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Insulin/FGF-binding Ciliary Membrane Glycoprotein from *Tetrahymena*

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Abstract. Triton X-100 extracted ciliary membrane protein from isolated cilia, prepared from the protozoon *Tetrahymena thermophila*, were fractionated by affinity chromatography on columns with covalently bound fibroblast growth factor (FGF), insulin, or concanavalin A (ConA), respectively. The eluted proteins were further analyzed by electrophoresis on sodium dodecyl sulfate polyacrylamide gels, isoelectric focusing, and by immunoblotting techniques using antibodies against the FGF receptor, platetelet derived growth factor (PDGF) receptor α-subunit, and insulin receptor β-subunit. The particular antibodies were chosen because the peptides PDGF, FGF, insulin, and ConA are chemoattractants in this organism and corresponding binding (receptor) proteins could be expected to be identified.

A 66 kDa protein fraction was eluted from the FGF-MiniLeak agarose, insulin-MiniLeak agarose and ConA sepharose. This fraction responded in Western immunoblots to an antibody against the β-subunit of the human insulin receptor, to an antibody against the PDGF receptor (PDGFR) and also to an antibody against the bovine FGF receptor (FGFR) that is known, in other systems, to inhibit FGF binding to its receptor. When analyzed by SDS-PAGE and stained with Coomassie blue the 66 kDa fraction appeared as a single component. However, in some experiments it appeared more heterogeneous when stained with silver indicating the presence of minor components that may be a procedural artifact or isoforms of the same glycoprotein. The 66 kDa protein(s) migrated in isoelectric focusing with a *pI* of 7.4.

The results are discussed in terms of the possible role of the 66 kDa glycoprotein as a protein involved in peptide-mediated cell signalling.

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Key words: FGF/insulin-binding protein — Ciliary membrane — *Tetrahymena*

Introduction

Rapidly swimming cells like the ciliated protozoa offer an interesting opportunity to study the chemosensory behavior involved in chemoattraction/repulsion in response to external chemical stimuli. The membrane ultrastructure of ciliates resembles that of the chemosensory neurons and the olfactory epithelium in mammals (Hufnagel, 1992; Menco, 1992). Ciliates may therefore be viewed as "swimming receptors" where correlations between cellular behavior and molecular signal transduction events can be studied experimentally because many ciliates are easy to grow and handle in the laboratory (Leick, Koppelhus and Rosenberg, 1994; Wheatley, Rasmussen and Tiedtke, 1994). Christopher and Sunderman (1995) found a protein in the ciliary membrane being immunologically similar to insulin. This 66 kDa protein was suggested to function both as an insulin precursor and membrane-bound binding site/receptor as has been suggested for TGF and EGF in mammals (Massagué, 1990). Dentler (1992) reported on a ciliary 66 kDa ConA-binding membrane protein in *Tetrahymena* that is exposed to the ciliary surface.

Previous studies of the chemosensory behaviour of *Tetrahymena* have shown that this ciliate is chemoattracted to a range of different peptides, proteins, and amino acids (Almagor, Ron and Bar-Tana, 1981; Leick, Grave and Hellung-Larsen, 1996). FGF, PDGF, insulin and concanavalin A (ConA) were found to act as growth promotors and/or chemoattractants in hormonal concentrations that are too low to act as food sources (Andersen, Flodgaard, Klenow and Leick, 1984; Leick, Bøg-Hansen, Christensen and Kaufman, 1996). Kuruvilla

and Hennessey (1998) found that lysozyme was a chemorepellent and identified its receptor in the plasma membrane as a 45 kDa protein. In order to gain insight in the cellular and molecular events leading to chemoattraction we have subjected isolated ciliary membranes to affinity column chromatography on agarose-coupled FGF, agarose-coupled insulin and ConA sepharose. The present report describes some biochemical and immunological properties of a 66 kDa protein fraction from the ciliary membrane that can be isolated to high purity using these affinity chromatography methods.

