SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 12-185230- -3 **FECHA**: 2013-09-30 14:17:45
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 7
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
LUZ CLEMENCIA SUAREZ DE PAEZ

Asunto: Radicación: 12-185230- -3
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 18631
Folios: 7

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/JP2011/056643				
Fecha de Presentación Internacional	18/03/2010				

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:	Rad. No. 12-185230-00000-0000	19/Octubre/2012
Reivindicaciones:	Rad. No. 12-185230-00000-0000	19/Octubre/2012
Dibujos:	Rad. No. 12-185230-00000-0000	19/Octubre/2012
Prioridades:	JP 2010-065089	19/Marzo/2010

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

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10)

reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Se aceptan para estudio todas las 12 reivindicaciones presentadas (Fls. 33 a 35 del Rad. No. 12-185230-00000-0000) porque las tasas pagadas cubren en su totalidad lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única.

Título de la solicitud: “Conjunto de agujas que comprenden proteoglicano” (Fl. 1 del Rad. No. 12-185230-00000-0000).

El problema planteado en esta solicitud consiste en la necesidad de suministrar una matriz de microagujas para la dosificación de principios activos que no causen dolor ni hemorragia en la capa superficial de la piel y presenten buena solubilidad debajo de la piel.

Para resolver este problema técnico, la solicitud en estudio proporciona métodos de formación de microagujas a partir de proteoglicano como material de base.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP) A61K 008/064, 009/000, 035/060, 038/000, A61M 037/000, A61P 017/016, 019/002, A61Q 019/000

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicaciones 1, 9 y 10 no son concisas porque las definiciones de substrato, polímero soluble en agua, propiedad medicinal cosméticamente o farmacéuticamente aceptable, son géneros funcionales, se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que han sido sintetizados o probados, es decir, que se encuentran debidamente sustentados.

La reivindicación 4 no es clara porque no se debería caracterizar el compuesto solamente por sus parámetros. Se sugiere definirlo por sus características esenciales, estructurales o funcionales.

La reivindicación 11 no es clara porque muestra dos métodos diferentes. Se sugiere hacer una reivindicación independiente para cada uno de los métodos.

5. 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 invocada, es decir, el 19 de marzo de 2010.

Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Item	Documento	Título	Fecha de Publicación
D1	EP 1844763	“Percutaneously absorbable preparation, percutaneously absorbable preparation holding sheet, and percutaneously absorbable preparation holding equipment”	17/Octubre/2007
D2	EP 1935438	“Biomaterial for regenerative medicine”	25/Junio/2008

El documento D1[1] divulga preparaciones absorbibles por vía percutánea autodisolventes en forma de aguja, la cual es absorbible en el cuerpo a través de la piel. (Pág. 2, Fig. 1, Pág. 3, Párr. 1, Pág. 6, Párr. 26, Pág. 9, Párr. 69)

El documento D2[2] divulga biomateriales utilizables para la regeneración de tejidos, particularmente los complejos de glicosaminoglicano/proteoglicano/colágenos formadas a través de técnicas de auto-organización. (Pág. 2, Párr. 1, Pág. 3, Párr. 9, Pág. 6, Párr. 16)

6. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art 18 D486)

La reivindicación 1 consiste en "*Un conjunto de microagujas caracterizado porque comprende una o más microagujas formadas en la superficie de un substrato…*" (Fl. 33 del Rad. No. 12-185230-00000-0000).

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

Características **esenciales**	**D1**	**D2**
Conjunto de microagujas caracterizado porque comprende una o más microagujas formadas en la superficie de un substrato	Preparaciones absorbibles auto-disolventes por vía percutánea que tiene una forma delgada, puntiaguda adaptado para su inserción en la piel (forma de tipo aguja o forma filamentosa), que incluyen una base de soporte y una sustancia activa. (Pág. 3, Párr. 1, Pág. 4, Párr. 13, Pág. 5, Párr. 19, Pág. 37, Fig. 10)	No se menciona
Las microagujas comprenden proteoglicano como un material base	No se menciona	Biomateriales utilizables para la regeneración de tejidos, particularmente los complejos de glicosaminoglicano/ proteoglicano/colágeno formadas a través de técnicas de auto-organización (Pág. 2, Párr. 1, Pág. 3, Párr. 9, Pág. 5, Párr. 11).

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 1, porque divulgan microagujas que incluyen una base de soporte y biomateriales utilizables para la regeneración de tejidos que pueden adaptarse para ser administrados en pacientes.

La diferencia entre la invención, definida en la reivindicación 1 y las microagujas del documento D1 consiste en que D1 no se menciona el proteoglicano.

La diferencia entre la invención, definida en la reivindicación 1 y el documento D2 consiste en que en D2 no se mencionan las microagujas.

El efecto que logra el incluir el proteoglicano como material de base para las microagujas es que permite suministrar un producto resistente, flexible, soluble en el cuerpo y adecuado para aplicaciones cosméticas o médicas de forma más eficiente.

_____
[1]http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=EP&NR=1844763A1&KC=A1&FT=D&ND=3&date=20071017&DB=EPODOC&locale=en_EP
[2]http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=EP&NR=1935438A1&KC=A1&FT=D&ND=3&date=20080625&DB=EPODOC&locale=en_EP

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así "¿cómo modificar las microagujas D1 para que sean un producto resistente, flexible, soluble en el cuerpo y puedan ser utilizadas para aplicaciones cosméticas o médicas de forma más eficiente?".

Sin embargo, la aplicación médica de proteoglicanos, ya está divulgado en el documento D2 que se refiere a biomateriales utilizables para la regeneración de tejidos, particularmente complejos de glicosaminoglicano/proteoglicano/colágeno y el método de su obtención (Pág. 2, Párr. 1, Pág. 3, Párr. 8 y 9, Pág. 5, Párr. 11).

En consecuencia, una persona normalmente versada en la materia incluiría los proteoglicanos de D2 como material de base para las microagujas de la anterioridad D1, esperando con certeza lograr el producto, como la de la reivindicación 1 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Puesto que las reivindicaciones dependientes 2 a 10 no mencionan características adicionales que hagan inventiva la solicitud, se considera que tales reivindicaciones tampoco tienen nivel inventivo ya que D1 divulga la forma de las agujas como un cono truncado, la forma de las agujas (microagujas) es de preparación sólida, con medidas de longitud que puede ser seleccionada entre 0,5 a 1500 micrómetros, el diámetro de la pieza que empuja está en un intervalo aproximado 0,1 a 500 micrómetros, valor máximo del diámetro es de 500 micrómetros, no hay ninguna limitación en el contenido de la sustancia activa en la preparación absorbible por vía percutánea, todas las preparaciones absorbibles percutáneas que tienen una forma delgada y puntiaguda constan de una base de material de polímero soluble en agua y soluble biológicamente y sobre esta base una sustancia activa, garantizando la seguridad higiénica al usar preparados farmacéuticos, la microaguja está hecha del material de base que se disuelve en el cuerpo (Pág. 4, Párr. 9 y 12, Pág. 5, Párr. 16 y 19, Pág. 6, Párr. 48, Pág. 9, Párr. 66, 67 y 69, Pág. 12, Párr. 87, Pág. 14, Párr. 99, Pág 29, Fig. 1, Pág. 30, Fig. 2); y asimismo D2 divulga que los glicosaminoglicanos unidos covalentemente a las proteínas forman proteoglicanos y que se clasifican en sulfato de condroitina, sulfato de dermatán, heparina, sulfato de heparán, en donde el proteoglicano se puede extraer de peces tales como tiburones y salmones, siendo conocido por la persona versada en la materia que los proteoglicanos son utilizados en estudios de interacción de células con una matriz extracelular, por lo que se pueden considerar como un sustituto en el material base de las microagujas[3] (Pág. 6, Párr. 13, Pág. 6, Párr. 16).

