K..R..R..R	WT	5′-TTGCGCACCCCTCGCGCCTGCCAAGTCAGC
K65A/K68G	A..G..R..R..R	K68G	5′-TTGCGCACCCCTCGCGCCTGCCGGGTCACC
K65A/R74A	A..K..R..A..R	R74A	5′-TTGCGCACCCCTCGCGCCTGCCAAGTCAGC
K65A/R77A	A..K..R..R..A	WT	5′-GTCCGTGCCCAGGCCCACACCGACA and 5′-TTGCGCACCCCTCGCGCCTGCCAAGTCAGC
K65A/R71X	A..K..X	WT	5′-TGCGCACCCCTCGCGCCTGCCAAGTCAGCT TGATCTGTCCGTGCCCAG-3′
K65A/K68G/R71X	A..G..X	K68G	5′-TGCGCACCCCTCGCGCCTGCCGGGTCAGCT TGATCTGTCCGTGCCCAG-3′
K68G	K..G..R..R..R	WT	5′-TCAAGCCTGCCGGGTCAGCTCGCTC
K68G/R71X	K..G..X	K68G	5′-CCGGGTCAGCTTGATCTGTCCGTGC
K68G/R71A	K..G..A..R..R	WT	5′-CCGGGTCAGCTGCCTCTGTCCGTGC
K68G/R74A	K..G..R..A..R	K68G	5′-GCTCGCTCTGTCGCTGCCCAGCGCC
K68G/R77A	K..G..R..R..A	K68G	5′-GTCCGTGCCCAGGCCCACACCGACA
R71A	K..K..A..R..R	WT	5′-CCAAGTCAGCTGCCTCTGTCCGTGC
R71A/R77A	K..K..A..R..A	WT	5′-CTGCCAAGTCAGCTGCCTCTGTCCGT GCCCAGGCCCACACCGACATGCC-3′
R74A	K..K..R..A..R	WT	5′-GCTCGCTCTGTCGCTGCCCAGCGCC
R74A/R77A	K..K..R..A..A	R74A	5′-CGCTGCCCAGGCCCACACCGACA
R77A	K..K..R..R..A	WT	5′-GTCCGTGCCCAGGCCCACACCGACA
R71X	K..K..X	WT	5′-CCAAGTCAGCTTGATCTGTCCGTGC
R74X	K..K..R..X	WT	5′-GCTCGCTCTGTCTGAGCCCAGCGCC
R77X	K..K..R..R..X	WT	5′-GTCCGTGCCCAGTCACACACCGACA



[illegible] **the proIGF-I-FLAG expression construct.** [illegible] FLAG peptide [illegible] Ala [illegible] and Gly [illegible] preproIGF-I cDNA [illegible] the pCMV expression [illegible] for preproIGF-I-FLAG [illegible] peptide; *FLAG*, FLAG peptide; [illegible] *Numbering* [illegible] the first [illegible] Cleavage [illegible] for signal peptidase and the [illegible] The R71X mutant was generated by converting the codon for Arg [illegible] to a stop codon. The monoclonal antibody M1 will recognize the FLAG peptide only when it is located at the amino terminus of a protein while the M2 monoclonal antibody will recognize the FLAG peptide in any location. Polyclonal antisera UBI 189 was generated using mature human IGF-I as antigen.

and a resolving gel of 10% [illegible] polyacrylamide. Gels were fixed in 10% acetic acid, 25% isopropyl alcohol for 30 min, and then soaked in a fluorographic solution (Amplify, Amersham) for 15 min. Dried gels were exposed to x-ray film (Kodak) with an intensifying screen.

Deglycosylation—7 ml of conditioned medium from [^{35}S]cysteine-labeled cells was concentrated on a Sep-Pak and lyophilized as described above. The lyophilized proteins were solubilized in 0.1% SDS, denatured by heating at 95 °C for 10 min, and placed on ice. [illegible] of deglycosylation buffer (200 mM NaH_2PO_4, pH 7.2, 5% Nonidet P-40) was added, and 100-µl aliquots were incubated with 2 milliunits of neuraminidase (sialidase), 2.5 milliunits of *O*-glycosidase, 0.4 unit of *N*-glycosidase F (Boehringer Mannheim), or combinations of these enzymes for 24 h at 37 °C. After incubation, 400 µl of IP buffer was added and epitope-tagged peptides were immunoprecipitated with M1 antibody and run on 10% polyacrylamide gels as described above.