Materials and Methods

Materials

The insulin MiniLeak column (agarose-coupled insulin) was a gift from Dr. Jan Markussen NOVO Nordisk Copenhagen Denmark (Markussen, Halstrøm, Wiberg and Schäffer, 1991). Basic and acidic FGF was a generous gift from Dr. Stephen Kaufman The University of Illinois, Urbana, Illinois. Divinyl sulfone-activated agarose, MiniLeak Medium was from Kem-En-Tek Copenhagen Denmark. ConA sepharose was from Pharmacia (Uppsala, Sweden). IRβ rabbit antibody (immunogen was generated from the human insulin receptor β-subunit C-terminal amino acids 1203–1382 and was from Transduction Laboratories, Catalog #I16630). Mouse FGFR monoclonal antibody immunogen was FGFR purified from bovine coronary venular endothelial cells and was from Biogenesis Ltd. England Catalog #4460-6006. Mouse monoclonal antibody to PDGF receptor α-subunit was from Biogenesis Ltd. England. Jack bean Concanavalin A was from Sigma, St. Louis, Mo, USA.

Growth of Cells

Tetrahymena thermophila (strain BIII) was grown at 21°–28°C to early or mid-stationary phase in complex growth medium, PPYS: 0.75% proteose peptone (Difco) containing 0.75% yeast extract (Difco) and 1.5% glucose, (in mM) 1 $MgSO_4$, 0.05 $CaCl_2$, and 0.l ferric citrate, in thin-layer (1–2 cm) cultures without agitation in Fernbach flasks to a cell concentration of 1.5–2 × 10^5 cells per ml for about 40 hr (2 days).

Isolation of Cilia by the Dibucain Method and Preparation of Ciliary Membranes by Triton-X-100

Cells from 1.6 liter of medium were collected by centrifugation for 5 min at room temp in 250 ml centrifuge tubes at 500 × g. Cells were resuspended in 100 ml 2% proteose peptone and PMSF and dibucain were added to a final concentration of 1 mM, respectively, and in 1× axoneme buffer (diluted from 10× stock: (in mM) 300 HEPES, pH 7.6, 200 KCl, 50 Mg-sulfate, 5 EDTA). Complete deciliation occurred in 10–15 min as followed by phase contrast microscopy. The process was stopped by centrifugation before cell lysis occurred. After deciliation cells were transferred to 50 ml tubes and centrifuged at 600 × g for 5 min and the supernatant transferred to new tubes and respun at 600 × g for 5 min to ensure that no cell bodies were left. After transfer of supernatant to new centrifuge tubes an aliquot was checked microscopically for presence of cilia. The supernatant was centrifuged at 8,000 × g for 10 min to pellet cilia. The pellet was resupended in 10 ml of 1× axoneme buffer and overlayed onto 20 ml of sucrose (1 M sucrose, 2 mM EGTA in 1× axoneme buffer). The interphase was lightly mixed in order to create a smoother transition between the two phases and then centrifuged for 15 min at 12,000 × g. The pellet was resuspended in 1 ml axoneme buffer and transferred to a new tube. 10 ml of axoneme buffer was added and the tube was centrifuged at 8,000 × g for 10 min. The pellet was washed again with 1 ml of axoneme buffer, resuspended in 10 ml axoneme buffer and centrifuged for 10 min at 8,000 × g. The last step was then repeated once in order to obtain the final pellet of washed cilia. Demembranation of the cilia was carried out by resuspending pellet in demembranation buffer (0.5% Triton-X-100 in 1× axoneme buffer). Incubation was at 0°C for 30 min. After centrifugation at 8,000 × g for 10 min, the supernatant was then concentrated by centricon ultrafiltration and defined as the membrane fraction. The pellet was defined as the axoneme fraction.

Affinity Chromatography of Ciliary Membrane Proteins on Insulin MiniLeak, FGF MiniLeak and ConA Sepharose

Affinity chromatography was carried out at 0°C or at room temperature and all steps were in the presence of protease inhibitors (Complete, EDTA-free, Boehringer Mannheim; 1 tablet in 50 ml final solution).