Conclusión:
Las reivindicaciones 1 a 10 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 11 consiste en "*Un método de producción del conjunto de microagujas de acuerdo con la reivindicación 1 a 10…"* (Fl. 34 del Rad. No. 12-185230-00000-0000).

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

Características **esenciales**	**D1**	**D2**
Método de producción del conjunto de microagujas de acuerdo con la reivindicación 1 a 10	Los métodos de fabricación de las preparaciones absorbibles por vía percutánea no están particularmente limitados y varios métodos son aplicables. (Pág. 12 Párr. 93)	No se menciona

1. Verter una solución acuosa en la cual se disuelve proteoglicano	No se menciona	La preparación de glicosaminoglicano-proteoglicano es en solución acuosa, aunque la solución no se limita al agua. (Pág. 6, Párr. 13 y 17).
para formar microagujas en un molde en el cual está ahuecada la forma de un conjunto de microagujas, de modo que se forman una parte de microaguja y una parte de substrato	Se utiliza un molde para la fabricación de las preparaciones absorbibles por vía percutánea, el cual se prepara al hacer agujeros cónicos en la parte delantera de la placa y la base que contiene una sustancia activa que se introduce en los orificios. (Pág. 12, Párr. 93, Pág. 13, Párr. 94, Pág. 36, Fig. 8A y 8B, Pág. 37, Fig. 9)	No se menciona
2) Evaporar la humedad a sequedad a temperatura ambiente o por calentamiento,	La elaboración de las preparaciones absorbibles por vía percutánea en forma tipo aguja después de moldearse, se dejan secar y solidificar. (Pág. 13, Párr. 95)	No se menciona
3) remover del molde el conjunto de microagujas formado.	Se retira el molde luego de obtener la forma tipo aguja y luego de secado y solidificación. (Pág. 13, Párr. 95)	No se menciona
1´) Verter una solución acuosa en la cual se disuelve proteoglicano	No se menciona	La preparación de glicosaminoglicano-proteoglicano es en solución acuosa, aunque la solución no se limita al agua. (Pág. 6, Párr. 13 y 17).
para formar microagujas en un molde en el cual está ahuecada la forma de una microaguja;	Se utiliza una placa y un palo, una base que contiene la sustancia activa se pone en un plato, se utiliza una varilla de vidrio que al levantarla la base se adhiere a la parte superior de ésta y se moldea en forma similar a una aguja. (Pág. 12, Párr. 93, Pág. 35, Fig.7A, 7B y 7C, Pág. 13, Párr. 96)	No se menciona
2´) Evaporar la humedad a sequedad a temperatura ambiente o por calentamiento, y	La elaboración de las preparaciones absorbibles por vía percutánea en forma tipo aguja después de moldearse, se dejan secar y solidificar. (Pág. 13, Párr. 95)	No se menciona
3´) laminar un substrato sobre la misma y combinar o unir la parte inferior de las microagujas y el substrato, y	Para sostener al menos una de las preparaciones absorbibles por vía percutánea se realizó un soporte similar a una lámina, donde éstas se llevan a cabo por un lado del cuerpo de soporte, así se insertan en la piel empujando la lámina portadora. (Pág. 13, Párr. 97, Pág. 37, Fig. 10)	No se menciona
4´) remover del molde las microagujas combinadas o unidas con el substrato	En la elaboración de las preparaciones absorbibles por vía percutánea luego de obtener la forma tipo aguja y luego de secado y solidificación se retira el molde. (Pág. 13, Párr. 95)	No se menciona

[3]Echtermeyer, et al. Syndecan-4 core proteins is sufficient for the assembly of focal adhesions and actin stress fibers. Journal of cell science, 1999. Vol. 112, Pág. 3437.

Los documentos D1 y D2 se consideran el estado de la técnica más cercano a la invención definida en la reivindicación 11, porque divulgan un método de fabricación de microagujas que incluyen una base de soporte y biomateriales utilizables para la regeneración de tejidos que pueden adaptarse para ser administrados en pacientes.

La diferencia entre la invención definida en la reivindicación 11 y la producción de las microagujas del documento D1 consiste en que en D1 no se menciona el uso del proteoglicano.

La diferencia entre la invención, definida en la reivindicación 11 y el documento D2 consiste en que en D2 no se menciona la producción de microagujas.

El efecto que logra el incluir proteoglicano como material de base para la formación de microagujas es que permite suministrar un producto resistente, flexible, soluble en el cuerpo y adecuado para aplicaciones cosméticas de forma más eficiente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así "¿cómo modificar las microagujas D1 para que sean un producto resistente, flexible, soluble en el cuerpo y puedan ser utilizadas para aplicaciones cosméticas o médicas de forma más eficiente?".

> Sin embargo, la aplicación médica de proteoglicanos, ya está divulgado en el documento D2 que se refiere a biomateriales utilizables para la regeneración de tejidos, particularmente complejos de glicosaminoglicano/proteoglicano/colágeno y el método de su obtención (Pág. 2, Párr. 1, Pág. 3, Párr. 8 y 9, Pág. 5, Párr. 11).

En consecuencia, una persona normalmente versada en la materia incluiría los proteoglicanos de D2 como material de base para la elaboración de las microagujas de la anterioridad D1, esperando con certeza lograr el método, como la de la reivindicación 11 de la solicitud, lo cual es considerado obvio y, por lo tanto, sin altura inventiva.

Puesto que la reivindicación dependiente 12 no menciona características adicionales que hagan inventiva la solicitud, se considera que tal reivindicación tampoco tiene nivel inventivo ya que D1 divulga preparaciones absorbibles percutáneas que contienen interferón como sustancia activa y albúmina de suero humano como base, en relación de 0,2 ml de agua destilada por 150 mg de albúmina de suero humano (Pág. 15, Párr. 105 y 106) y D2 divulga preparaciones de glicosaminoglicano/proteoglicano en donde el proteoglicano se prepara en solución acuosa de 0,1 a 0,5mg/ml y siendo conocido por la persona versada en la materia que los proteoglicanos son utilizados en estudios de interacción de células con una matriz extracelular, por lo que se pueden considerar como un sustituto en el material base de las microagujas[3] (Pág. 6, Párr. 17).

Conclusión:
Las reivindicaciones 11 y 12 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

7. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	EP 1844763	1 a 12	Nivel inventivo
D2	EP 1935438	1 a 12	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 del día siguiente 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.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título 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 2013-10-08

Elaboró: Jorge Eduardo Cortazar Gomez
Revisó: Rosa Patricia Tellez Chavarro

Journal of Cell Science 112, 3433-3441 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JCS4670

Syndecan-4 core protein is sufficient for the assembly of focal adhesions and actin stress fibers

Frank Echtermeyer, Peter C. Baciu*, Stefania Saoncella, Yimin Ge and Paul F. Goetinck‡

Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Building 149, 13th Street, Charlestown, Massachusetts 02129, USA

*Present address: Allergan Inc., 2525 Dupont Drive, Irvine, CA 92612, USA

‡Author for correspondence (e-mail: paul.goetinck@cbrc2.mgh.harvard.edu)

Accepted 20 April; published on WWW 30 September 1999

SUMMARY

The formation of focal adhesions and actin stress fibers on fibronectin is dependent on signaling through β1 integrins and the heparan sulfate proteoglycan syndecan-4, and we have analyzed the requirement of the glycosaminoglycan chains of syndecan-4 during these events. Chinese hamster ovary cells with mutations in key enzymes of the glycanation process do not synthesize glycosaminoglycan chains and are unable to assemble actin stress fibers and focal contacts when cultured on fibronectin. Transfection of the mutant cells with a cDNA that encodes the core protein of chicken syndecan-4 leads to the production of unglycanated core protein. The overexpression of syndecan-4 core protein in these mutant cells increases cell spreading and is sufficient for these cells to assemble actin stress fibers and focal adhesions similar to wild-type cells seeded on fibronectin and vitronectin matrices. Syndecan-4 core protein colocalizes to focal contacts in mutant cells that have been transfected with the syndecan-4 core protein cDNA. These data indicate an essential role for the core protein of syndecan-4 in the generation of signals leading to actin stress fiber and focal contact assembly.