RESULTS

The FLAG Peptide Does Not Interfere with Processing of PreproIGF-I—Our initial attempts to study the processing of proIGF-I were hampered by difficulties in immunoprecipitating proIGF-I from conditioned medium. Commercially available antisera to IGF-I have been generated using mature IGF-I as antigen, and the E-domain of proIGF-I may interfere with epitope recognition. To circumvent this problem we designed a preproIGF-I expression vector that contained an epitope tag, the 8-amino-acid FLAG peptide, between Ala [illegible] of the signal peptide and Gly [illegible] of the B domain (Fig. 2). Two monoclonal antibodies to the FLAG peptide have been generated. The M1 antibody requires calcium for binding and will only recognize a FLAG peptide with an exposed amino terminus. The M1 antibody can therefore be used to determine whether the signal peptide has been removed from proIGF-I-FLAG. The M2 antibody will recognize the FLAG peptide in any location and should therefore immunoprecipitate all epitope-tagged peptides. To obtain an accurate molecular weight marker for IGF-I-FLAG, we mutated Arg^{71} of proIGF-I-FLAG to a stop codon. This R71X mutant directs synthesis of the 70-amino acid mature IGF-I molecule with the FLAG peptide at the amino terminus (Fig. 2 and Table I).

Before proceeding with biosynthetic studies, it was necessary to determine if the presence of the FLAG peptide would alter processing of the IGF-I prohormone. 293 cells were transfected with IGF-I, R71X, and IGF-I-FLAG expression vectors, and [^{35}S]cysteine-labeled proteins were immunoprecipitated from conditioned medium using UBI-189 anti-human IGF-I antisera (Fig. 3). Two proteins of 7–8 kDa were immunoprecipitated from conditioned media of cells transfected with CMVigf1. Two proteins were also immunoprecipitated from conditioned media of cells transfected with CMVigf1-FLAG. These peptides have a molecular mass of 9–10 kDa and represent the epitope-tagged homologues of the IGF-I peptides synthesized from the CMVigf1 vector. The 9-kDa band generated from the IGF-I-FLAG transfection migrates at the same position as the R71X mutant, which has a predicted molecular mass of 8910 daltons, indicating that proIGF-I-FLAG is processed at Arg [illegible]. The larger molecular mass bands of the IGF-I and IGF-I-FLAG doublets may represent carboxyl-extended forms of mature IGF-I.

When conditioned medium from R71X-transfected 293 cells was immunoprecipitated with M1, M2, and UBI-189 antisera, a predominant band was seen at 9 kDa (Fig. 4). The slightly larger band recognized by the M1 antibody may be an oxidized form of IGF-I. When conditioned medium from IGF-I-FLAG-transfected 293 cells was immunoprecipitated with these antisera, several peptides were visualized. The 9-kDa band generated from the R71X transfection was also present in IGF-I-FLAG-transfected medium and was recognized by anti-FLAG and anti-human IGF-I antisera, indicating that this band represents mature IGF-I-FLAG. The 10-kDa band from the IGF-I-FLAG-transfected media was also recognized by all three antisera and may represent a carboxyl-extended form of mature IGF-I-FLAG. In addition to the 9–10-kDa doublet, the M1 and M2 antibodies immunoprecipitated peptides of 14 kDa and 16 kDa, as well as a broad band from 19–21 kDa. Since these peptides were recognized by both M1 and M2, they do not represent forms of IGF-I containing a signal peptide and thus the FLAG peptide does not interfere with signal peptidase activity. The higher molecular weight bands, which were not immunoprecipitated with the UBI-189 antisera, may be forms of proIGF-I.

ProIGF-I and N-Glycosylated ProIGF-I Are Secreted by Transfected 293 Cells—Selective amino acid labeling was employed in order to determine whether the high molecular weight proteins immunoprecipitated from IGF-I-FLAG-transfected conditioned media by anti-FLAG antibodies were unprocessed or partially processed proIGF-I molecules. Human proIGF-IA contains two histidine residues, both of which are located in the E domain. Therefore histidine labeling should be specific for proIGF-I as opposed to oligomers or aberrantly processed mature IGF-I. Fig. 5 shows the results of [^{35}S]cysteine and [^{14}C]histidine labeling of cells transfected with the IGF-I-FLAG expression vector. The 9–10-kDa doublet migrated as a single band on this 10% polyacrylamide gel and was clearly visible when cells were labeled with [^{35}S]cysteine but was not present when labeled with [^{14}C]histidine. In contrast, 14-kDa and 19–21-kDa proteins were visible from both [^{35}S]cysteine and [^{14}C]histidine labeling, and the [^{14}C]histidine-labeled 16-kDa band could be seen after overexposure (data not shown).