Insulin MiniLeak material was prepared by coupling selectively the insulin through the B1 amino acid to divinyl sulfone-activated agarose (Markussen et al., 1988). The column material (1 ml) was transferred to a plastic column and washed with 3 × 5 ml of loading buffer. One ml of ciliary membrane extract (appr. 50 μg protein) was diluted with 5 ml loading buffer and applied on the column. After loading, the column was washed twice with 5 ml of the same buffer. Elution was with 10 ml Elution buffer at pH 5 (see below). Eluted fractions from the affinity chromatography columns were neutralized and were concentrated 5–10 times by ultrafiltration through centricon yM-10 (cutoff at 10 kDa) at 6000 rpm before further analysis.

FGF MiniLeak preparation and chromatography were carried out as follows. Three to 4 ml MiniLeak Medium was soaked dry on a filter and washed with 30 ml loading buffer. 0.8 ml of acidic FGF solution (5 mg/ml dissolved in 10 mM Tric-HCl, 1 mM EDTA and 1.8 M NaCl, pH 7.0) was added to 1 ml of washed MiniLeak material and incubated for 25 hr at 4°C using slow rotation. The column material (1 ml) was transferred to a plastic column and washed and incubated with 3 ml blocking buffer for 1 hr followed by washing with 3 ml citrate solution and 15 ml of loading buffer. Affinity chromatography was carried out as described above for the insulin MiniLeak column. Column regeneration was done at room temperature by washing the column with regeneration buffer followed by 3 ml loading buffer. Blank MiniLeak column material was prepared as described for the FGF columns. In this case ethanolamine (5 mg/ml) was used as crosslinking amino group providing blank MiniLeak column material.

Affinity chromatography on ConA sepharose was carried out as follows: 2 ml ciliary membrane extract in 1% Triton X-100 in 0.1 M HEPES buffer pH 7.2 in 20 ml Buffer A was incubated with 1.5 ml Con A sepharose for 60 min and centrifuged for 20,000 × g for 10 min. After pouring the suspension onto a column, elution was carried out with 5 ml 0.3 M alpha-methyl-mannose in buffer A. Eluted fractions were concentrated 5–10 times by ultrafiltration through centricon yM-10 at 6000 rpm before further analysis.

Buffers used in insulin and FGF affinity chromatography: *Washing buffer:* 0.1 M $NaHCO_3$, 1 mM EDTA, 1.8 M NaCl, pH 7 (FGF column only). *Blocking buffer:* 0.1 M ethanolamine pH 9 (FGF column only). *Loading buffer:* 0.1 M Tris/HCl, 0.01 M $MgCl_2$, 0.5 M NaCl, 0.1% Triton X-100. *Elution buffer:* 0.2 M Na_3-citrate, 0.4 M NaCl, 3 M Urea, pH 5 with HCl. *Regeneration buffer:* 0.2 M Na_3-citrate, 0.4 M NaCl, 8 M urea, pH 5 with HCl. *Citrate solution:* 0.01 M Na_3-citrate pH

2.5 (FGF column only). Buffer A: 100 mM HEPES, 1 mM $CaCl_2$, 1 mM $MnCl_2$.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were boiled in 20 μl SDS-sample buffer (0.06 M Tris-HCl (pH 6.8), 10% glycerol, 2% (w/v) SDS, 1% DTT, 0.002% bromophenol blue) for 5 min. They were then loaded and run on Pre-Cast 12% TRIS-Glycine Gels (EC6005, NOVEX) or NuPAGE 10% Bis-Tris Gels according to standard minigel procedures for the NOVEX XCell II Mini-Cell (E19001) system. Due to a better performance, NuPAGE gels only were used in experiments where electroblotting to membrane filters were carried out. SeeBlueTM Pre-Stained Standard markers (NOVEX) were used as molecular weight markers. Coomassie blue staining was by a standard procedure; silver staining was carried out with the SilverXpress Staining protocols (NOVEX Experimental Technology, CA, USA).

