Isolation and Characterization of a 60–70-kD Plasma Membrane Glycoprotein Involved in the Contact-dependent Inhibition of Growth

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Abstract. Previous studies have shown that plasma membrane compounds are involved in the contact-dependent inhibition of growth of human diploid fibroblasts. The purification of the active plasma membrane glycoprotein is described in this report. The glycoprotein has an apparent molecular mass of 60–70 kD and, due to differential sialylation, isoelectric points between pH 5.5. and 6.2. Treatment with sialidase yielded one spot in two-dimensional gel electrophoresis with an isoelectric point of 6.3. After removal of the N-glycosidically linked oligosaccharide chains, the apparent molecular mass is reduced by ~22 kD. Treatment was diluted NaOH, which removes the O-glycosidically linked portion of oligosaccharides, resulted in a reduction of the apparent molecular mass by ~5 kD. The addition of 50 ng/ml of this glycoprotein—for which the term "contactinhibin" is proposed—in immobilized form to sparsely seeded human fibroblasts resulted in a reversible 70–80% inhibition of growth. The inhibition was not confined to human fibroblasts as other cells were also inhibited, with the exclusion of transformed cells, which are refractory to contactinhibin. The inhibitory activity was abolished by treatment with β-galactosidase or glycopeptidase F, indicating that the glycan moiety is the biologically active part of the molecule.

Confluent cultures treated with antibodies raised against contactinhibin were released from the contact-dependent inhibition of growth. In addition to enhanced saturation density, these cultures exhibited a crisscross growth pattern and the formation of foci. Immunocytochemical studies showed that contactinhibin was associated with vimentin. Furthermore, contactinhibin was found to be not expressed in a species- or organ-specific manner.

NORMAL diploid mammalian cells are characterized by their density-dependent regulation of growth in vitro (Holley, 1975). Growth regulation is thought to be the result of the interplay between the diffusible microenvironment (e.g., growth factors and hormones) and the contact environment comprising extracellular matrix compounds and the cell membrane of neighboring cells. In transformed cells, the balance between the two compartments seems to be disturbed, thereby conferring a growth advantage to transformed cells over nontransformed cells. It has been suggested that enhanced synthesis of growth factors or growth factor receptors (Goustin et al., 1986; Heldin et al., 1987; Klein and Klein, 1986), and/or altered cell–cell or cell–matrix adhesion (Bolscher et al., 1988; Brackenbury et al., 1984; Burger, 1971; Vollmers et al., 1985) results in uncontrolled growth or unbalanced differentiation (Wille and Scott, 1986).

Confluent cultures of human diploid cells show a strongly reduced proliferation rate compared with sparsely seeded cells (Bard and Elsdale, 1986; Augenlicht and Baserga, 1974). In cultured cells the addition of isolated plasma membranes, or of membrane proteins, reduced the growth rate in a concentration-dependent fashion (Natraj and Datta, 1978; Kinders et al., 1980; Raben et al., 1981; Fritze et al., 1985; Heimark and Schwartz, 1985; Nilsson et al., 1983; Pereira-Smith et al., 1985; Stein and Atkins, 1986; Yaoi, 1984). Using a similar approach in hepatocytes, a correlation between the degree of inhibition of proliferation and the induction of differentiated functions has been found (Nakamura et al., 1983). From other studies it is well known that cell–cell contacts induce certain mature biochemical properties (Archeson and Rutishauser, 1988; Hatten, 1987; Saadat and Thoenen, 1986). In cultures of sparsely seeded human fibroblasts, growth is strongly inhibited by the addition of glutaraldehyde-fixed fibroblasts and isolated plasma membranes (Wieser et al., 1985) or immobilized plasma membrane proteins (Wieser and Oesch, 1986, 1987). In this and several other systems, the glycan moiety has been found to participate in the biological activity. In previous studies, we have shown that a crude detergent extract of isolated plasma membranes inhibited the growth of human diploid fibroblasts in a reversible, nontoxic manner (Wieser and Oesch, 1986). The inhibitory activity was strongly dependent on the immobilized state of the proteins. Here we describe the iso-

lation and characterization of the active compound, a 60–70-kD plasma membrane glycoprotein, for which the term "contactinhibin" is proposed. In addition, immunochemical data is presented as well as data on the effects on cultured cells of anti-contactinhibin antibodies.

Materials and Methods

Cells, Immunization, and Antibody Purification

The human diploid fibroblasts used in this study have been described elsewhere (Wieser et al., 1985). Rabbits (New Zealand White) were immunized by subcutaneous injection of contactinhibin (10 μg) adsorbed to nitrocellulose powder (Diano et al., 1987). After two boosts, at an interval of 2 wk, the rabbits were bled, and IgGs were isolated by protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography. Affinity-purified antibodies were obtained by incubation with nitrocellulose-bound contactinhibin and subsequent elution with glycine/HCl (0.1 M), pH 2.3, followed by dialysis against PBS at 4°C. Fab fragments were prepared by digestion of IgGs with immobilized papain (Pierce Chemical Co., Munich, FRG) according to the manufacturer's instructions, followed by affinity chromatography over protein A–Sepharose to separate Fab fragments from Fc fragments. IgGs and Fabs were stored at −30°C at a protein concentration of 1 mg/ml.

Isolation of Contactinhibin

Plasma membranes isolated according to Scott (1976) were concentrated by filtration over XM 300 filters (Amicon Corp., Witten, FRG) and precipitated by the addition of cold acteone. The precipitate was resuspended in SB buffer (PBS, 4 mM CHAPS, 1 mM PMSF). After 30 min at 4°C the sample was centrifuged (30,000 *g* for 30 min) and the proteins of the supernatant were precipitated by cold acetone. The pellet was solubilized in PB buffer (50 mM sodium phosphate, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% β-mercaptoethanol, 5% SDS, 1 mM PMSF) and heated for 10 min at 96°C. The solubilized sample was applied onto a gel permeation chromatography column (96 × 1.6 cm), packed with Fractogel HW 65 F (Merck, Darmstadt, FRG), which had been equilibrated with RB buffer (PB buffer containing 0.1% β-mercaptoethanol and 0.5% SDS). The sample was eluted at a flow rate of 25 ml/h. 2.3-ml fractions were collected and assayed for protein content and the presence of the growth inhibitory compounds. The active fractions were combined and the proteins were precipitated by the addition of cold acetone.

Immobilization of Solubilized Proteins and Growth Assays

Covalent binding of solubilized proteins to derivatized silica beads was done as described (Wieser and Oesch, 1987).

For measurement of DNA synthesis, 5×10^3 cells were seeded per well of a microtiter plate in DME/0.5% FCS. After 24 h membrane proteins bound to silica beads in DME/10% FCS were added. Controls received silica beads which had been reacted with DME or a control protein (asialofetuin or BSA). After 24 h, 0.25 μCi of [^{3}H]thymidine (New England Nuclear, Bad Homburg, FRG) was added to each well and the cells were cultured for an additional 4 h. The cultures were processed for measurement of incorporated radioactivity as described (Rinderknecht and Weiler, 1983). Net cell growth was measured by seeding 10^5 cells in 2 ml DME/10% FCS in 35-mm culture dishes and culturing them for 5 d in the presence of control beads, or beads bearing contactinhibin. In some dishes the beads were removed by repeated flushing with culture medium and all cultures were continued until day 8. Cells were counted after trypsinization using a hemocytometer.