Key words: Syndecan, Heparan sulfate proteoglycan, Cell attachment, Actin cytoskeleton, Chinese hamster ovary cell

INTRODUCTION

Interactions between extracellular matrix (ECM) molecules and cell surface receptors in vivo regulate various important aspects of cell behavior such as adhesion, proliferation, migration, survival and differentiation (Adams and Watt, 1993; Ashkenas et al., 1996; Giancotti, 1997; Gumbiner, 1996; Howe et al., 1998). Fibroblasts seeded on a fibronectin or vitronectin substrate attach, spread and assemble actin stress fibers and focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996). The latter are specialized sites of adhesion that contain integrins and syndecan-4 as transmembrane receptors, cytoplasmic structural proteins such as talin, vinculin, alpha-actinin, tensin and paxillin and the signaling proteins focal adhesion kinase (FAK), Src and protein kinase C (PKC) (Burridge and Chrzanowska-Wodnicka, 1996; Clark and Brugge, 1995). The assembly of these macromolecular complexes requires signals from both integrins and heparan sulfate proteoglycans (HSPGs) (Hynes, 1992; Woods et al., 1986). Recently we identified syndecan-4 as the cell surface HSPG that acts cooperatively with integrins in generating signals necessary for the assembly of actin stress fibers and focal adhesions in cells plated on fibronectin (Saoncella et al., 1999). Syndecan-4 is a member of the syndecan family of transmembrane heparan sulfate proteoglycans, which are characterized by a highly conserved short cytoplasmic domain, a transmembrane domain and an extracellular domain bearing heparan sulfate glycosaminoglycan (GAG) chains at specific serine residues (Bernfield et al., 1992; Rapraeger and Ott, 1998). The GAG chains are post-translationally linked to the core protein via a linkage region composed of xylose-galactose-galactose-glucuronic acid (Fig. 1). The transfer of xylose to serine residues in the core protein is catalyzed by xylosyltransferase and the addition of the two galactose residues is catalyzed, respectively, by galactosyltransferase-I and galactosyltransferase-II. There have been no reports of naturally occuring syndecan core proteins without GAG chains (Carey, 1997). The majority of the interactions of the syndecans with ECM molecules, growth factors and cell adhesion molecules is thought to be mediated by their heparan sulfate side chains (Bernfield et al., 1992; Stringer and Gallagher, 1997). Only a few examples of molecular interactions involving the ectodomain of the core protein have been documented (McFall and Rapraeger, 1998). A number of mutant Chinese hamster ovary (CHO) cell lines with defects that affect different stages of the glycanation of proteoglycan core proteins have been described (Esko et al., 1985, 1986, 1987). Among those were mutants with a defective xylosyltransferase (CHO-745) and galactosyltransferase-I (CHO-618) respectively. The CHO-745 mutation affects the transfer of xylose, the first sugar of the neutral sugar linkage region necessary for GAG addition, leading to an absence or

highly reduced amounts of GAG chains on the HSPG core proteins (Esko et al., 1985). CHO-745 cells plated on fibronectin attach and spread, but show a reduced number of focal contacts and they are not able to form typical actin-containing stress fibers (LeBaron et al., 1988). In the present study we have used these cells to analyze the function of the syndecan-4 core protein during cytoskeletal rearrangements and the process of adhesion and focal contact formation. Our results indicate that the GAG-deficient syndecan-4 core proteins are sufficient for the assembly of focal adhesions and actin stress fibers in these cells.

MATERIALS AND METHODS

Cell lines, antibodies and materials

Chinese hamster ovary wild-type (CHO-K1) and xylosyltransferase (CHO-745) and galactosyl transferase-I mutant (CHO-618) cells were obtained from J. D. Esko. The cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 50 mM Hepes, 250 μg/ml streptomycin and 250 i.u./ml penicillin in a humidified atmosphere of 5% CO_2 at 37°C. Medium and supplements were from Life Technologies (Gaitersburg, MD).

Wild-type and mutant cell lines were transfected with the full-length chicken syndecan-4 cDNA cloned into the expression vector pcDNA 3 (Invitrogen; Carlsbad, CA) or with the pcDNA 3 vector only and geneticin (G418) (700 μg/ml) (Life Technologies, Gaitersburg, MD)-resistant stable transfectants were selected. Polyclonal antibodies directed against the cytoplasmic domain of murine syndecan-1 (MS-1-C) and against a unique region of the extracellular domain of chicken syndecan-4 (CS-4-E) have been described (Baciu et al., 1994). The monoclonal anti-human vinculin antibody (hVIN-1) was obtained from Sigma (St Louis, MO). Anti-mouse and anti-rabbit antibodies conjugated with fluorescein isothiocyanate (FITC) were from Pierce (Rockford, IL) and anti-mouse antibodies conjugated with Cy5 were from Amersham Life Science Inc. (Arlington Heights, IL). The BODIPY 581/591 phalloidin was obtained from Molecular Probes (Eugene, OR). Human fibronectin and human vitronectin were obtained from Becton Dickinson (Bedford, MA).

Immunoprecipitation

Cell labeling and immunoprecipitation were performed as previously described (Baciu et al., 1994). Briefly, 10^7 cells were incubated for 4 hours at 37°C with 1 mCi of a ^{35}S-labelled methionine/cysteine mixture (Amersham Pharmacia Biotech, Piscataway, NJ) in methionine- and cysteine-free DMEM containing 10% FCS. PBS-washed cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.0; 150 mM NaCl; 5 mM EDTA; 1 mM EGTA; 1% NP-40; 1% sodium desoxycholate; 0.1% SDS) supplemented with a mixture of EDTA-free proteinase inhibitors (Boehringer Mannheim Cooperation, IN) for 10 minutes on ice and scraped. The lysate was passed several times through a 20 G-1 1/2 needle and microcentrifuged for 1 hour at 4°C. The resulting supernatant was incubated overnight at 4°C with 50 μl of protein-G beads preloaded with the MS-1-C antibody. Immunoprecipitated complexes were washed for 20 minutes each in RIPA buffer, RIPA buffer containing 0.5 M NaCl, a RIPA/TNE buffer mixture and TNE buffer alone (10 mM Tris-HCl, pH 7.0; 150 mM NaCl; 5 mM EDTA). SDS-polyacrylamide gel electrophoresis buffer (4×) was added, and the samples were size-fractionated by electrophoresis in a 4%-20% SDS-polyacrylamide gel under denaturing conditions (Laemmli, 1970). Gels were incubated for 30 minutes each in fixation solution (50% methanol, 7% acetic acid), and in amplifying solution (Amplify, Amersham Life Science), dried on 3 MM paper under vaccuum and exposed to autoradiography films (Kodak, BIOMAX MS) with intensifying screens overnight at –70°C.

Labelling of GAG chains was achieved by incubation of 10^7 cells with 360 μCi $H_2{}^{35}SO_4$ for 4 hours in 5 ml of F12 medium supplemented with 10% FCS. Cells were lysed in RIPA buffer and syndecan-4 was precipitated with 50 μl of protein-G beads preloaded with CS-4-E antibodies as described above. After washing with a series of RIPA buffers, the immunoprecipitated material was rinsed in heparitinase buffer (50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, 0.1% TritonX-100, pH 7.4) and than resuspended in 60 μl heparitinase buffer. 2 milliunits of heparitinase (Seikagaku, Rockville, MD) were added and the mixture was incubated for 1 hour at 37°C. Another 2 milliunits of heparitinase were added and the digestion was continued for an additional 2 hours at 37°C. The amount of radiolabed GAG chains cleaved from precipitated syndecan-4 was determined by measuring the supernatant of heperitinase digested mixture by liquid scintillation in a Beckman LS60001C counter.