The predicted molecular mass of proIGF-I-FLAG is 13.[illegible] kDa, in close agreement with the 14-kDa band seen in [^{14}C]histidine labeling. The proIGF-IA E-domain contains one potential *N*-glycosylation site at Asn[illegible] and several serine and threonine residues which could be used for *O*-glycosylation. To determine if the 16-kDa and 19–21-kDa peptides were glycosylated forms of proIGF-I, peptides from conditioned medium of proIGF-I-FLAG-transfected 293 cells were subjected to treatment with various glycosidases. As shown in Fig. 6, neuraminidase and *O*-glycosidase had no effect on the electrophoretic mobility of proIGF-I peptides. However, the 19–21-kDa band completely disappeared after digestion with *N*-glycosidase while the intensity of the 14-kDa and 16-kDa peptides increased dramatically. It is therefore likely that the 19–21-kDa peptide is *N*-glycosylated proIGF-I-FLAG, the 14-kDa peptide is proIGF-I-FLAG, and the 16-kDa peptide may be partially glycosylated proIGF-I-FLAG.

Conversion of ProIGF-I to Mature IGF-I Occurs Intracellularly—Since there are examples of growth factors that can be proteolytically activated intra- or extracellularly, it was of interest to determine the location of conversion of proIGF-I to mature IGF-I. Cell lysates from R71X or IGF-I-FLAG-transfected 293 cells were immunoprecipitated with the M1 antibody, and peptides were resolved on 16.5% polyacrylamide gels. The 9–10-kDa processed IGF-I-FLAG doublet, as well as proIGF-I-FLAG and [illegible]-glycosylated proIGF-I-FLAG, were clearly visible in cell lysates, indicating that conversion can occur intracellularly (Fig. [illegible]). We have also demonstrated that proIGF-I-FLAG peptides are not processed to mature IGF-I-FLAG when incubated with conditioned media from 293 cells or 50% fetal calf [illegible] mouse serum for up to 24 h at 37 °C. However, [illegible] of proIGF-I-FLAG occurs when [illegible]

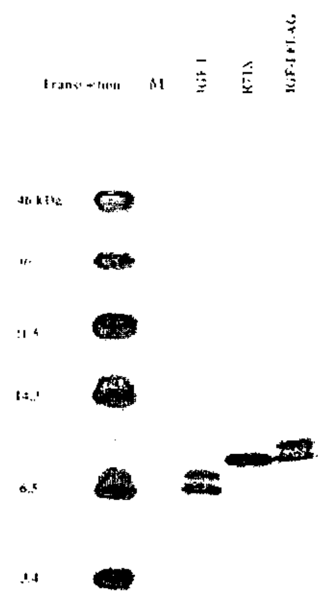


FIG. 3. **The FLAG peptide does not interfere with proIGF-I processing.** 293 cells were transfected with IGF-I, R71X, or IGF-I-FLAG expression vectors, and [^{35}S]cysteine-labeled IGF peptides were immunoprecipitated using the UBI-189 antisera and resolved on Tricine-buffered 16.5% polyacrylamide gels, as described under "Experimental Procedures." *M* indicates mock transfection. The molecular masses of [^{14}C]methylated protein markers are indicated.

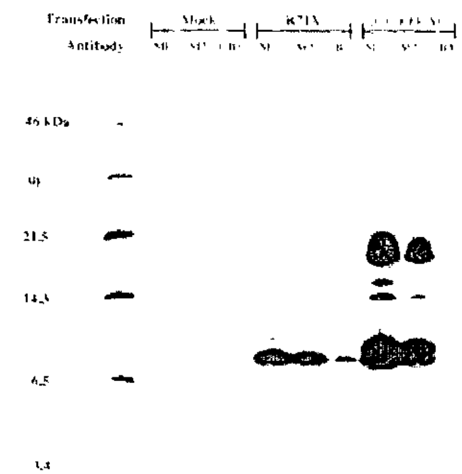


FIG. 4. **PreproIGF-I-FLAG is processed to mature IGF-I-FLAG.** 293 cells were transfected with R71X or IGF-I-FLAG expression vectors, and [^{35}S]cysteine-labeled peptides were immunoprecipitated from aliquots of reconstituted conditioned medium with M1, M2, or UBI-189 antisera. Immunoprecipitates were resolved on Tricine-buffered 16.5% polyacrylamide gels as described under "Experimental Procedures." The molecular masses of the [^{14}C]methylated protein markers are indicated.