For studies with anti-contactinhibin antibodies, the growth assays were performed as described above in the presence of anti-contactinhibin IgGs or Fabs (10 μg/ml) or, as controls, of IgGs or Fabs from rabbit preimmune sera. For determination of net cell growth, 10^5 cells were seeded in wells of a 24-well microtiter plate.

Glycosidase Treatments

Contactinhibin (1 μg) was incubated with neuraminidase (*Arthrobacter ureafaciens*, Boehringer Mannheim GmbH, Mannheim, FRG) in incubation buffer (0.25 M sodium acetate, pH 6.0, 1 mM PMSF, 2 mM CHAPS) for 16 h at 37°C, heated for 5 min at 96°C, after addition of SDS sample buffer, and analyzed by SDS-PAGE followed by silver staining or Western blotting.

For glycopeptidase F (Boehringer Mannheim GmbH) digestion, contactinhibin was dissolved in GF buffer (0.25 M sodium acetate, pH 7.6, 20 mM EDTA, 1 mM PMSF, 10 mg/ml CHAPS, 1 mg/ml SDS) by heating for 10 min at 96°C and then cooled to 37°C. Digestion was performed for 16 h at 37°C.

Beads bearing contactinhibin were incubated for 12 h in 0.5 ml PBS, 0.5 mM $CaCl_2$, 5 μl β-mercaptoethanol, and 5 U β-galactosidase (from bovine testis, Boehringer Mannheim GmbH) at 37°C.

For removal of O-glycosidically linked oligosaccharides, contactinhibin (1 μg) was incubated for 16 h at 37°C in 50 mM NaOH/0.1 M $NaBH_4$ in a toluene atmosphere.

Electrophoresis and Western Blotting

Samples solubilized in Laemmli buffer (Laemmli, 1970) were electrophoresed on 5–20% gradient SDS-PAGE gels and either visualized by silver staining or transferred to Immobilon membranes (Millipore, Eschborn, FRG) according to Towbin et al. (1979). After transfer, membranes were blocked with PBS/0.5% gelatine for 1 h. Anti-contactinhibin antibodies were added in PBS/0.5% gelatine/0.1% Triton X-100 at a 1:5,000 dilution and incubated for 90 min. After washing, peroxidase-conjugated anti-rabbit antibodies (DAKOPATTS, Copenhagen, Denmark) were added at a dilution of 1:1,000 and incubated for 90 min. After washing, antibody binding was visualized by incubation with 4-chloronaphthol.

Two-dimensional gel electrophoresis was performed as described by Rodemann and Bayreuther (1986).

Miscellaneous Procedures

Protein content was estimated according to Lowry et al. (1951) using BSA as a standard.

Concentration of dilute protein samples was performed by precipitation according to Wessel and Flügge (1984).

Results

The Active Compound-exerting Contact-dependent Inhibition of Growth Is a 60–70-kD Plasma Membrane Protein

The procedure developed for the isolation of the glycoprotein involved in the contact-dependent inhibition of growth of human diploid fibroblasts is outlined in Fig. 1. We started with plasma membranes which were enriched 20–30-fold over the homogenate with respect to the plasma membrane marker enzymes, alkaline phosphatase, and phosphodiesterase, respectively. The activities of enzymes of intracellular organelles (acid phosphatase as marker enzyme for lysosomes, iodonitrotetrazolium formazan-succinate dehydrogenase for mitochondria, and lactate dehydrogenase for cytosol) were reduced by ~90%, compared with the activities present in the homogenate (data not shown). Subsequent purification steps led to a strong increase in the specific growth inhibitory activity; altogether a 20-fold increase in total activity was obtained over that of the starting material (Table I). Although this increase was not studied further, it could have resulted from the loss of growth-stimulating compounds which have been shown to be present on plasma membranes (Lieberman, 1984). Separation of the last acetone precipitate by gel permeation chromatography yielded three major inhibitory fractions of 370–350, 80, and 50 kD, respectively (Fig. 1). It should be mentioned that calibration had been performed with globular marker proteins, which may behave differently

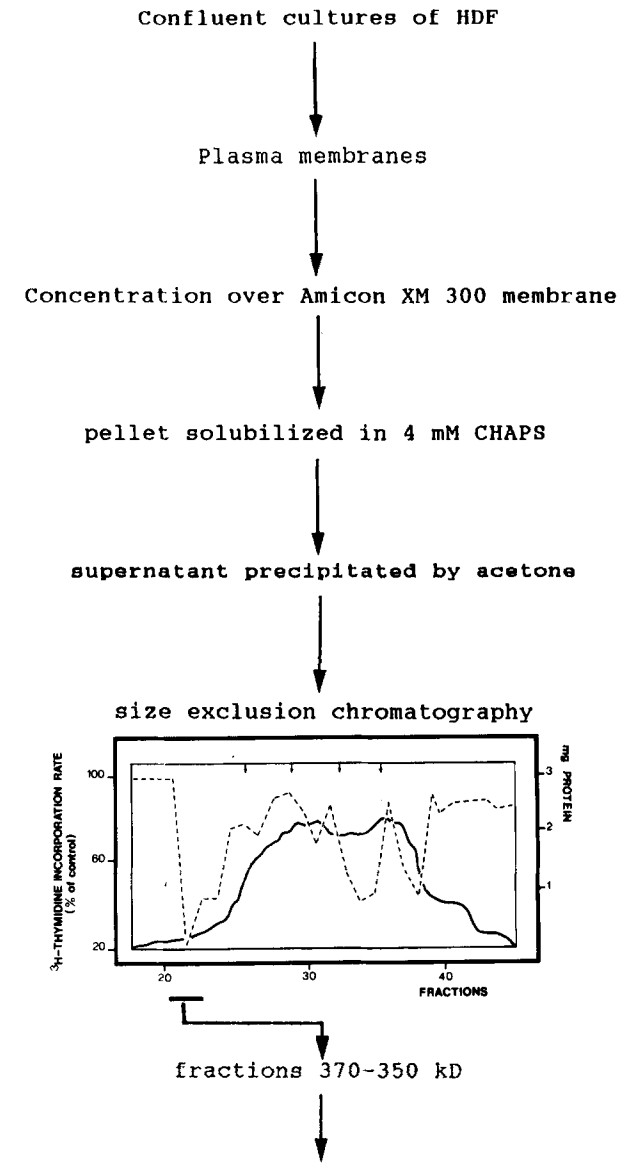

Figure 1. Purification scheme of contactinhibin. (*Insert*) Fractionation of solubilized plasma membrane glycoproteins by size exclusion chromatography. (*Broken line*) Influence on proliferation rate ([^{3}H]thymidine incorporation rate given as a percentage of controls) of the individual fractions in immobilized form. (*Solid line*) Protein content of the individual fractions. The arrows on top of the figure point to the elution position of the following marker proteins (from left to right): catalase (230 kD), aldolase (158 kD), β-galactosidase (reduced form, 116 kD), BSA (67 kD).