Cell adhesion assay

Cell adhesion assays were performed as described earlier (Echtermeyer et al., 1996). Briefly, 96-well plates (Maxisorb, Nunc) were coated for 1 hour at 37°C with serial dilutions of fibronectin or vitronectin solutions at initial concentrations of 100 μg/ml or 50 μg/ml, respectively. Residual protein binding sites were then blocked with heat-treated (10 minutes at 80°C) 2% BSA (Sigma; St Louis, MO) in PBS overnight at 4°C. Cells were seeded at a density of 5×10^4 cells per well in 100 μl of DMEM, 0.5% BSA. After 1 hour of incubation at 37°C unattached cells were removed by washing with PBS and the attached cells were assayed by colorimetric determination of endogenous hexosaminidase activity (Goodman et al., 1987; von der Mark et al., 1991).

Immunofluorescence

Indirect immunofluorescence analysis was performed by coating glass coverslips with 40 μl of a fibronectin (100 μg/ml) or vitronectin (50 μg/ml) solution for 1 hour at 37°C followed by washing three times with PBS. Cells were allowed to attach for indicated times and then fixed for 5 minutes with 3.7% paraformaldehyde in PBS containing 0.1% Tween 20. These cells were then washed for 10 minutes with PBS, quenched for 10 minutes with NH_4CL, and washed for 10 minutes with PBS before the addition of the primary antibody (Woods and Couchman, 1994). Nonspecific binding was blocked by incubating the cells with 1% goat serum in PBS for 15 minutes at room temperature or overnight at 4°C. The vinculin antibody was used at a 1/500 dilution in PBS containing 1% goat serum for 1 hour at 37°C for the detection of focal contacts. Samples were washed three times with PBS, incubated for 45 minutes at 37°C with FITC-conjugated anti-mouse antibodies, and washed three times with PBS. Actin fibers were visualized by staining with BODIPY 581/591 phalloidin following the manufacturer's instructions.

Detection of syndecan-4 in focal adhesions was achieved by plating cells on fibronectin-coated coverslips for 3 hours, washing with PBS and incubating with deionized water containing 1% PBS for 20 minutes at 37°C. After intensive washing with PBS, samples were fixed as described above and incubated for one hour with CS-4-E antibodies diluted 1/20 and hVIN-1 antibodies diluted 1/500 in PBS containing 1% goat serum. Samples were washed three times with PBS and incubated with FITC-conjugated anti-rabbit antibodies for syndecan-4 detection and Cy5-labeled anti-mouse antibodies for vinculin detection. With both techniques coverslips were then washed three times with PBS and mounted using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Antibody staining was observed using a Leica TCS NT4D confocal imaging system (Leica, Heidelberg, Germany). During double-labeling experiments there was no evidence of bleed-through from one channel to the other. All experiments were repeated at least three times and the cells shown

represent typical staining patterns observed at the indicated time points.

RESULTS

Characterization of generated cell lines

Wild-type CHO cells (CHO-K1) and glycosaminoglycan-deficient mutant CHO cells defective in the xylosyltransferase (CHO-745) or in galactosyltransferase-I (CHO-618) (Fig. 1) were used to investigate the function of the syndecan-4 core protein in cell adhesion, actin stress fiber assembly and focal contact formation. These various cell lines were stably transfected with pcDNA 3 containing full-length chicken syndecan-4 cDNA or vector alone. The presence of chicken syndecan-4 proteoglycan or core protein was identified in immunoprecipitation assays from cells that had been incubated with ^{35}S-labeled methionine and cysteine (Fig. 2A). The MS-1-C antibody, which recognizes the cytoplasmic domain of syndecan-1 and -4 of different species, was used in these experiments. A diffuse band of approximately 200 kDa, typical for fully glyconated syndecans, was observed only in the immunoprecipitates of CHO-K1 that had been transfected with the chicken syndecan-4 core protein construct (Fig. 2A, CHO-K1/syn4; arrowhead). Identification of the 200 kDa band as a heparan sulfate proteoglycan is indicated from treatment of the immunoprecipitates with heparitinase. Such treatment eliminates the 200 kDa band completely and results in the appearance of a 34 kDa band. A large amount of syndecan-4 core protein, but not its glycanated form, was evident in the xylosyltransferase mutant transfected with the chicken syndecan-4 core protein construct (Fig. 2A, CHO-745/syn4; arrow). The radiolabeled bands in the 46 to 70 kDa range seen in all the lanes have not been identified. These bands are not detected in western blots with antibodies directed against chicken syndecan-4 (data not shown). These results are interpreted to mean that the xylosyltransferase mutant cells (CHO-745) do not glycanate the chicken syndecan-4 core protein. This conclusion is further supported by measuring the incorporation of $^{35}SO_4$ into heparan sulfate. Syndecan-4 was immunoprecipitated from $^{35}SO_4$-labeled cells with the chicken syndecan-4 ectodomain specific antibody CS-4-E and heparan sulfate was quantified as heparitinase-sensitive material in the immunoprecipitates. In these experiments heparitinase-sensitive material (Fig. 2B) was detected only in the wild-type cells transfected with the chicken syndecan-4 cDNA (CHO-K1/syn4) and not in the similarly transfected xylosyltransferase mutant cells (CHO-745/syn4). Together these results indicate that the xylosyltransferase mutant cells do not detectably glycanate proteoglycan core proteins even when they express large amounts as a result of transfection with the chicken syndecan-4 core protein cDNA construct. These results are consistent with those reported by Esko et al. (1987) and LeBaron et al. (1988).

In contrast to absence of glycanation of the chicken syndecan-4 core protein by the xylosyltransferase mutant CHO-745 cells, a small amount of glycanated syndecan-4 protein could be detected in the galactosyltransferase-I defective mutant CHO-618 cells, indicating that the addition of GAG chains to syndecan core proteins is not completely abolished in this mutant (data not shown). For this reason we used only vector and syndecan-4 transfected wild-type and xylosyltransferase defective CHO-745 cells for further analysis.

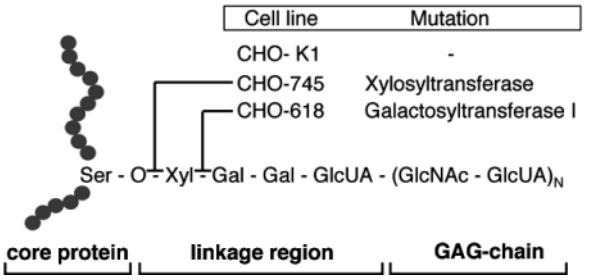

Fig. 1. Attachment of glycosaminoglycan chains to proteoglycan core proteins. Glycosaminoglycan chains (GAG-chains) consist of repeats of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA) and are attached to serine (Ser) residues of the core protein via a short linkage region in wild-type CHO cells (CHO-K1). CHO cells with mutations in enzymes that catalyze the transfer of xylose (Xyl) to serine (CHO-745 cells) or galactose (Gal) to xylose (CHO-618 cells) cannot build the linkage region and therefore are lacking glycanated proteoglycans.

Cell attachment on fibronectin and vitronectin substrates

The presence of GAG chains at all three attachment sites of the syndecan-1 core protein is critical for the adhesion of ARH-77 cells to collagen (Langford et al., 1998). As CHO-745/syn4 cells have no GAG chains linked to the syndecan-4 core protein and as syndecan-4 is a component of focal contacts formed on fibronectin and vitronectin, we examined if these cells have changed their adhesion capabilities for fibronectin and vitronectin. We found that CHO-K1/pc and CHO-745/pc cells had similar profiles of attachment to increasing concentrations of fibronectin and vitronectin (Fig. 3). This is consistent with previous data demonstrating no difference in the attachment of CHO-K1 and CHO-745 cells to fibronectin (LeBaron et al., 1988). The expression of the chicken syndecan-4 core protein is not sufficient to increase the adhesion rate of CHO-745/syn4 cells to fibronectin or vitronectin (Fig. 3). CHO-K1/syn4 cells, however, seemed to achieve a slightly better adherence to fibronectin and vitronectin than the CHO-K1/pc cells, indicating that fully glycanated syndecan-4 might improve the adhesion process. On the other hand these data demonstrate that the lack of GAG chains on the syndecan-4 core protein does not impair the ability of both CHO-745/pc and CHO-745/syn4 cells to attach to fibronectin and vitronectin.