Immunoblotting

Resolved profiles and molecular weight markers were electrophoretically transferred from SDS-NuPAGE gel onto nitrocellulose sheets (Advantec MFS, CA, USA) according to the NOVEX Western transfer apparatus instructions for the NOVEX blot module (El9051). Electrotransfer to nitrocellulose sheets was for 2 hours in 25 mM bicine and 25 mM Bis-Tris buffer (pH 7.2) containing 1 mM EDTA and 10% methanol. Membranes were blocked by overnight incubation in 3% bovine serum albumin (BSA), 0.1% Tween 20 in PBS, pH 8.9 at 0°C, followed by incubation for 60 min at room temperature with the antibody in the recommended concentration. Antibody-binding was detected using HRP-conjugated secondary antibodies diluted 1:2500 as recommended by the manufacturer (Kem-En-Tec, Copenhagen, Denmark). HRP-catalyzed chemoluminescence was visualized by the ECL+/TM method (Amersham Pharmacia, UK, Western blotting protocol), and detected by exposure to autoradiography X-ray film.

Isoelectric Focusing (IEF)

Isoelectric focusing was done using Novex pI 3-10 IEF gels using the electrophoretic conditions and buffers recommended by the manufacturer (IEF argine/lysine cathode buffer and phosphoric acid anode solution) and using Serva pI 3-10 IEF protein markers. Before application, the assayed proteins were mixed with an equal volume of NOVEX sample buffer containing 1% Triton-X-100. After electrofocusing the IEF gels were silver stained.

Results

Ciliary Membrane Proteins from Fractions Solubilized by Triton X-100

More than 30 different polypeptides can be resolved by silver staining when using 1% solutions of the non-ionic detergent Triton X-100 to solubilize membranes from isolated cilia followed by analysis by NOVEX 12% SDS-PAGE under reducing conditions in Tris-glycine buffer (Fig. 1). A characteristic major family of heavily stained membrane protein bands was consistently observed to migrate in the 45–50 kDa region. This family of bands was also observed in whole cilia but was virtually absent in the axoneme fraction as seen in Fig. 1.

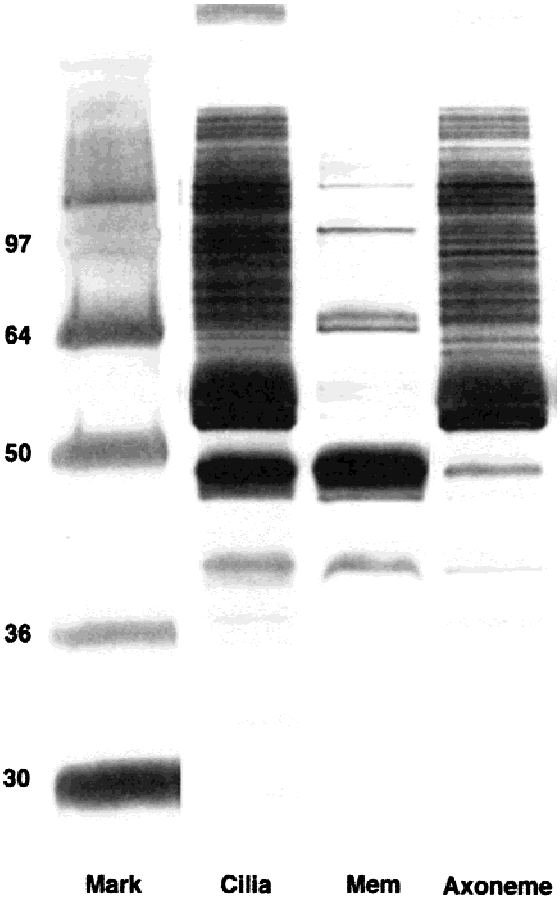

Fig. 1. SDS-PAGE of ciliary proteins electrophoresed under reducing conditions in Tris/glycine 12% gels and then stained by silver. Ciliary membrane and axoneme fractions were prepared by incubation of cilia in 1% Triton X-100 at 0°C. The appropriate molecular weights in kDa of the marker proteins (*Mark*) are indicated by numbers. Isolated cilia (*Cilia*). Ciliary membranes (*Mem*). Axonemes (*Axoneme*).