from glycosylated proteins. The three minor proteins as well as the 60–70-kD component, which have been observed after SDS-PAGE of the fractions 21–23, were isolated by preparative SDS-PAGE and tested for biological activity. Only the 60–70-kD form, contactinhibin, showed strong growth-inhibitory activity. Upon reelectrophoresis, contactinhibin migrated as a broad diffuse band, characteristic for microheterogeneous glycoproteins, and migration was reduced with increasing concentrations of β-mercaptoethanol (Fig. 2), which may be indicative of the presence of intramolecular disulfide bridges. When the proteins of the fractions 21–23

Table I. Purification of Contactinhibin

Preparation	Activity recovered	Protein recovered	sp act
	U	*mg*	*U/mg*
Plasma membranes	4,500	150	34
CHAPS-extract	90,000	30	3,300
Size exclusion chromatography	30,000	0.3	100,000
SDS-PAGE	10,000	0.02	500,000

1 U is defined as the amount of immobilized protein (mg) in 200 μl of culture medium which inhibited the proliferation rate by 50%.

were electrophoresed under nonreducing conditions, three additional proteins of 320, 240, and 180 kD were observed. Upon isolation of these forms followed by SDS-PAGE under reducing conditions, they were found to be oligomers of contactinhibin. Upon prolonged storage of contactinhibin, a 50-kD fragment has been found to appear. The inhibitory peaks observed after gel permeation chromatography have been shown to contain material cross-reacting with anti-contactinhibin antibodies in dot blots, suggesting that they contain aggregates and the 50-kD fragment of contactinhibin (data not shown).

Functional Characterization of Contactinhibin

The influence of immobilized contactinhibin on the proliferation of sparsely seeded fibroblasts was measured either after 28 h by the [^{3}H]thymidine incorporation assay, or by counting the cell number after a cultivation period of 8 d. In the presence of ~10 ng/ml of immobilized contactinhibin the thymidine incorporation of human fibroblasts was reduced by 50% compared with cultures grown in the presence of control beads. Control beads had no significant effect on the growth behavior of the cells. A 70–80% inhibition was achieved at a concentration of ~50 ng/ml of contactinhibin (Fig. 3). Variations in concentration were achieved by binding various amounts of contactinhibin to a constant number

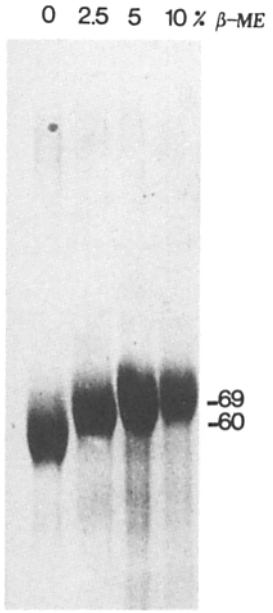

Figure 2. SDS-PAGE analysis of contactinhibin isolated by preparative SDS-PAGE followed by electroelution and treated with increasing concentrations of β-mercaptoethanol. The positions of reduced BSA (67 kD) and catalase (60 kD) are shown on the right.

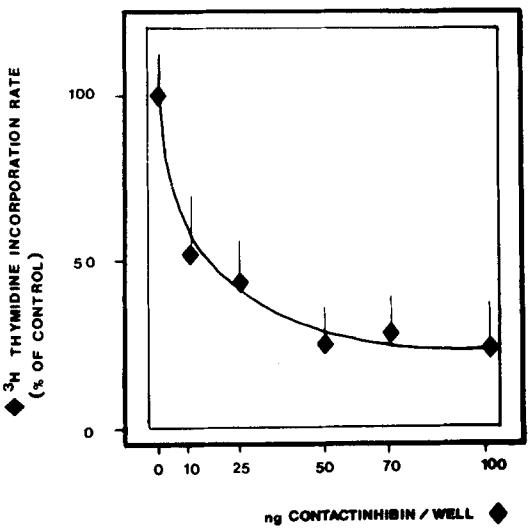

Figure 3. Effect of immobilized contactinhibin on the [^{3}H]thymidine incorporation rate of sparsely seeded fibroblasts. Various amounts of immobilized contactinhibin (5–100 ng/well) were added to sparsely seeded cells and proliferation rate was determined as described in Materials and Methods. Values are given as a percentage of controls ± SD (n = 3) from three independent experiments.

of beads. Culturing human fibroblasts in the presence of immobilized contactinhibin over a period of 8 d resulted in a reduction of the cell number by 70%, compared with control cultures (Fig. 4). Removal of the beads after 5 d resulted in a substantial increase in cell number. According to Table I, the purification led to a 16,000-fold enrichment of specific activity of the growth-inhibitory glycoprotein. It should be pointed out however that this value may be overestimated because of the likely presence of growth-stimulating compounds in the starting material, which would partially mask the real inhibitory activity.

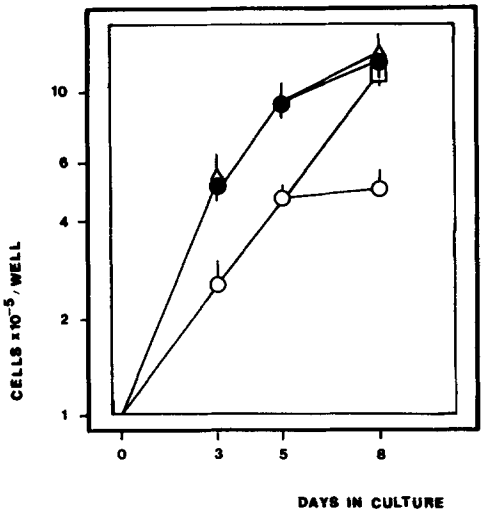

Figure 4. Effect of immobilized contactinhibin on net cell growth of sparsely seeded fibroblasts. Net cell growth was measured over an 8-d period as described in Materials and Methods. (◇) Cells grown without beads; (●) cells grown in the presence of control beads; (○) cells grown in the presence of immobilized contactinhibin; (□) cells grown for 5 d in the presence of immobilized contactinhibin, after which time beads were removed.

Table II. Effects of Various Treatments of Contactinhibin on Growth-inhibitory Activity

Preparation	[^{3}H]Thymidine incorporation rate
	% of control
Soluble contactinhibin (1 μg/ml)	69 ± 13 (9)*
Immobilized contactinhibin (0.1 μg/ml)	19 ± 9 (14)*
β-Galactosidase-treated immobilized contactinhibin (0.1 μg/ml)	87 ± 7 (4)*
Glycopeptidase F-treated immobilized contactinhibin (0.1 μg/ml)	78 ± 11 (3)*

The data are given as a percentage ± SD of controls.
* Number of independent experiments performed (n = 3).

The addition of contactinhibin in soluble form even at concentrations of up to 1 μg/ml had, surprisingly, only a low inhibitory activity (Table II). This is in agreement with our findings that the proteins of a crude membrane extract in soluble form had a 50-fold lower inhibitory activity than in immobilized form (Wieser and Oesch, 1986).

A special feature of contactinhibin, together with the previous preparations such as glutaraldehyde-fixed fibroblasts, isolated plasma membranes, and immobilized plasma membrane proteins (Wieser et al., 1985; Wieser and Oesch, 1986), was the observation that β-galactosidase treatment led to a complete inactivation of the growth-inhibitory activity (Table II). While other exoglycosidases (α-mannosidase, α-fucosidase, β-*N*-acetylglucosaminidase, and sialidase) were without effect, glycopeptidase F treatment also abolished the inhibitory activity of contactinhibin (Table II).