Focal contact and stress fiber formation on fibronectin

For the analysis of actin cytoskeleton and focal contact formation, cells were seeded on fibronectin-coated coverslips and fixed after different time intervals. Focal contacts were visualized by immunofluorescence staining with antibodies against vinculin and by interference reflection microscopy (IRM). Actin-containing cytoskeleton was identified by staining with phalloidin-rhodamine.

Thirty minutes after plating, the GAG chain-deficient CHO-

745/pc and CHO-745/syn4 cells displayed a more rounded cell shape and did not spread as well as the wild-type CHO-K1/pc and CHO-K1/syn4 cells (Figs 4, 5). After 3 hours, CHO-745/syn4 cells were bigger in size and their cell body was flatter and better spread than the CHO-745/pc cells.

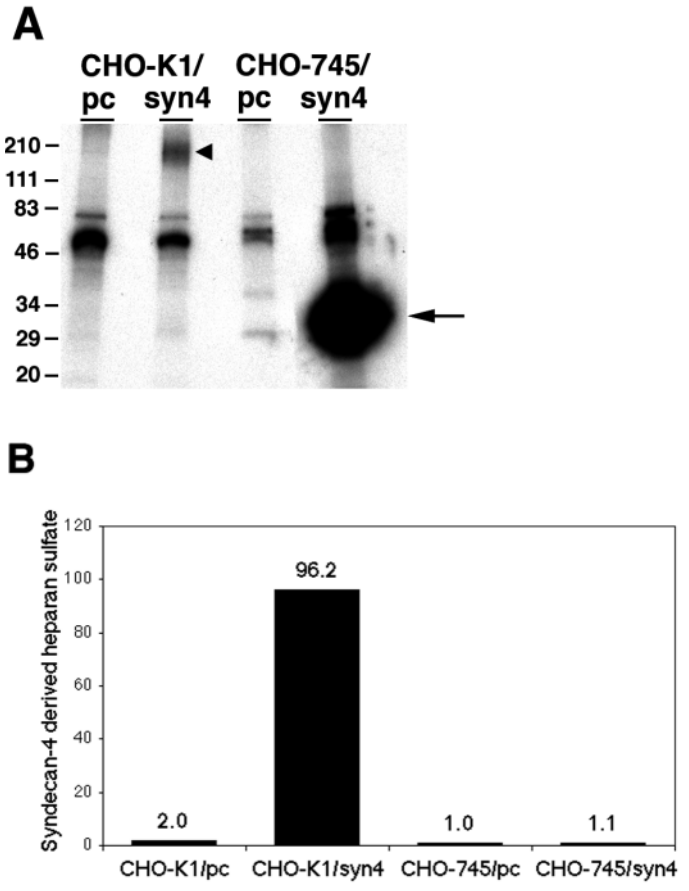

Fig. 2. Xylosyltransferase mutant CHO cells do not glycanate syndecan-4 core protein. (A) The ability of wild-type and mutant CHO cells to glycanate syndecan core protein was determined by immunoprecipitation of ^{35}S-labeled cell lysates with antibodies recognizing the cytoplasmic domain of all syndecans (MS-1-C). A band with a molecular mass of 200 kDa, characteristic for glycanated syndecan-4, is detected in wild-type cells transfected with the syndecan-4 construct (CHO-K1/syn4, arrowhead, lane 2). A band of lower intensity is also visible in that molecular mass range in the wild-type cells transfected with the vector (CHO-K1/pc, lane 1). However, no bands in this range are detectable in vector-transfected CHO-745/pc (lane 3) or in the syndecan-4 transfected CHO-745/syn4 cells (lane 4). A large amount of unglycanated syndecan-4 core protein can easily be detected in the CHO-745/syn4 cells (arrow, lane 4). The radiolabeled bands in the 46 to 70 kDa range have not been identified. SDS-PAGE standards in kDa are shown on the left side. (B) Incorporation of $^{35}SO_4$ into heparan sulfate chains of immunoprecipitated syndecan-4. $^{35}SO_4$-labeled cell lysates were immunoprecipitated with the chicken-specific syndecan-4 antibody (CS-4-E) and digested with heparitinase. Heparitinase-sensitive material could only be detected in chicken syndecan-4 transfected wild-type cells (CHO-K1/syn4) and not in the syndecan-4 transfected xylosyltransferase mutant cells (CHO-745/syn4). Untransfected wild-type (CHO-K1/pc) and mutant cells (CHO-745/pc) were used as negative controls. Values are counts per minute, with actual values above each column.

The assembly of F-actin fibers was evident in CHO-K1/pc and CHO-K1/syn4 cells at 30 minutes (Fig. 4B,D). The filamentous actin initially extended from the cortical actin to the center of the cell. These fibers increased in length and diameter after 1 hour and became typical stress fibers after 3 hours. Actin stress fiber formation was accompanied by extensive cell spreading (Fig. 4K,M). In CHO-745/pc cells, actin was localized exclusively at the periphery of the cell at all time points. In these cells the staining for actin intensifies over time, but no typical stress fibers spanning the cells could be detected at any of the time points. In contrast, transfection of the syndecan-4 cDNA into these cells resulted in their ability to assemble F-actin into stress fibers (Fig. 5D,H,M). The beginning of stress fiber formation in CHO-745/syn4 cells was delayed in comparison to wild-type CHO cells. In the mutants it was first observed after 1 hour whereas the CHO-K1/pc and CHO-K1/syn4 cells initiated actin fiber formation at 30 minutes. Nevertheless, fully developed stress fibers could be detected in CHO-745/syn4 cells after 3 hours. These were similar in size to the stress fibers seen in the wild-type CHO cells (Fig. 5M).

Vinculin-containing focal contacts at the end of actin stress fibers were detected in CHO-K1/pc and CHO-K1/syn4 cells 30 minutes after plating. The number and size of focal contacts in these cells increased over time as the actin stress fibers grew in length and size. However, in CHO-745/pc cells vinculin staining was localized mainly to long stretches at the cell border matching the actin staining. The number of vinculin-containing focal contacts in these mutant cells is dramatically reduced at all time points examined in comparison with wild-type cells. In contrast, vinculin-containing focal contacts in CHO-745/syn4 cells could be detected 1 hour after plating. These, however, were fewer in number and smaller in size compared to those of CHO-K1/pc and CHO-K1/syn4 cells. 3 hours after plating the vinculin staining in focal contacts in CHO-745/syn4 cells becomes more prominent and approximates the size and number of those of CHO-K1/pc and CHO-K1/syn4 cells.