Fractionation of Ciliary Membrane Proteins by FGF, Insulin and ConA Affinity Chromatography; Recovery of 66 kDa Material

Triton X-100-soluble ciliary membrane extracts were subjected to affinity chromatography on insulin MiniLeak, FGF MiniLeak and ConA sepharose. After concentration of the eluted fractions by centricon ultrafiltration the protein fractions were subjected to SDS-PAGE and then to silver staining. It is seen in Fig. 2*A* that a 66 kDa protein is eluted from all three columns although a heterogeneity was observed in the material eluted from both the insulin and FGF column. A blank MiniLeak column preincubated and crosslinked with eth-

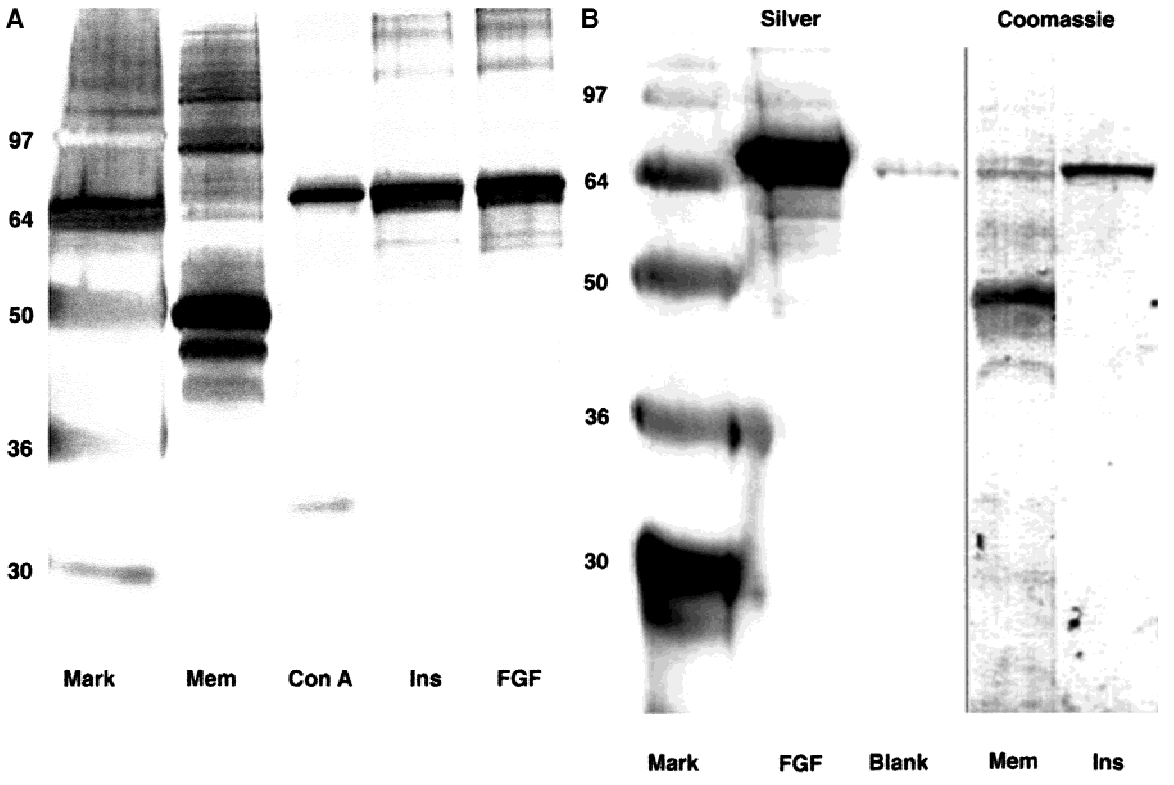

Fig. 2. (*A*) SDS-PAGE of affinity chromatography fractionated proteins electrophoresed on Tris/glycine 12% gels under reducing conditions and then stained by silver. Ciliary membrane fractions were prepared by incubation of cilia in 1% Triton X-100 at 0°C. Affinity chromatography purification from ciliary membrane fractions of insulin/FGF- and ConA-binding proteins was carried out as described in Materials and Methods. (*B*) This panel shows various controls. A blank MiniLeak column where the linker was coupled to ehanolamine was included as a control (*Blank*). A Coomassie blue stain of the material eluted from the insulin MiniLeak column only revealed one band at 66 kDa. The appropriate molecular weights in kDa of the marker proteins (*Mark*) are indicated by numbers. Ciliary membranes (*Mem*). Material purified by affinity chromatography on ConA sepharose (*ConA*), insulin MiniLeak column (*Ins*), FGF MiniLeak column (*FGF*).