The biological activity of contactinhibin was not restricted to human fibroblasts, as other normal cells were inhibited to a similar extent (Table III). One the other hand, it has been shown that some transformed cells were refractory to the presence of contactinhibin and, with the exception of the transformed hamster fibroblasts V79, they have never been found to be inhibited by more than 50% compared with the controls.

Biochemical and Immunochemical Characterization of Contactinhibin

Since isolation of contactinhibin by the procedure described

Table III. Relative Inhibition of Various Cells by Immobilized Contactinhibin

Cell line	Relative inhibition
FH109	1.0
3T3	1.0
C3H/10T1/2	0.9
C3H/10T1/2 MC transformed	0.1
HT1080	0.2
HepG2	0.5
V79	0.6

The amount of contactinhibin used gave a 70–80% inhibition of proliferation rate of human diploid fibroblasts (FH109). Values are expressed as

$$\frac{\%\text{ inhibition of growth of tested cells}}{\%\text{ inhibition of growth of FH109}}.$$

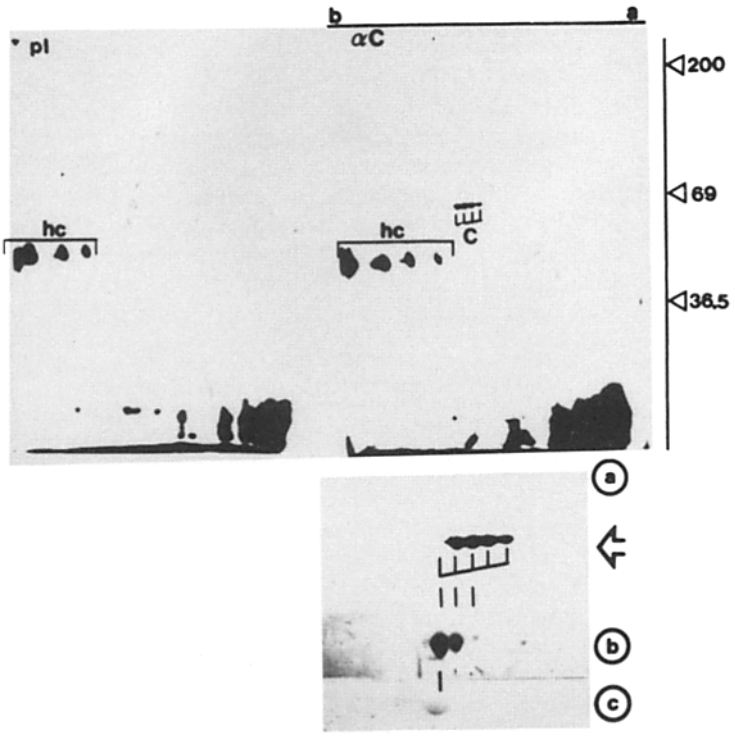

Figure 5. Molecular analysis of contactinhibin. (*a*) A detergent extract of human fibroblasts has been incubated with preimmune serum (left; *pI*) or with anti-contactinhibin antiserum (right; *αC*) followed by isolation of the immune complexes with protein A–Sepharose. The immune complexes were analyzed by two-dimensional gel electrophoresis followed by silver staining. For optimal comparison, both the pI and αC gels from the IEF step were applied together on one SDS slab gel. On the top of the αC gel, the basic and acidic ends of the IEF gel are indicated. On the right, the position of the molecular mass marker proteins myosin (200 kD), BSA (69 kD), and lactate dehydrogenase (36.5 kD) is indicated. *pI*, Immunoprecipitation using preimmune serum; *αC*, immunoprecipitation using anti-contactinhibin antiserum; *C*, contactinhibin; *hc*, heavy chains of immunoglobulins. (⇳) enlarged picture of contactinhibin. (*b* and *c*) Two-dimensional gel electrophoretic analysis of isolated contactinhibin after partial (*b*) and complete digest with sialidase (*c*). Isolated contactinhibin has been treated with sialidase as described in Materials and Methods and analyzed by two-dimensional gelelectrophoresis followed by silver staining.

is relatively expensive because of the minute amounts which can be obtained (~2–4 μg from ~10^9 cultured cells), some of the characterizations mentioned here were performed after immunoprecipitation or by Western blots using crude membrane extracts and anti-contactinhibin antibodies.

Immunoprecipitation followed by two dimensional gel electrophoresis and silver staining showed that contactinhibin consisted of five discrete spots with pIs in the pH 5.5–6.2 range (Fig. 5). An identical picture has been observed by separating isolated contactinhibin (not shown). After complete digestion of contactinhibin with sialidase from *A. ureafaciens*, the protein pattern shifted towards the most basic spot, indicating that contactinhibin consisted of an unsialylated form and several more forms that were increasingly higher sialylated (Fig. 5, *b* and *c*). In addition, this experiment clearly showed that the isolated contactinhibin was present in homogeneous form.

Treatment of contactinhibin with glycopeptidase F, which removes N-linked oligosaccharide side chains with broad specificity, resulted in a molecular mass shift of ~22 kD, although the diffuse migration behavior remained unchanged (Fig. 6, *GP-F*, incomplete digest; for a complete digest please see the Western blot in Fig. 8, lane *C*). On the other hand,

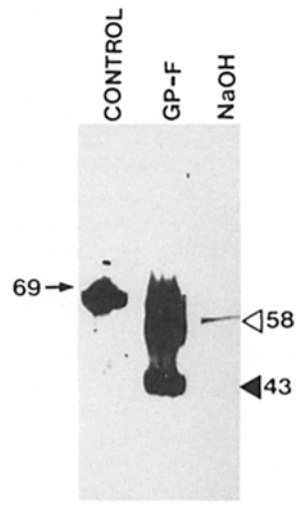

Figure 6. Glycosylation analysis of contactinhibin. Treatment of isolated contactinhibin with glycopeptidase F to remove N-linked (*GP-F*) and with dilute alkali solution to remove the O-linked glycan chains (*NaOH*), respectively. (*CONTROL*) Untreated contactinhibin; (*GP-F*) contactinhibin treated with glycopeptidase F; (*NaOH*) contactinhibin treated with dilute NaOH.

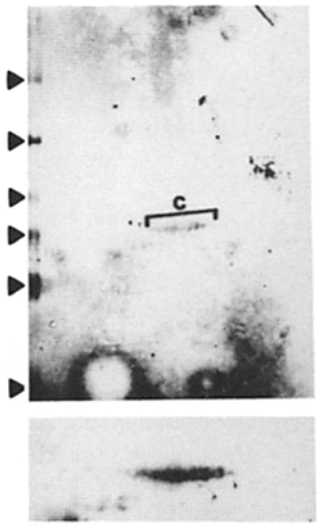

Figure 7. Two-dimensional gel electrophoresis of a detergent extract of human fibroblasts followed by Western blotting. The proteins of the detergent extract were separated by two-dimensional gel electrophoresis, blotted onto nitrocellulose, and reacted with anti-contactinhibin antibodies and peroxidase-conjugated anti-rabbit antibodies. The position of the prestained molecular mass marker proteins (Sigma Chemical Co., Munich, FRG) α-macroglobulin (180 kD), β-galactosidase (116 kD), fructose-6-phosphate kinase (84 kD), pyruvate kinase (58 kD), fumarase (48 kD), and lactic dehydrogenase (36.5 kD) is shown on the left side. At the bottom, an enlarged picture of the immunoreactive protein is shown.