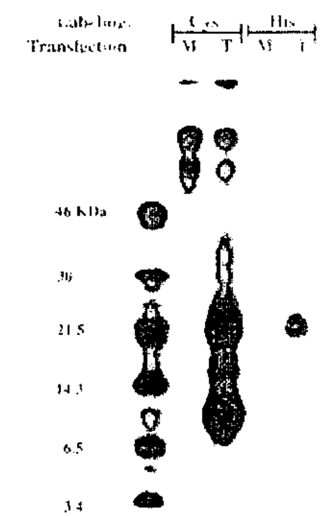


FIG. 5. **Multiple forms of unprocessed proIGF-I are secreted.** 293 cells were transfected with the IGF-I-FLAG expression vector and metabolically labeled with [^{35}S]cysteine (*Cys*) or [^{14}C]histidine (*His*). Peptides were immunoprecipitated from reconstituted conditioned medium with the M2 antibody and resolved on Tricine-buffered 10% polyacrylamide gels. *M*, mock; *T*, transfected. The molecular masses of [^{14}C]methylated protein markers are indicated.

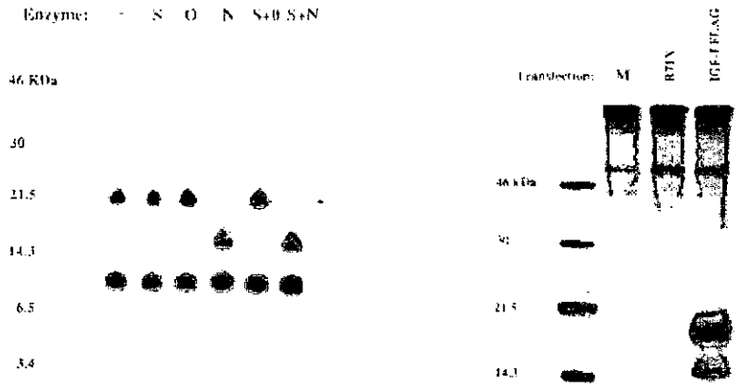


FIG. 6. ProIGF-I is *N*-glycosylated. 293 cells were transfected with the IGF-I-FLAG expression vector and metabolically labeled with ^{35}S-cysteine. Conditioned medium was concentrated on a Sep-Pak cartridge, reconstituted, and denatured. One-tenth volume of 10× deglycosylation buffer was then added, and aliquots were treated in the absence of enzyme (-), or with neuraminidase (N), *O*-glycosidase (O), *N*-glycosidase (S) alone or in combinations for 24 h at 37 °C. Peptides were immunoprecipitated with M1 antibody and run on Tricine-buffered 10% polyacrylamide gels. The molecular masses of the ^{14}C-methylated protein markers are indicated.

FIG. 7. Conversion of proIGF-I to mature IGF-I occurs intracellularly. 293 cells were transfected with the R71X or IGF-I-FLAG expression vector, and ^{35}S-cysteine-labeled peptides were immunoprecipitated from cell lysates with the M1 antibody and resolved on Tricine-buffered 16% polyacrylamide gels, as described under "Experimental Procedures." *M* indicates mock transfection. The molecular masses of the ^{14}C-methylated protein markers are indicated.

huted with trypsin (data not shown).

Processing of ProIGF-I Mutants in 293 Cells—To determine the importance of each of the basic residues in the pentabasic proIGF-I processing motif for prohormone conversion activity, we systematically mutated each basic residue to an uncharged residue (Table I). Single, double, and triple mutants were generated. Since our initial experiments with the R71X and IGF-I-FLAG expression vectors indicated that proIGF-I was being cleaved at Arg^{71} and a downstream site, we also made two additional stop codon mutants for use as molecular weight markers. Arg^{71} and Arg^{77} were mutated to stop codons, generating R74*X* and R77*X*, respectively. In several cases, substitution of a basic residue with an uncharged amino acid changed the electrophoretic mobility of the protein. In some of these instances it was necessary to make additional stop codon mutants in order to correctly identify the processing site.