anolamine was included as a control (Fig. 2*B*) in order to show that the appearance of the 66 kDa band is dependent on crosslinking the MiniLeak agarose material to either insulin or FGF.

When fractionated on insulin MiniLeak and FGF MiniLeak the eluted material sometimes appears as a heterogeneous band in SDS-PAGE under reducing conditions in silver stained Tris/glycine gels as seen in Fig. 2*A*. This heterogeneity is even more conspicuous when electrophoresis was applied on NuPAGE gels before electrotransfer to nitrocellulose filters (*data not shown*). However, in SDS-PAGE experiments stained with Coomassie blue only one protein is found in the 66 kDa position as seen in Fig. 2*B*. This indicates that the heterogeneity observed is due to a minor contamination that is only resolved when staining with the more sensitive silver method or when carrying out Western blot analysis (Fig. 3).

A 66 kDa fraction also eluted with 0.3 M alpha-methyl mannoside when using ConA sepharose affinity chromatography as seen in Fig. 2*A*. However, with this method, at least one additional component eluted in the 32 kDa position (Fig. 2*A*). Driscoll and Hufnagel (1999) found two major fractions eluting with mannose that they designated 28 and 50–55 kDa fractions, respectively. It is rather likely that these bands are identical to the material that elutes from our ConA sepharose columns.

When calculating the protein recovery, 66 kDa material represents a minute fraction of cellular protein (less than 10^{-5}) as calculated from protein recovery yields: In a typical experiment 5×10^8 cells (about 1 g of total cellular protein) gave rise to 2.5 mg of ciliary protein yielding about 2.4 mg of axonemal protein and 350 μg of ciliary membrane protein. Following affinity chromatography on the insulin MiniLeak column approx. 30 μg of 66 kDa material was recovered from the eluate.

Western/ECL Immunoblot Analysis of the Proteins Isolated by FGF, Insulin and ConA Affinity Chromatography

In order to characterize further the products eluted from the three different affinity chromatography columns

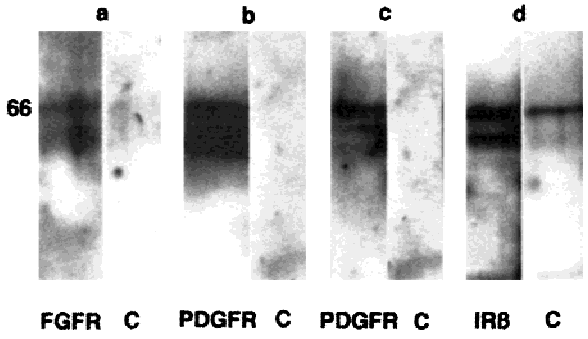

Fig. 3. ECL Western blot analysis of material using anti IRβ (*IR*β), antiFGFR (*FGFR*) and antiPDGFR (*PDGFR*) as primary antibodies was done on ciliary membrane extracted material eluting from the FGF MiniLeak (*a* and *b*) ConA sepharose (*c*) and insulin MiniLeak column (*d*). Electrophoretic separation was carried out under reducing conditions by SDS-PAGE on Novex NuPAGE gels in order to facilitate electrotransfer of the protein material to the nitrocellulose membrane. Electroblotting was onto nitrocellulose filter membrane paper before incubation with primary and/or secondary antibody. In the control lanes (*C*) the ECL was carried out only in the presence of secondary antibody i.e. in the absence of the primary antibody. Number indicates the position of 66 kDa material. The Table shows a summary of all Western blot experiments.