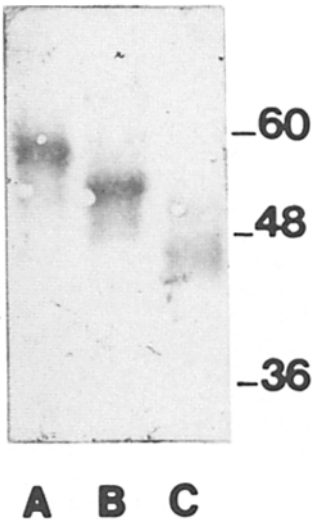

Figure 8. Western blot analysis of contactinhibin. (*A*) Plasma membrane proteins were electrophoresed under nonreducing conditions and, after blotting onto nitrocellulose, reacted sequentially with anti-contactinhibin antibodies (1:5,000) and peroxidase-conjugated anti-rabbit antibodies (1:1,000). (*B*) Western blot of sialidase-treated or (*C*) glycopeptidase F–treated contactinhibin.

contactinhibin ran as a focussed band after treatment with dilute alkali solutions, which leads to the removal of O-glycosidically linked oligosaccharide chains (Fig. 6, *NaOH*). This may be indicative of the presence of sialic acids exclusively on the O-linked glycans. We have previously found that plasma membrane proteins isolated from sparse cells had strongly reduced growth inhibitory potency, compared with the proteins isolated from confluent cultures (Wieser and Oesch, 1988*b*). The degree of inhibition could be fully restored after sialidase treatment. In addition, we have shown that the N-linked oligosaccharides are indispensible for an effective inhibition of growth (Wieser et al., 1985). In agreement with the data shown above, this would imply that the N-linked oligosaccharide chains are sialylated only in sparsely growing cells, while in confluent cultures sialic acids are restricted to the O-linked carbohydrate moieties.

In Western blots of plasma membrane proteins separated by two-dimensional gel electrophoresis, again only one protein consisting of several spots with the same apparent molecular masses and isoelectric points as contactinhibin itself was recognized by the anti-contactinhibin antibodies (Fig. 7). The antibodies also reacted specifically with glycopeptidase F– or sialidase-treated plasma membrane proteins separated by SDS-PAGE followed by Western blotting (Fig. 8).

Contactinhibin is most likely an integral plasma membrane glycoprotein, as shown by the following experiments: (*a*) It was solubilized only by detergents and was not released from isolated plasma membranes by high/low pH or salt (not shown). (*b*) It was not released by trypsin treatment in the presence of either Ca^{++} or EDTA (not shown). (*c*) After extraction with detergents, a portion of contactinhibin remained associated with the cytoskeleton (Fig. 9). In general, in confluently grown cells a larger portion was found in the detergent-insoluble fraction than in sparse cells, although the total amount is the same in both cell types.

Cytoskeletal association has been confirmed by double immunofluorescence studies. Cells were fixed with paraformaldehyde and incubated with anti-contactinhibin antibodies. After several wash steps the cells were permeabilized by Triton X-100 and incubated with mouse anti-vimentin antibodies. Binding of the two antibodies was visualized by incubation with rhodamine anti–rabbit and FITC anti–mouse antibodies, respectively. As shown in Fig. 10, contactinhibin shows a fibrillar distribution which exactly correlates with the distribution of vimentin. Previous immunocytochemical studies on the cytoskeletal components of the fibroblasts used in these studies excluded the association of contactinhibin with other components (not shown here). On the electronmicroscopical level, a distinct patchy binding of the anti-contactinhibin antibodies to the cell membrane has been found (Fig. 11). As shown in Table IV, contactinhibin has been found to be expressed in almost all of the cells and tissues studied until now.

Anti-contactinhibin Antibodies Release Confluent Cultures of Human Fibroblasts from Contact-dependent Inhibition of Growth

According to our working hypothesis that the growth of confluent cultures of human fibroblasts is mainly inhibited by the interaction of contactinhibin with postulated receptors on neighboring cells, the presence of anti-contactinhibin antibodies in confluent cultures should prevent this interaction. This was tested by adding affinity-purified antibodies or Fab fragments (10 μg/ml culture medium) to cells seeded with increasing densities from 1,000 to 80,000 per well and culturing them for 24 h with an additional 4 h in the presence of [^{3}H]thymidine. The proliferation rate was found to increase over controls grown in the presence of preimmune IgGs or Fabs with increasing cell densities, culminating at the highest cell density in a two- to threefold higher proliferation rate (Fig. 12). On the other hand, the growth of sparsely seeded cells was not affected by the presence of the antibodies, thus excluding an intrinsic activity of the antibodies in a manner similar to some anti–growth receptor factor antibodies. Highest stimulation of proliferation has been found with 10 μg/ml of anti-contactinhibin antibodies or fragments, with a slight stimulation found at 5 μg/ml. Higher concentrations of IgGs led to a slight inhibition of proliferation, which may be explained by reinforcement of cell–cell contacts. Increasing the concentration of Fab fragments was without effect. Culturing cells, which have been seeded at a density of 10^5 cells/cm^2 (confluency) for 4 d in the presence of anti-contactinhibin antibodies, resulted in a 1.8-fold increase in cell number over control cultures ($3.4 + 0.4 \times 10^5$ cells/cm^2 vs. $1.8 + 0.3 \times 10^5$ cells/cm^2). The exact cell number of the anti-contac-

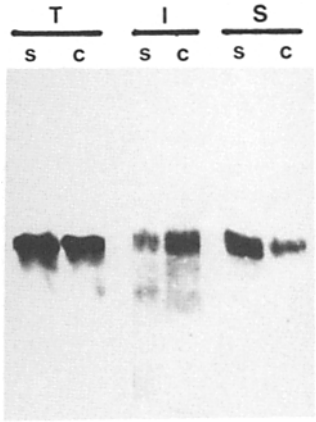

Figure 9. Cytoskeletal association of contactinhibin as shown by immunoblotting. Total proteins (*T*), detergent-soluble (*S*), or -insoluble (*I*) proteins of sparsely seeded cells (*s*), or confluent cultures (*c*) were separated by SDS-PAGE and after blotting onto nitrocellulose reacted sequentially with anti-contactinhibin antibodies and anti–rabbit antibodies.