Fig. 8*A* shows the results of expression of the single substitution mutants in 293 cells. On Tricine-buffered 20% polyacrylamide gels, the R71*X* mutant migrates as a single band. The R74*X* mutant migrates as a poorly resolved doublet, and the R77*X* mutant migrates as a clearly defined doublet. Thus gel electrophoresis system is therefore capable of resolving IGF-I-FLAG peptides of 78, 81, and 84 amino acids in length. It is also interesting to note that both the R74*X* and R77*X* peptides are processed at Arg^{71}, indicating that as few as two residues on the carboxyl terminus of Arg^{71} are sufficient for processing activity.

As shown previously (Figs. 3 and 5), wild-type IGF-I-FLAG is processed at two sites yielding a peptide that migrates with the R71*X* standard at 9 kDa as well as a 10 kDa peptide. Comparison of these peptides with the pattern produced by expression of R74*X* and R77*X* indicates that the 10-kDa IGF-I-FLAG peptide is generated by cleavage at Arg^{77} (Fig. 8*A*). In several different transfection experiments, we have observed that approximately 50% of proIGF-I-FLAG is cleaved at Arg^{71} and 50% at Arg^{77}. We have not observed evidence for cleavage at Arg^{74} (Table II).

Mutation of Arg^{71} to alanine (R71A) abolishes processing at this site, indicating that the basic residue is required at the P1 site. Processing at Arg^{77} is not qualitatively affected (Fig. 8*A*). Mutations at either Arg^{74} (R74A) or Arg^{77} (R77A) eliminate processing at Arg^{71} without qualitatively affecting cleavage at Arg^{77} (Fig. 8*A*).

The K65G mutant processing pattern is qualitatively similar to that of wild type proIGF-I processing except that all bands migrate slightly faster on Tricine-buffered gels (Fig. 8*A*). This band shift may be attributed to size and/or charge differences of the lysine and glycine side chains. When K68G and K68G/R71X peptides are run together on Tricine-buffered 20% gels, it is clear that processing does not occur at Arg^{71}. The K68G peptide migrates more slowly than the K68G/R71*X* stop codon mutant (data not shown) and is thus processed at a downstream site such as Arg^{77}. The P4 lysine residue is therefore required for processing at Arg^{71}.

When Lys^{65} is mutated to Ala (K65A), the peptide is processed at two sites (Fig. 8*B*). The smaller band migrates with the K65A/R71*X* mutant, indicating that it is generated by cleavage at Arg^{71}. Since the larger band of the doublet is well resolved, it is likely that it represents processing at Arg^{77}.

Three K65A double mutants were generated. The K65A/R74A and K65A/R77A mutants migrate as a single band with the same molecular weight as the K65A/R71*X* mutant (Fig. 8*B*). These mutants are therefore cleaved at Arg^{71} but not Arg^{77}. The processed K65A/K68G mutant has a higher molecular weight than the corresponding K65A/K68G/R71X mutant, indicating that the two upstream Arg-Arg residues are required for cleavage at Arg^{71} (Fig. 8*C*). It is likely that cleavage of K65A/K68G occurs at Arg^{77} since the K65A/K68G and K65A/K68G/R71A mutants are clearly resolved.

While it was not possible to definitively assign cleavage locations for the K68G double mutants, it is clear that these mutants are processed very poorly, if at all, at Arg^{71}. Only 10% of the K68G/R71A mutants and 10% of the K68G/R77A mutants were processed (Fig. 8*D* and Table II). 70% of the K68G/R71A/R77A triple mutant was processed, and it is likely that cleavage occurred at Arg^{77} since this site is preferred in Arg^{77} in proIGF-I-FLAG. When Arg^{77} is ablated by substitution of alanine for Arg^{71} and Arg^{74} (R71A/R74A), only 8% of the precursor is cleaved. When Arg^{71} and Arg^{77} are changed to alanine residues (R74A/R77A), approximately 50% of the precursor is cleaved at Arg^{71} (Fig. 8*D* and Table II).

Processing of ProIGF-I in RIN 40 Cells—The data on processing of proIGF-I mutants indicated that processing at Arg^{71} was prevented in all mutants having a substitution at either Arg^{71} or Arg^{74} (Fig. 8 and Table II), suggesting that this sequence might be a cleavage site for SPC1, which has minimally been defined as -RXXR-. These data also demonstrate that processing at Arg^{71} requires both Lys^{65}-P4 and Arg^{71}-P1. To see if SPC1 can process precursors containing a Lys-

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TABLE II
Summary of processing of wild-type and mutant proIGF-I

The radioactivity in gels used for autoradiography in Fig. 8 was measured using a PhosphoImager. The percentage of proIGF-I and IGF-I processed at Arg[71], Arg[74], and Arg[77] was calculated. N-Glycosylated proIGF-I was not included in this analysis. Amino acid substitutions are in boldface type. Residues mutated to stop codons are represented by X. Nonbasic residues in mutant sequences are represented by dots.