Focal contact and stress fiber formation on vitronectin

Wild-type and mutant CHO cells seeded on vitronectin exhibit delayed cell spreading, stress fiber and focal contact formation compared to cells seeded on fibronectin. Cell spreading was apparent 1 hour after plating, but the area covered by the cells after attachment to vitronectin at 1 and 3 hours was less than that on fibronectin. In CHO-K1/pc and CHO-K1/syn4 cells, actin stress fiber formation and the localization of vinculin to fiber termini was evident 1 hour after plating on vitronectin and at increased levels after 3 hours (data not shown). In the glycanation mutant CHO-745/pc cells, actin was primarily localized to the cell periphery and stress fibers were either small or absent (Fig. 6B,F,K). In contrast, in the mutant cells that express the syndecan-4 core protein (CHO-745/syn4), stress fiber formation was evident 1 hour after plating on vitronectin and actin stress fibers resembling those seen in wild-type CHO-K1 cells were visible after 3 hours. Focal contacts, identified by vinculin staining, were prominent after 3 hours (Fig. 6G) and already visible after 1 hour (Fig. 6H). Therefore, expression of the core protein of syndecan-4 in the glycanation-defective CHO-745 cells restores their ability to

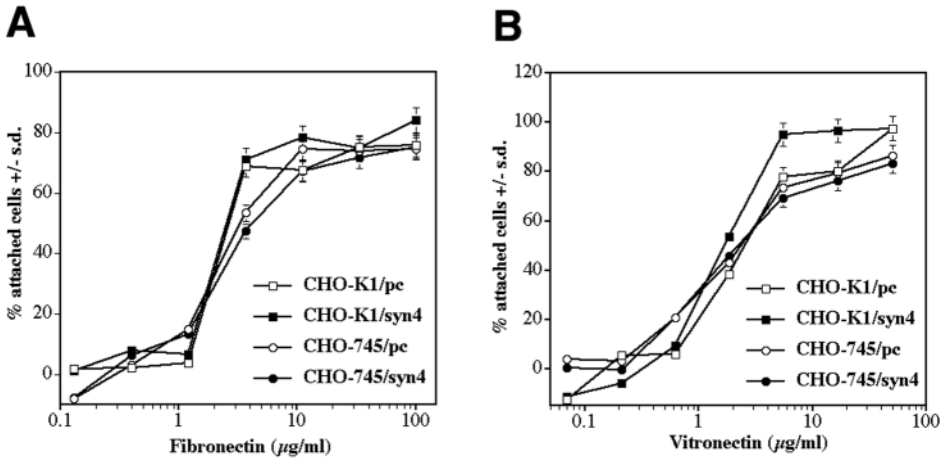

Fig. 3. Attachment of wild-type (CHO-K1) and mutant (CHO-745) cells transfected with vector (CHO-K1/pc and CHO-745/pc) or with syndecan-4 core protein (CHO-K1/syn4 and CHO-745/syn4) to serial dilutions of fibronectin (A) and vitronectin (B).

assemble actin stress fibers and focal adhesion on fibronectin as well as on vitronectin. However, the differences between the CHO-745/pc and CHO-745/syn4 cells are more dramatic on fibronectin.

Syndecan-4 staining of focal contacts

Recently we have shown that glycanated syndecan-4 is an essential component for the formation of focal adhesions (Saoncella et al., 1999). We wanted to determine if the ability of CHO-745/syn4 cells to form focal adhesions is correlated with the localization of syndecan-4 core protein to these structures. Syndecan-4 could not be visualized in focal adhesion with conventional immunofluorescence because the overexpression of syndecan-4 resulted in bright staining of the cytosol. However, syndecan-4 could be visualized in focal contacts when CHO-745/syn4 cells were plated for 3 hours on fibronectin-coated coverslips, washed with PBS and incubated for 20 minutes at 37°C with deionized water containing 1% PBS. Treatment with this highly hypotonic solution caused the disruption of the cells and only focal contact proteins remained attached to fibronectin. Focal contacts were identified by interference reflection microscopy and staining for vinculin. In CHO-745/pc cells syndecan-4 staining of focal contacts was absent although some focal contacts were positive for vinculin (Fig. 7B,C). The presence of vinculin staining demonstrates that the focal adhesion were not totally removed during the hypotonic treatment. In sharp contrast, all focal contacts in CHO-745/syn4 cells displayed intensive staining for both syndecan-4 and vinculin. Syndecan-4 and vinculin staining colocalized in most cases, although some focal contacts contained only syndecan-4 or vinculin (Fig. 7E,F). These results indicate that the expressed chicken syndecan-4 core protein is transported to the cell membrane and is incorporated in focal contacts in these cells.

DISCUSSION

The interaction of cells with the extracellular matrix involves both integrins and cell surface proteoglycans. The latter can either be chondroitin sulfate or heparan sulfate proteoglycans. Chondroitin sulfate proteoglycans have been shown to act cooperatively in α4β1 integrin-mediated cell adhesion to fibronectin (Iida et al., 1998; Moyano et al., 1999). The cooperativity in these cases can result from either an interaction of the chondroitin sulfate chains with the α4 integrin chain (Iida et al., 1998) or also with the heparin III domain of fibronectin (Moyano et al., 1999).

Interaction of cells with ECM proteins in cell culture occurs at focal adhesions, which are specialized macromolecular complexes that contain the transmembrane receptors syndecan-4 and integrins and a variety of intracellular structural and signaling molecules. These focal adhesions are points of attachment for filamentous actin to the inner cell surface. Assembly of focal adhesions and actin stress fibers in cells plated on fibronectin require interactions of the cell with both the cell-binding and heparin-binding domains of fibronectin (Woods et al., 1986). The interactions with the cell binding domain involve integrins. Focal adhesion and stress fiber assembly in the context of integrins requires receptor clustering, ligand occupancy, tyrosine phosphorylation and an intact cytoskeleton (Miyamoto et al., 1995a,b). Fibroblasts plated on a substrate of either the cell-binding domain of fibronectin or of an anti-β1 integrin antibody do not assemble focal adhesions and actin stress fibers. When such cells are supplemented with an antibody to the ectodomain of syndecan-4, full focal adhesion and actin stress fiber assembly occurs. Thus, syndecan-4, as the second transmembrane receptor of focal adhesions, acts cooperatively with integrins in generating the signals necessary for the formation of focal adhesions and actin stress fibers. This cooperativity between syndecan-4 and integrins occurs in a Rho-dependent manner (Saoncella et al., 1999).

In the present study we sought to extend our investigations on the role of syndecan-4 in adhesion and the assembly of focal adhesions and actin stress fibers by evaluating the respective roles of its core protein and GAG chains in cells plated on fibronectin and vitronectin. This study was possible as a result of the availability of CHO cell lines that are defective in enzymes that catalyze the addition of carbohydrate residues to proteoglycan core proteins. A cDNA that encodes the core protein of chicken syndecan-4 core protein was introduced in these cells. The expression of the syndecan-4 core protein in either the wild-type CHO-K1 or the xylosyltransferase mutant CHO-745 cells did not alter the adhesion of these cells to fibronectin or vitronectin. These results suggest that syndecan-4 is not critical for the adhesion of cells to ECM substrates and this adhesion is likely mediated primarily by integrins.

In contrast to the results of the adhesion experiments, the expression of syndecan-4 core protein had a dramatic effect on the assembly of focal adhesions and actin stress fibers in the mutant but not in the wild type cells. The wild-type CHO cells (CHO-K1) constitutively assembled focal adhesions and organized their actin cytoskeleton when plated on fibronectin