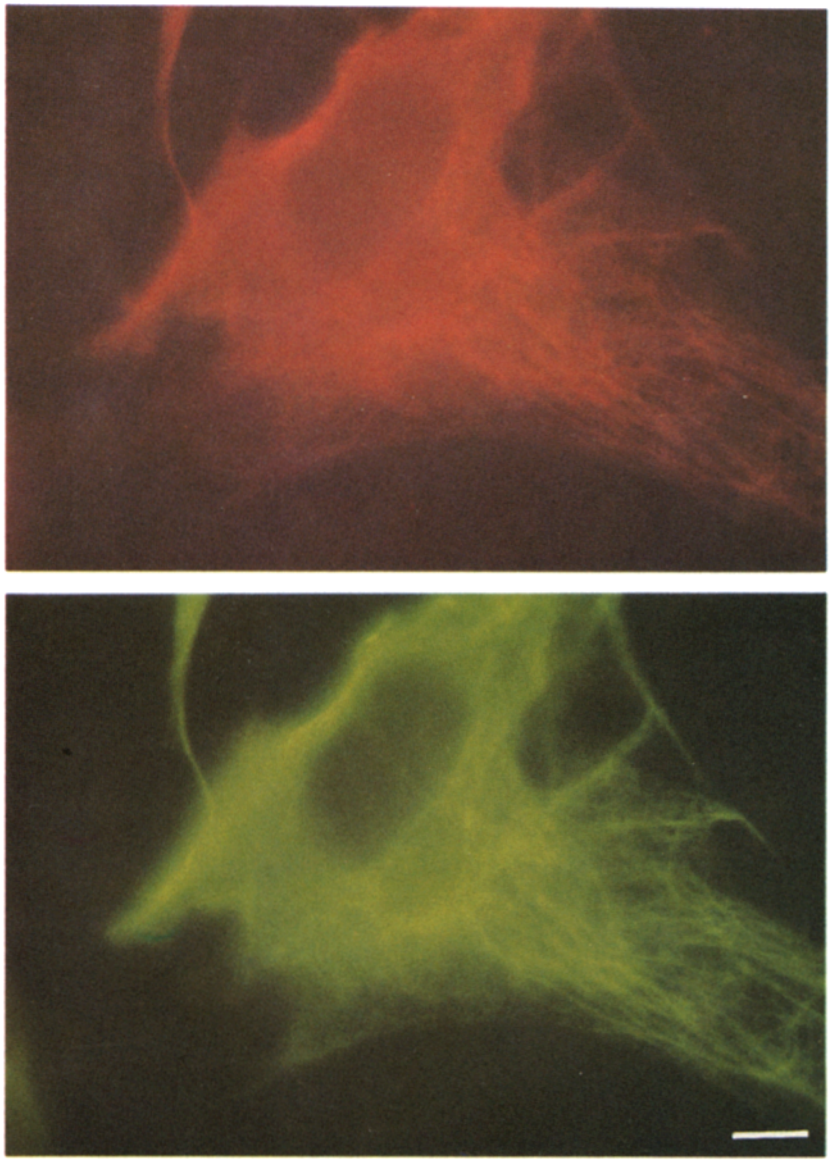

Figure 10. Demonstration of the cytoskeletal association of contactinhibin by double immunofluorescence. After fixation by paraformaldehyde, human fibroblasts have been incubated with rabbit anti-contactinhibin antibodies (*top*). After several wash steps, cells were permeabilized by Triton X-100 and incubated with mouse anti-vimentin antibodies (*bottom*). Binding of the antibodies was visualized by FITC-labeled anti-mouse antibodies and by rhodamine-labeled anti-rabbit antibodies, respectively. Bar, 2 μm.

tinhibin antibody-treated cultures may in reality be slightly higher because it was not possible to obtain single cell suspensions without aggregates, even when both trypsin and collagenase were used for dissociation. In addition to enhanced proliferation rate and saturation density, the anti-contactinhibin antibody-treated cultures showed an extensive crisscross growth pattern and the massive emergence of foci (Fig. 13).

Incubation of detergent extracts of plasma membranes with Sepharose-bound anti-contactinhibin antibodies showed that the growth-inhibitory activity was found exclusively in the bound fraction, while the nonbound fraction was almost inactive with respect to growth inhibition (Table V).

Discussion

Molecular Properties of Contactinhibin

We have described the isolation of a 60–70-kD plasma membrane glycoprotein, which in immobilized form inhibits the growth of human diploid fibroblasts in a reversible, nontoxic manner at picomolar concentrations. The purification led to a 16,000-fold enrichment of the specific activity of the isolated glycoprotein. In SDS-PAGE, it migrated as a broad band, characteristic for glycosylated proteins, of 50–60 kD in nonreduced form, while in fully reduced form it migrated slower with an apparent molecular mass of 60–70 kD. This may be indicative of the presence of intramolecular disulfide bridges. The smeared running behavior was still present after digestion of contactinhibin with glycopeptidase F, indicating that the microheterogeneity might reside on the O-glycosidically linked oligosaccharide side chains. This has been confirmed by removal of the O-linked glycans, which led to a reduction in apparent molecular mass by ~5 kD and a focusing of the protein band. In two-dimensional gel electrophoresis, contactinhibin has been shown to consist of five distinct forms which, after treatment with sialidase from *A. ureafaciens*, were converted to only one form. These results give evidence for the presence of sialic acids on O-linked