Construct	Sequence (65 68 71 74 77)	%Arg[71]	%Arg[74]	%Arg[77]	%Pro
Wild-type (WT)	K P A K S A R S V R A Q R	55	0	41	4
K65A	**A** . . K . . R . . R . . R	28	0	57	15
K68G	K . . **G** . . R . . R . . R	0	(5)[a]	(87)	8
R71A	K . . K . . **A** . . R . . R	—[b]	0	83	17
R74A	K . . K . . R . . **A** . . R	74	—	0	26
R77A	K . . K . . R . . R . . **A**	91	0	—	9
K65A/K68G	**A** . . **G** . . R . . R . . R	0	(0)	(86)	14
K65A/R74A	**A** . . K . . R . . **A** . . R	68	—	0	32
K65A/R77A	**A** . . K . . R . . R . . **A**	92	0	—	8
K68G/R71A	K . . **G** . . **A** . . R . . R	—[b]	(0)	(79)	21
K68G/R74A	K . . **G** . . R . . **A** . . R	(0)	—	(10)	90
K68G/R77A	K . . **G** . . R . . R . . **A**	(0)	(16)	—	84
R71A/R77A	K . . K . . **A** . . R . . **A**	—[b]	8	—	92
R74A/R77A	K . . K . . R . . **A** . . **A**	56	—	—	44
K65A/R71X	**A** . . K . . **X**	100	—	—	—
K65A/K68G/R71X	**A** . . **G** . . **X**	100	—	—	—
K68G/R71X	K . . **G** . . **X**	100	—	—	—
R71X	K . . K . . **X**	100	—	—	—
R74X	K . . K . . R . . X	ND[c]	ND	—	—
R77X	K . . K . . R . . R . . **X**	69	0	31	—

[a] Assignment of cleavage site is tentative.
[b] —, residue eliminated by site-directed mutagenesis.
[c] Not determined.

for Arg substitution in the P4 position. In order to determine if SPC1 is involved in proIGF-I processing, we expressed IGF-I-FLAG in CHO-K1 and RPE.40 cells. RPE.40 cells were derived from CHO.K1 cells exposed to *Pseudomonas* exotoxin A (PEA) and selected for resistance (21). RPE.40 cells fail to process precursor membrane glycoproteins of several viruses, as well as the insulin proreceptor. This processing deficiency can be corrected by transfection with SPC1 cDNA (22, 23).

When CHO-K1 cells were transfected with the R71*X* and R77*X* stop codon mutants, peptides corresponding to cleavage products at Arg[71] and Arg[77] were immunoprecipitated from conditioned media (Fig. 9). Transfection with IGF-I-FLAG produced the Arg[71] and Arg[77] doublet, as well as proIGF-I and glycosylated proIGF-I. Expression of R71*X* and R77*X* in RPE.40 cells resulted in the same pattern of immunoprecipitable peptides as was seen in transfected CHO-K1 cells. However, when IGF-I-FLAG was expressed in RPE.40 cells, a single band of mature IGF-I, corresponding to IGF-I-FLAG processed at Arg[71], was observed. This indicates that RPE.40 cells process proIGF-I at Arg[71] but not Arg[77]. We have also observed a single band corresponding to Arg[71] cleavage when IGF-I-FLAG was transfected into LoVo cells (data not shown). LoVo cells do not process the MET protooncogene or the insulin proreceptor (24), and this processing deficiency has been attributed to a defective SPC1 gene (25).

DISCUSSION

We have used the pCMVigf1-FLAG expression vector and human embryonic kidney 293 cells to study proteolytic processing of human proIGF-I. Using the UB3–189 anti-human IGF-I antisera, we have shown that the pCMVigf1 and pCMVigf1-FLAG expression vectors direct synthesis and secretion of processed IGF-I and IGF-I-FLAG, respectively (Fig. 3). Using the M1 and M2 monoclonal antibodies, we have demonstrated that the FLAG epitope does not interfere with removal of the signal peptide (Fig. 4). PreproIGF-I-FLAG is therefore faithfully processed to mature IGF-I-FLAG and can be used to study post-translation processing of proIGF-I.