Western immunoblot analysis was carried out using three different immunological probes: An IRβ antibody to the β-subunit of the insulin receptor, an FGF receptor antibody that interferes with the FGF binding site, and an antibody against platelet-derived growth factor receptor raised against its α-subunit. These immunological receptor antibody probes were selected because FGF, PDGF, and insulin previously were found to be active in this organism as chemoattractants/growth signals (review by Leick et al., 1996). As seen in Fig. 3, the 66 kDa protein material responded to all three primary antibodies in the Western blot analysis. However, a heterogeneity was observed as at least one band migrating slightly faster than 66 kDa also responded to the antibodies used. In this experiment electrophoresis on NuPAGE gels was used before electrotransfer to the nitrocellulose membrane because these gels have a high efficiency of electrotransfer of proteins to the filter membrane. NuPAGE gels also resolve the microheterogeneity in the 66 kDa region (primary gel not shown) better than the Tris/glycine gels. However, the latter gels give much sharper bands as shown in Figs. 1 and 2.

To test whether or not the material purified by the three different affinity columns is the same protein material (Fig. 2), two aditional types of analysis were carried out. Firstly, the cross-reactivity was tested in Western immuno-assay and secondly, the homogeneity of the 66 kDa was tested by isoelectric focusing (IEF) on the proteins eluting from all three columns.

Using Western analysis the immunological cross-reactivity was tested as shown in the Table and Fig. 3.

Table. Cross reactivity of affinity chromatography purified material towards IRβ, FGFR and PDGFR antibodies, respectively, as determined by Western blot analysis.

Antibody	Affinity chromatography purification method		
	Con A sepharose	FGF MiniLeak	Insulin MiniLeak
IR-β-antibody	+(3)	+(3)	+(3)
PDGFR-antibody	+(2)	+(2)	+(2)
FGFR-antibody	+(1)	+(1)	+(3)

The experiments were performed as shown in the legends to Fig. 3 and in Materials and Methods. In parentheses, the number of individual experiments.

There is moderate cross-reactivity of the IRβ antibody to the material purified by FGF MiniLeak and ConA sepharose and there is moderate cross-reactivity of the FGFR antibody to material affinity-column-purified by ConA sepharose and FGF-MiniLeak chromatography, as well as cross-reactivity to the PDFGR antibody. This suggests that it is the same protein material that is purified by the three types of affinity chromatography.

The analysis of the protein material by isoelectric focusing (IEF) is shown in Fig. 4 and indicates that the 66 kDa material purified by affinity chromatography on insulin- as well as FGF MiniLeak is homogeneous and has a *pI* of 7.4. This supports the assumption that it is one major glycoprotein that is isolated by both types of affinity columns.

Discussion

In this work we have identified a ciliary membrane protein that elutes from MiniLeak affinity chromatography columns covalently linked with three different peptides. We describe some biochemical and immunological properties of this 66 kDa protein. One per cent Triton X-100 extracts from ciliary membrane protein fractions prepared from *Tetrahymena thermophila* by the dibucain method were fractionated by FGF, insulin or ConA affinity chromatography, respectively, and we showed that out of more than 20 ciliary membrane proteins identified by silver staining in SDS-PAGE, a major 66 kDa protein fraction eluted from all of these affinity columns. The 66 kDa protein in all three eluates responded in Western immunoblots to an antibody against the β-subunit of the human insulin receptor and antibodies against FGFR and PDGFR, respectively.

The immunochemical data are best explained as follows. The IR-β antibody recognizes a tyrosine kinase-like amino acid sequence close to the C-terminal of this 66 kDa protein as the Ir-β antibody was raised using part of the tyrosine kinase domain of the insulin receptor