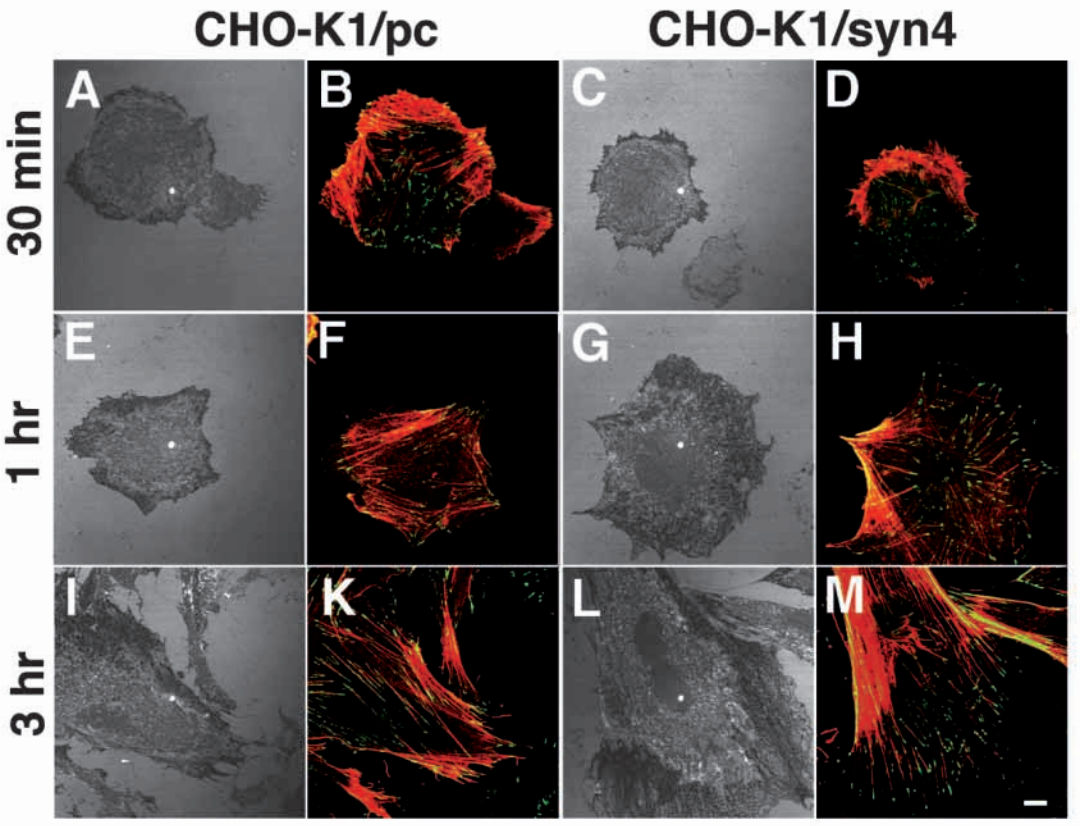

Fig. 4. Focal adhesion and actin stress fiber formation of vector (CHO-K1/pc) and syndecan-4 (CHO-K1/syn4) transfected wild-type cells seeded on fibronectin-coated coverslips. Cells were incubated for the indicated times, fixed and analyzed for focal contacts and actin stress fibers. Focal contacts and actin stress fibers were visualized by interference reflection microscopy (A,C,E,G,I,L) and double-labeling for actin and vinculin (B,D,F,H,K,M). Bar, 10 μm.

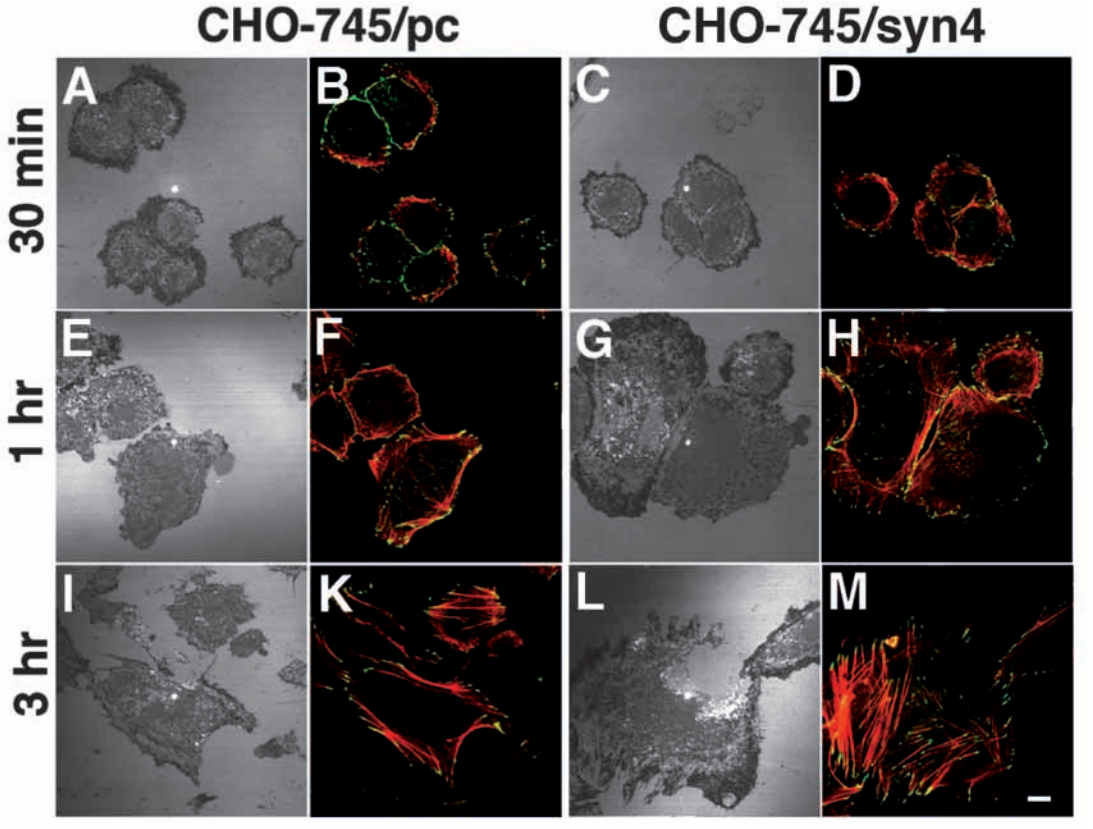

Fig. 5. Focal adhesion and actin stress fiber formation of vector (CHO-745/pc) and syndecan-4 (CHO-745/syn4) transfected glycanation defective cells seeded on fibronectin-coated coverslips. Focal contacts and actin stress fibers were visualized by interference reflection microscopy (A,C,E,G,I,L) and double-labeling for actin and vinculin (B,D,F,H,K,M). Fully developed actin stress fibers and focal contacts were detected in CHO-745/syn4 cells after 3 hours (M) whereas actin remained cortical and focal contacts are smaller and fewer in CHO-745/pc cells (K). Bar, 10 μm.