When expressed in 293 cells, pCMVigf1-FLAG directs synthesis of multiple forms of high molecular weight proIGF-I-FLAG peptides which are converted to proIGF-I-FLAG by incubation with *N*-glycosidase (Figs. 5 and 6). Although glycosylated forms of human proIGF-I have not been described previously, Simmons *et al.* (26) have shown that rat proIGF-I can be *N*-glycosylated in an *in vitro* translation system. Interestingly, peptides generated from initiation of translation at Met[−22] or Met[−32], but not Met[−48], were substrates for *N*-glycosylation. The pCMVigf1-FLAG expression vector contains translation initiation codons at Met[−22] and Met[−25], but Met[−48] has been eliminated. *N*-Glycosylated proIGF-I has not been identified *in vivo*, and the significance of this post-translational modification is unknown. However, it has been demonstrated that glycosylation can affect proteolytic processing of some precursor proteins. For instance, *N*-glycosylation of the human influenza virus hemagglutinin precursor at Asn[22] can inhibit processing at Arg[329] (27).

Proprotein precursors can be converted to mature forms intracellularly, at the cell surface membrane, or extracellularly. Pulse-chase experiments have shown that the E-domain of proIGF-II is cleaved at several sites during transport through the cell. Most of these cleavages, including the final cleavage to produce the 7-kDa mature IGF-II, appear to occur very late in the secretory pathway and may also occur extracellularly (28). We detect mature IGF-I-FLAG in 293 cell lysates, indicating that conversion can occur intracellularly (Fig. 7). We have not seen evidence for processing of proIGF-I-FLAG to mature IGF-I-FLAG when proIGF-I-FLAG peptides were incubated with conditioned media from 293 cells or serum from mouse, rat, or fetal calves (data not shown).

Expression of wild-type and mutant proIGF-I-FLAG constructs in 293 cells indicates that the IGF-I prohormone can be cleaved at two sites, Arg[71] and Arg[77]. The Arg[77] cleavage site conforms to the minimal SPC1 cleavage site defined by Molloy *et al.* (15) with Arg[74] as the P4 residue and Arg[77] as the P1 residue. All mutants containing alanine in place of Arg[74] or Arg[77] eliminate processing at Arg[77] (R74A, R77A, K65A/R74A, K65A/R77A, K68G/R77A, R71A/R77A, R74A/R77A). In all mutants containing substitutions of nonbasic residues for Lys[65], Lys[68], or Arg[71], the percentage of precursor processing at Arg[77]

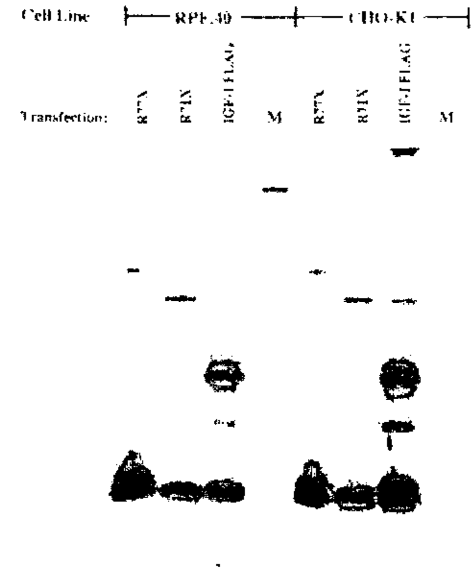

FIG. 9. **Processing of proIGF-I in RPE.40 cells.** CHO-K1 and RPE.40 cells were transfected with R71*X*, R77*X* or IGF-I-FLAG expression vectors and cells were metabolically labeled with [35S]cysteine. Peptides were immunoprecipitated with the M2 antibody and resolved on Tricine-buffered 16.5% polyacrylamide gels, as described under "Experimental Procedures." *M* indicates mock transfection. The molecular masses of the [14C]-methylated protein markers are indicated.