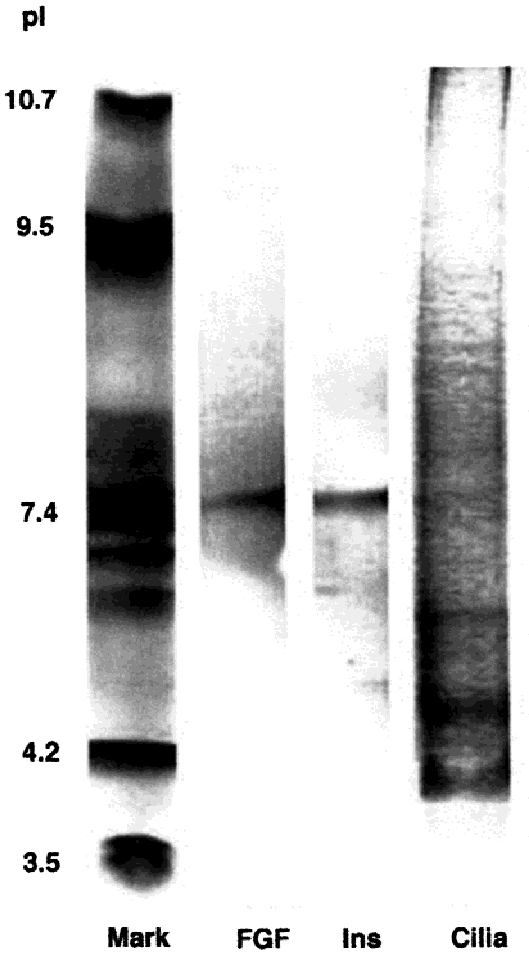

Fig. 4. Isoelectric focusing in a pH gradient between 3 and 10 of affinity chromatography-purified proteins followed by silver staining. Ciliary membrane fractions were prepared by incubation of cilia in 1% Triton X-100 at 0°C. Affinity chromatography purification of proteins from ciliary membrane fractions on insulin MiniLeak and FGF MiniLeak, respectively, was carried out as described in Materials and Methods. *pI*-values are indicated by numbers (*Mark*). Isolated cilia (*Cilia*). Material purified by affinity chromatography on insulin MiniLeak column (*Ins*), FGF MiniLeak column (*FGF*).

close to the C-terminus of the β-subunit. It has been observed by Christensen that the 66 kDa protein also binds PY20 antibody towards phosphotyrosine (*personal communication*). The FGFR antibody and PDGFR antibody probably bind to an N-terminal extracellular domain of the 66 kDa material that may contain immunoglobulin-like structure elements as has been shown for the PDGF and FGF receptors. Recently an insulin-binding protein that belongs to the immunoglobulin superfamily has been found in insects (Andersen, Hansen, Schäffer and Kristensen, 2000). This could explain the fact that binding of the secondary antibody to the 66 kDa material is observed in some control experiments in the Western blots where no primary antibody was included (Fig. 3, IRβ control experiment). A microheterogeneity was observed in the affinity purified material from all three columns that could be a procedural artifact due to proteinase activity but it could be also due to a variation of the sugar moiety of glycoproteins giving rise to different isoforms of the same glycoprotein. In particular, the Western blot analysis of Fig. 3 shows immunoresponding material with a molecular weight lower than 66 kDa.

The biological implications of these findings are presently not clear. Studies of mammalian peptide growth factors that stimulate chemotaxis, cell growth and cell survival in the ciliate protozoan *Tetrahymena* have shown that PDGF, FGF and insulin have significant biological effects on this organism, when used in hormonal (nano- to picomolar) concentrations. PDGF was shown to induce chemotaxis and nucleic acid synthesis (Andersen et al., 1984). Insulin has an effect on glucose uptake (Csaba and Lantos, 1975) and cell growth/survival as well as on chemotaxis (Leick et al., 1996). FGF and concanavalin A were shown to be chemoattractants (Leick et al., 1996). Further experiments are planned to show whether or not the 66 kDa material binds the above peptides under in vitro conditions and perhaps performs the initial step in a signal transduction cascade. A possible localization of the initiation of a signal transduction cascade to the ciliary membrane would be a new observation for ciliates as Kuruvilla and Hennesey (1998) previously showed that the repellent receptor protein responding to lysozyme is found in the plasma membrane in *Tetrahymena*. Our results obtained so far show the presence of a ciliary membrane 66 kDa receptor protein with a rather broad peptide-binding specificity. As this organism originated rather early in evolution and responds biologically to a variety of peptides (Leick et al., 1996), one might expect to find peptide receptor proteins with broader specificity than in higher cells.

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