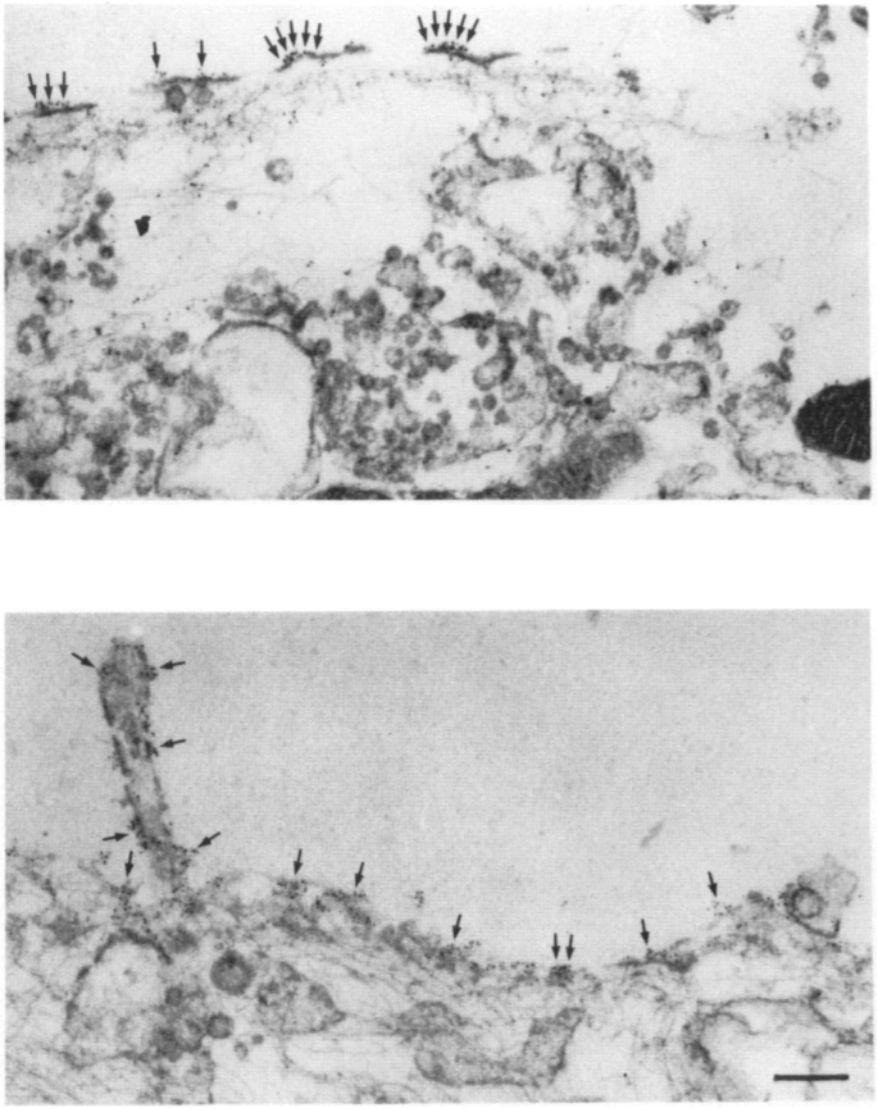

Figure 11. Immunoelectronmicroscopical localization of contactinhibin. Cells were fixed in situ with a mixture of periodate lysin paraformaldehyde and permeabilized by Triton X-100 treatment. After incubation with anti-contactinhibin antibodies cells were incubated with gold-labeled secondary antibodies, dehydrated, and embedded in Epon. The ultrathin sections were counterstained with uranylacetate and lead citrate. Note the intense decoration of the plasma membrane with gold particles (*arrows*). Bar, 60 nm.

glycans. We have previously found that plasma membrane proteins isolated from sparse cells had strongly reduced growth inhibitory potency, compared with the proteins isolated from confluent cultures (Wieser and Oesch, 1988*b*). The degree of inhibition could be fully restored after sialidase treatment. In addition, we have shown that the N-linked oligosaccharides are indispensible for an effective inhibition of growth (Wieser et al., 1985). In agreement with the data shown above, this would imply that the N-linked oligosaccharide chains are sialylated only in sparsely growing cells, while in confluent cultures sialic acids are restricted to the O-linked carbohydrate moieties. The different sialylation pattern could either reflect the actual growth state of the cells, or could be due to the action of plasma membrane-bound sialidases, which would remove sialic acids upon the establishment of cell-cell contacts. The higher degree of sialylation of contactinhibin in sparsely seeded proliferating cells is consistent with our findings (Renauer et al., 1987) and those of other groups on the higher content of plasma membrane glycoprotein-bound sialic acids on growing cells, compared with resting cells (Muramatsu et al., 1983; Sasak et al., 1983). It is not known whether the different sialylation pattern on contactinhibin has a functional importance or not, which is for example the case with N-CAM (Hoffman and Edelman, 1983). While the N-linked glycans have clearly been shown to be implicated in the biological activity, the O-linked glycans could be responsible for the determination of the level of cell surface expression (Reddy et al., 1989).

Are There Similar Compounds Identified or Is Contactinhibin a Unique Functional Plasma Membrane Glycoprotein?

Although cell-cell contacts have clearly been shown to be involved in the homeostasis of a cell population in vitro, only rudimentary data is available on the molecular properties of the compounds involved. The molecules which have been described as being involved in the regulation of growth of normal, and in some cases transformed cells, have molecular masses which differ greatly from that of contactinhibin (for review see Miyazaki and Horio, 1989). These molecules include TGF-β (25 kD; Massague, 1987), the FRGs (13 kD; Hsu and Wang, 1986) and the IDF_{45} (45 kD; Blat et al.,

Table IV. Expression of Contactinhibin by Various Tissues and Cells

	Expression of contactinhibir
Human Tissue	
Skin	+++
Sarcoma	−
Muscle	+++
Kidney	+
Renal cell carcinoma	+
Adrenal gland	−
Intestine	+++
Colon carcinoma	++
Pancreas	+++
Liver	++
Lung	+++
Spleen	++
Lymphoma	+++
Mammary carcinoma	+++
Cells	
Wi 38 SV40 transformed	+++
HT1080	+++
3T3	+++
C3H/10T1/2	+++
C3H/10T1/2 MC transformed	+++
Rat liver fibroblasts	+++
Rat liver epithelial cells	+++
Mouse splenocytes	+++
Human osteocytes	+++
Human erythrocytes	−
Others	
Human serum	+++
Serum from tumor patients	+++

The analyses have been performed by Western blotting.
−, No reaction.
+, Weak reaction.
++, Good reaction.
+++, Strong reaction.

1989), growth inhibitors from bovine milk (13 kD; Herrmann and Grosse, 1986), and mammary-derived growth inhibitors (13 kD, Böhmer et al., 1987) from V79 cells (2 kD; Koga et al., 1986), from mouse cerebral cortex (18 kD; Kinders et al., 1980), from melanocytes (160 kD; Lipkin et al., 1978), and from rat liver (26 kD; McMahon et al., 1982). In addition to different molecular masses, these compounds are all active as solutes. This is also true for heparin and heparan sulfate, which have been shown to inhibit the proliferation of smooth (Fritze et al., 1985; Castellot et al., 1986) and skeletal muscle cells (Kardami et al., 1988) of human fibroblasts (Matuoka and Mitsui, 1981) and of endothelial cells (Imamura and Mitsui, 1987). Recently, mammary cell-specific growth-inhibitory proteins with molecular masses of 47 and 65 kD have been described (Ervin et al., 1989). In contrast, contactinhibin has been shown to be also active on cells other than human fibroblasts.

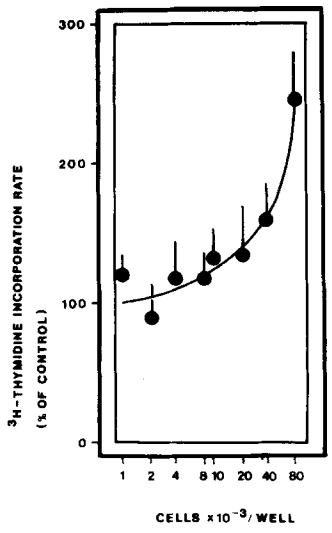

Figure 12. Release of confluent cultures of human diploid fibroblasts from contact-dependent inhibition of growth by anti-contactinhibin antibodies. Increasing numbers of human fibroblasts were seeded in wells of a microtiter plate and cultured for 28 h in the presence or absence of anti-contactinhibin antibodies (10 μg/ml). Proliferation rate was determined by the measurement of incorporation of [^{3}H]thymidine into DNA. Values are given as a percentage ± SD (n = 5) of controls which were cultured in the presence of antibodies from preimmune serum.

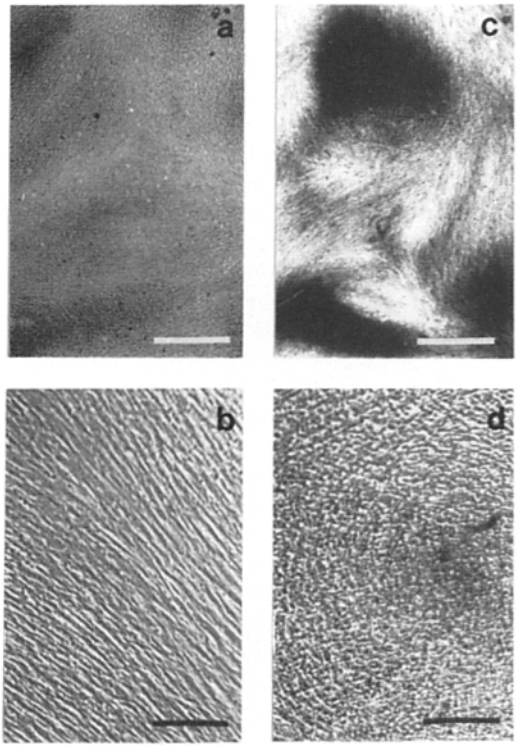

Figure 13. Morphological appearance of cultures grown in the absence (*a* and *b*) or presence (*c* and *d*) of anti-contactinhibin antibodies. (*a* and *c*) Overview of cultures stained with methylene blue. (*b* and *d*) Phase-contrast microphotographs of unstained cultures. Bars: (*a* and *c*) 100 μm; (*b* and *d*) 36 μm.