is increased (K65A, K68G, R71A, K65A/K68G, K68G/R71A) (Table II). One explanation for the increased processing at Arg[77] in these mutants is that the secondary structure of the peptide may have been modified in a way that allows for more favorable enzyme-substrate interactions. However, each of the Lys[65], Lys[68], and Arg[71] mutants also decreases or eliminates processing at Arg[71]. It is therefore likely that the increased processing at Arg[77] is a result of the creation of a less efficient Arg[71] cleavage site. In addition to mutational analysis, data obtained from the expression of wild-type proIGF-I-FLAG in SPC1-deficient cell lines also implicate SPC1 as the Arg[77] cleavage enzyme. When wild-type proIGF-I-FLAG is expressed in CHO-K1 cells, cleavage occurs at Arg[71] and Arg[77]. However, when expressed in RPE.40 cells, a CHO-K1 derivative lacking SPC1 activity, cleavage of proIGF-I-FLAG occurs at Arg[71] but not Arg[77] (Fig. 9). Processing at Arg[71], but not Arg[77], was also observed when proIGF-I-FLAG was expressed in the SPC1-deficient LoVo cell line (data not shown). It therefore appears that SPC1 is required for cleavage of proIGF-I-FLAG at Arg[77] but not Arg[71].

Since IGF-I isolated from human serum is 70 amino acids long, it is likely that final maturation occurs by cleavage of the precursor at Arg[71]. The presence of a second cleavage site at Arg[77] which affects processing efficiency at Arg[71] makes [illegible]

[illegible] Arg[77] [illegible] seems paradox[illegible] the K65A/K68G and K65A/R77A double mutants [illegible] than the K65A single mutant [illegible] (Table II). A likely explanation, however, [illegible] mutants eliminate the P4 and P[illegible] SPC1 cleavage site, therefore making [illegible] for the Arg[71] cleaving enzyme. Arg[71] alone is not sufficient for processing since the K68G/R74A mutant [illegible] very poorly (Fig. 8*D*, Table II).

The optimal SPC1 cleavage site has been defined as RXK/RR (29). SPC1 will also cleave some precursors with an RXXR motif with reduced efficiency (15, 16, 29) while other precursors with this motif are not cleaved (30). It is unclear whether SPC1 will cleave substrates containing a lysine residue in the P4 position. The fusion glycoprotein precursor (F_0) of the Ulster strain of Newcastle disease virus contains the KQGR cleavage motif. The F glycoprotein of Newcastle disease virus strain Ulster was not cleaved when coexpressed or incubated *in vitro* with SPC1 (30). A virulent avian influenza virus hemagglutinin containing the RKKR cleavage motif was processed efficiently by SPC1 in both coexpression and *in vitro* incubation experiments. When the P4 arginine of this motif was mutated to lysine to create KKKR, 75% of the hemagglutinin was cleaved when coexpressed with SPC1, and the hemagglutinin was not cleaved when incubated with SPC1 *in vitro* (31). Our data on expression of proIGF-I-FLAG in RPE.40 and LoVo cells indicate that SPC1 is not necessary for final maturation of IGF-I. The identity of the proIGF-I converting enzyme is unknown. SPC4 is a candidate because it is expressed in many tissue types. However, this enzyme apparently has a more strict requirement for a basic residue in the P2 position than does SPC1 (16, 17). For many proprotein precursors that are processed at basic residues, the scissile bond is on the carboxy terminus of the P1 residue. After cleavage of the scissile bond by a specific endoprotease, the basic residues are removed by carboxypeptidases. Recently, a metalloendopeptidase which cleaves peptide bonds on the amino terminus of arginine residues was cloned from a rat testis cDNA library (32). Although the proIGF-I cleavage site is similar to the motif recognized by enzymes of the SPC family, it is possible that a different class of enzyme may convert proIGF-I to mature IGF-I.

It is interesting that the R74*X* and R77*X* stop codon mutants are cleaved at Arg[71]. This indicates that as few as two amino acids on the carboxyl terminus of the P1 residue are sufficient for precursor processing by the enzyme. Processing of these mutants, as well as mutants containing alanine substitutions at Arg[74] or Arg[77], also demonstrates that cleavage at Arg[77] need not precede cleavage at Arg[71]. Pulse-chase experiments have shown that proIGF-II is cleaved at multiple sites in the E-domain (28). Similar experiments will be necessary to determine if proIGF-I is cleaved at Arg[77] before final maturation. At this time there is no evidence for the existence of IGF-I (1–77) *in vivo* and the SPC1-mediated cleavage observed at Arg[77] in our experiments may be a consequence of overexpression. Nonetheless, this observation is interesting in terms of SPC1 site selection specificity, and proIGF-I may be a useful model for studying this aspect of prohormone processing. Three repetitive motifs of basic-*X*-*X*-basic residues are contained within only 10 amino acids. SPC1 recognizes the motif R[74]XXR[77]. The