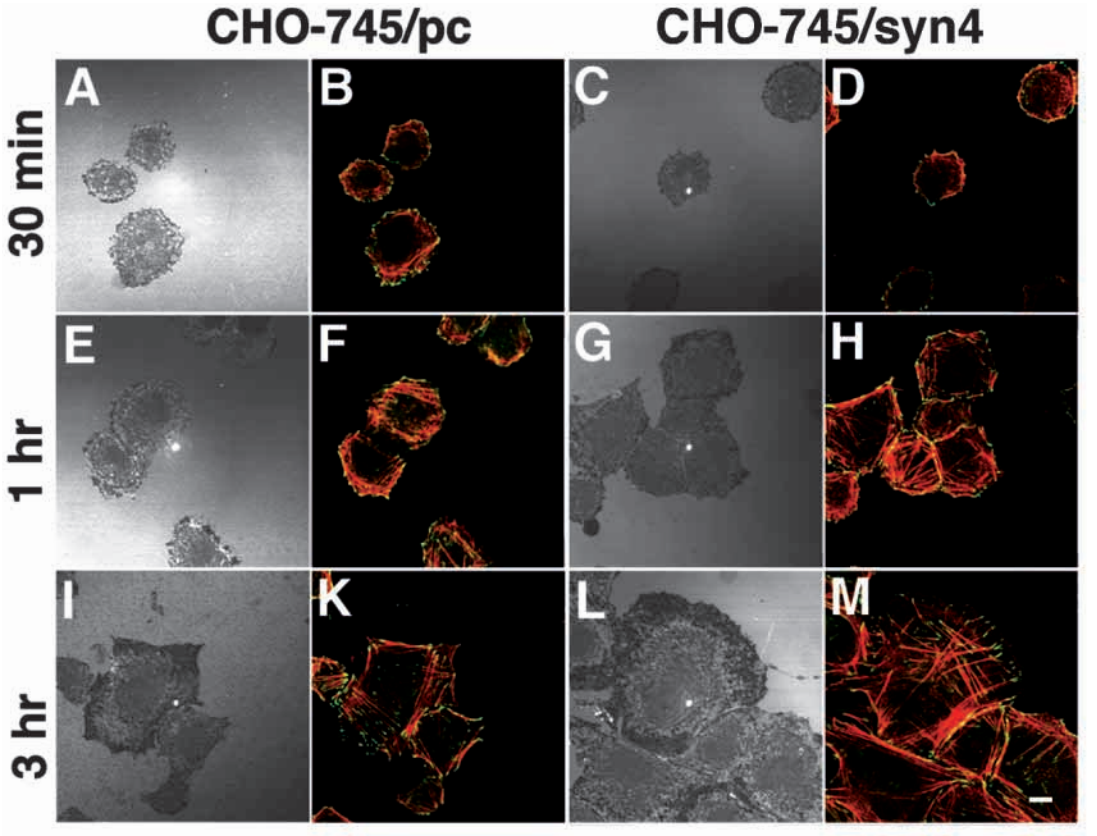

Fig. 6. Focal adhesion and actin stress fiber formation of vector (CHO-745/pc) and syndecan-4 (CHO-745/syn4) transfected glycanation-defective cells seeded on vitronectin-coated coverslips. Focal contacts and actin stress fibers were visualized by interference reflection microscopy (A,C,E,G,I,L) and double-labeling for actin and vinculin (B,D,F,H,K,M). Bar, 10 μm.

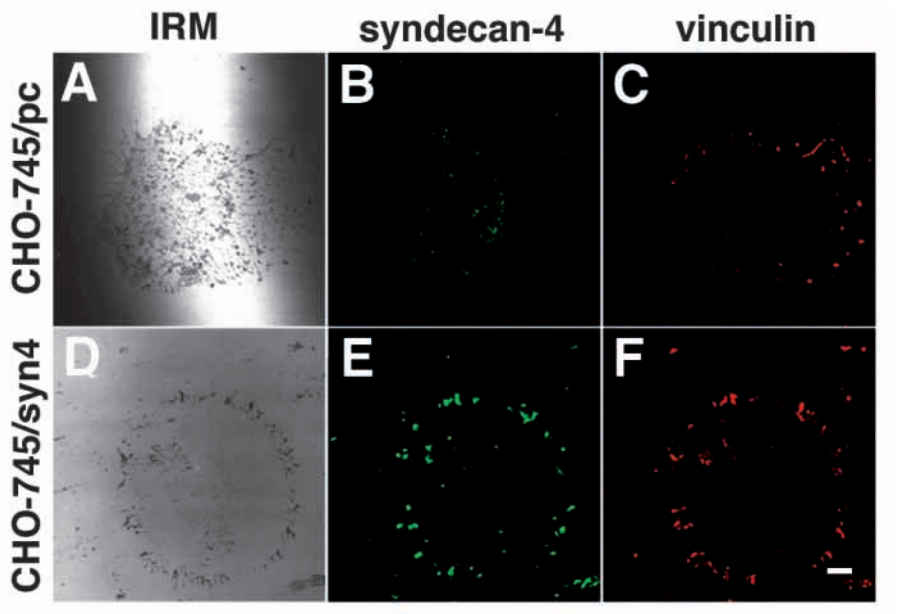

Fig. 7. CDNA-encoded syndecan-4 core protein localizes to focal contacts in glycanation-defective CHO-745. Mutant CHO cells transfected with empty vector (CHO-745/pc) (A-C) or with syndecan-4 (CHO-745/syn4) (D-E) were plated on fibronectin-coated coverslips for 3 hours and incubated for 20 minutes with deionized water containing 1% PBS. Focal contacts were visualized by interference reflection microscopy (A,D) and stained for syndecan-4 with CS-4-E antibodies (B,E) and vinculin (C,F). Bar, 10 μm.

or vitronectin. In contrast, when mutant CHO cells with defective xylosyltransferase (CHO-745) are plated on fibronectin or vitronectin, they attach but do not spread to the same extent as the wild-type cells. Similarly, CHO-745/pc cells did not assemble focal adhesions, but maintained cortical actin. Upon transfection of the xylosyltransferase mutant cells with the cDNA for chicken syndecan-4 core protein, the cells spread dramatically and they assemble extensive focal adhesions and actin stress fibers. No glycanation of the transfected syndecan-4 core proteins in the mutant cells could be detected. Similar results were obtained when syndecan-4 was overexpressed in CHO cells that were defective for galactosyltransferase-I (CHO-618) (data not shown). Thus, two CHO cell lines with enzymatically defective glycanation of proteoglycan core proteins do not assemble focal adhesions and actin stress fibers properly. Overexpression of syndecan-4 core protein in these glycanation defective cells restores the assembly of focal adhesions and actin stress fibers. The mechanism by which this occurs is not clear. The CHO-745 cells were derived from

the CHO-K1 and if the only difference between the two cell lines is a defective xylosyltransferase activity in the mutant, it is not clear why the mutant cells do not organize their focal adhesions to the same extent as the wild-type cells. Two explanations are possible. Either unglycanated core proteins are not present on the surface of the cells or the amount of unglyconated syndecan-4 core protein in the mutant CHO cells is not sufficient for the assembly of focal adhesions and actin stress fibers. Whatever the explanation, our results clearly indicate the presence of chicken syndecan-4 core protein in the focal adhesions of the CHO-745 cells transfected with the syndecan-4 construct. Therefore, the expression of unglycanated syndecan-4 core proteins in the mutant cells is sufficient for the assembly of focal adhesions and actin stress fibers and these core proteins become components of focal adhesions.

Longley et al. (1999) transfected a syndecan-4 cDNA construct into the CHO-K1 wild-type cells and reported increased focal adhesion formation in these cells. These authors also reported that cDNAs for syndecan-4 with partial and complete deletion of the cytoplasmic domain or cDNAs for syndecan-2 did not show the affect of the full-length syndecan-4 cDNA. Thus the effect seen in the present experiments with the xylosyltransferase mutant and those with CHO-K1 cells by Longley et al. (1999) are likely to result from signaling specifically through syndecan-4.

PKC is involved in the formation of focal adhesions and it regulates the recruitment of syndecan-4 into focal adhesions (Woods and Couchman, 1992; Baciu and Goetinck, 1995). Interactions of the variable region of the cytoplasmic domain of syndecan-4 have been shown to activate PKC-α (Oh et al., 1997b; Horowitz and Simons, 1998). Multimerization of the cytoplasmic domain of syndecan-4 is mediated by the cooperative activity of PKC-α and phosphotidylinositol 4,5-biphosphate (Oh et al., 1997a). These results would indicate that multimerization of syndecan-4 is essential for its function and it is possible that overexpression of unglycanated core proteins facilitates the multimerization needed for its function. A consequence of this speculation would be that the heparan sulfate side chains in wild-type cells may play a role in the multimerization process. This multimerization process may also be important for interaction of the cytoplasmic domain of syndecan-4 with cytoplasmic proteins such as syntenin (Grootjans et al., 1997) or CASK-LIN-2 (Cohen et al., 1998; Hsueh et al., 1998).

The results of the present study are consistent with those of previous studies that demonstrate that syndecan-4 signals in a cooperative manner with integrins in the assembly of focal adhesions and actin stress fibers (Saoncella et al., 1999). They extend these results in that they indicate that the unglycanated core protein of syndecan-4 is effective in that signaling.

The authors thank So Hyung Lee, Fabienne Denhez, Osamu Shimizu and Sarah Wilcox-Adelman for helpful discussions and Christine Herzog for critical reading of the manuscript and for support during this work. We thank Jeffrey Esko for providing us with the various CHO cell lines. This work was supported in part by grants HD-22016 and HD 37490 from the Child Health and Human Development Institute and from the Cutaneous Biology Research Center through the MGH/Shiseido Company Ltd. agreement to P.F.G. F.E. was supported by Research Fellowship EC 178/2 from the Deutsche Forschungsgemeinschaft.

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