Table V. Removal of Growth-inhibiting Compounds from Crude Plasma Membrane Extract (100 μg Protein) by Affinity Chromatography on Immobilized Anti-contactinhibin Antibodies

Human fibroblasts cultured in the presence of immobilized proteins	Proliferation rate
	% of controls
Untreated detergent extract	34 ± 5
Fraction not bound to Sepharose-bound anti-contactinhibin antibodies	93 ± 8
Fraction bound to Sepharose-bound anti-contactinhibin antibodies	43 ± 8

With respect to human fibroblasts, one report has been published on some molecular properties of plasma membrane proteins inhibiting the growth by contact-dependent mechanisms (Stein and Atkins, 1986). In agreement with our findings, the inhibitory activity has been found to be sensitive to periodate treatment, suggesting the involvement of glycans in the biological activity. On the other hand, the inhibitory activity has been described to be abolished after heat or trypsin treatment, which agrees with findings in the 3T3 cell system (Raben et al., 1981), but is contrary to our data. The differences in these findings could be ascribed to the different test systems. In growth-arrested and senescent human fibroblasts, a 57-kD protein has been described (Wang, 1985) that is confined to nuclei. Furthermore, the appearance of a 240-kD glycoprotein has been described that accumulates in contact inhibited and aging fibroblasts (Poot et al., 1986), but no functional studies have been done with these compounds. In 1987, a meeting abstract on a few purification steps of detergent extracts of human diploid fibroblasts with growth-inhibiting activity appeared in *In Vitro*, again without further molecular data (Smith, J. R., A. L. Spiering, and O. M. Pereira-Smith, unpublished observations).

In other systems, such as in mouse fibroblasts (Whittenberger and Glaser, 1977; Natraj and Datta, 1978; Yaoi, 1984; Raben et al., 1981), in endothelial cells (Heimark and Schwartz, 1985), and in hepatocytes (Nakamura et al., 1983, 1984) growth regulation via cell–cell contacts has been studied so far only with isolated plasma membranes, with crude membrane extracts or with partially enriched preparations.

Recently, a new family of cell adhesion molecules has been described, i.e., the "addressins" (Stoolman, 1989). It has been suggested that these endothelial cell surface glycoproteins represent the ligands for several lectinlike proteins on recirculating lymphocytes, enabling the adhesion to, and extravasation of, the endothelium of high endothelium venules. One addressin has been characterized as being a glycoprotein of 58–66 kD with a characteristic shift in molecular weight upon reduction (Streeter et al., 1988). In contrast to contactinhibin, it has been shown by immunohistochemistry that the tissue-specific expression of this glycoprotein is highly restricted. In addition, it has been shown that contactinhibin has no function as a cell adhesion molecule (Janik-Schmitt, 1989).

A further family that has been shown to be involved in the regulation of cell behavior is the cell surface proteoglycan family (for review see Ruoslahti, 1989). Again, no component has been described with molecular properties similar to contactinhibin, although human diploid fibroblasts have been studied extensively with respect to proteoglycans.

How Could Contactinhibin Exert its Inhibitory Activity?

From the various experiments performed to elucidate the biologically active part of contactinhibin, it is postulated that terminal, β-glycosidically-linked galactose residues on N-linked glycans are the crucial moiety. This has been deduced from the following observations: (*a*) Treatment of glutaraldehyde-fixed fibroblasts, of isolated plasma membranes (Wieser et al., 1985), of a crude membrane extract (Wieser and Oesch, 1986), and of contactinhibin itself (shown here) with β-galactosidase resulted in an almost complete loss of growth inhibitory activity. (*b*) Plasma membranes isolated from cells grown in the presence of tunicamycin were without effect on the growth of sparsely seeded fibroblasts (Wieser et al., 1985). (*c*) Removal of the N-glycosidically linked oligosaccharide chains by glycopeptidase F treatment abolished the growth-inhibitory activity of contactinhibin (shown here). (*d*) Plasma membranes isolated from pronase-treated cells lost their inhibitory activity, while the released compounds, even after exhaustive digestion, had still growth-inhibitory activity (Wieser and Oesch, 1986). This is in agreement with data obtained by Yaoi (1984). (*e*) Growing cells in the presence of β-galactosidase or α-lactalbumin, which acts as a modulator of the activity of galactosyltransferases (Morrison and Ebner, 1971), resulted in an enhanced proliferation rate and saturation density (Wieser and Oesch, 1988*a*). In this context, it might be interesting to mention that fibroblasts from patients with certain lysosomal storage diseases, leading for example to impaired sialylation, are deficient with respect to density-dependent regulation of growth (Oohira et al., 1987).

The mechanism of interaction of contactinhibin with neighboring cells could be of a homophilic or a heterophilic nature. Homophilic interaction has been implicated in N-CAM-mediated cell adhesion (Hoffman and Edelman, 1983), and in the form of carbohydrate–carbohydrate interactions between various cells of the nervous system (Keilhauer et al., 1985; Cole et al., 1986), as well as during embryogenesis and organogenesis (Eggens et al., 1989). Initial experiments with an aggregation assay, where contactinhibin was bound to fluorescently labeled acrolein beads, clearly showed that there was no homophilic binding between contactinhibin, while there was strong binding to immobilized proteins of a plasma membrane extract (data not shown). It could be speculated therefore that the interaction of contactinhibin with neighboring cells might occur by a receptor-mediated process. This could occur in a lectin/carbohydrate-like fashion (Zalik and Milos, 1986), as shown in the adhesion between lymphocytes and endothelial cells, or in the binding of sperms to the fertile egg (Bleil and Wassarman, 1988; Peterson and Hunt, 1989). A further possibility could be the interaction of contactinhibin with a plasma membrane–localized galactosyltransferase (Bayna et al., 1988). Another crucial mechanism regulating the function of contactinhibin could be the connection with the cytoskeleton, most likely with vimentin. Although we have no further proof, the relative inability of soluble contactinhibin to effectively inhibit in vitro growth suggests that contactinhibin not connected to vimentin might be without biological activity. Cytoskeletal connection has been shown for many cell–substrate and cell–cell adhesion proteins (Tarone et al., 1984; Lacy and Underhill, 1987; Horwitz et al., 1986; Nelson et al., 1990). In addition, concentration at contact sites of these compounds has been described together with an increased insolubility to detergents, which indicates cytoskeletal binding. This is in agreement with the findings that contactinhibin is concentrated at cell–cell contact sites and that in confluent cells, compared to sparse cells, a larger portion of contactinhibin is detergent resistant.

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