Glycoconjugate Journal (1995) **12**: 672–679

Cell-contact mediated modulation of the sialylation of contactinhibin

RAIMUND J. WIESER*, CLAUDIA E. BAUMANN and FRANZ OESCH

Institute of Toxicology, Obere Zahlbacherstraße 67, 55131 Mainz, Germany

Received 20 January 1995, revised 28 March 1995

Contactinhibin was found to be involved in contact-dependent inhibition of growth. The growth inhibitory activity of contactinhibin is mediated by N-linked oligosaccharides with desialylated β-glycosidically linked, terminal galactose residues. Here we show that in sparse human fibroblasts contactinhibin was expressed in a biologically inactive, highly sialylated form both on the plasma membrane and intracellularily, while in confluent cells plasma membrane localized contactinhibin was present in a biologically active, low sialylated form. Plasma membranes were shown to contain a glycoprotein sialidase which is suggested to be engaged in the activation of contactinhibin in a cell contact-dependent manner.

Keywords: sialylation, contact-inhibition

Introduction

Growth of non-transformed mammalian cells *in vitro* is regulated by density-dependent mechanisms, leading eventually to cessation of proliferation of cells at confluency. Density-dependent inhibition of growth can be elicited by the addition of isolated plasma membranes or plasma membrane extracts to sparsely seeded cells [1–5]. Previous studies have shown that the addition of glutardialdehyde-fixed fibroblasts to sparsely seeded fibroblasts also resulted in a marked contact-mediated inhibition of growth [6]. Most interestingly, growth inhibitory activity of the fixed cells varied with cell density of the cultures from which they had been isolated and correlated inversely with the proliferative potential of the source cells. These results have been confirmed by the addition of immobilized plasma membrane proteins [7] instead of fixed cells to sparsely seeded cells. In this case, full growth inhibitory activity of plasma membrane proteins from sparse cells could be restored by pretreatment with sialidases [8].

We have previously described the isolation of the plasma membrane glycoprotein, referred to as contactinhibin, which is responsible for the above mentioned contact-dependent inhibition of growth of human diploid fibroblasts [9]. For full growth inhibitory activity of contactinhibin the presence of terminal, β-glycosidically linked galactose-residues on N-linked glycans has been found to be indispensible. Together, these results point to a switch from inactive, sialylated contactinhibin in sparse cells to active contactinhibin with unmasked galactose residues in confluent cells.

Here we provide evidence that in human diploid fibroblasts contactinhibin is synthesized in a highly sialylated form and that the conversion to the active, less sialylated forms occurs in a cell density-dependent fashion, most likely by the action of a sialidase identified in isolated cell membranes.

Materials and methods

Cell culture

FH109, human diploid lung fibroblasts, and HaCat, human keratinocytes, were cultured as described [6] except that CG-medium (Vitromex, Vilshofen, FRG) with 0.5% FCS (Roth, Karlsruhe, FRG) was used instead of DMEM/10% FCS.

Isolation of intracellular and plasma membrane localized contactinhibin

In order to separate plasma membrane localized from intracellular contactinhibin most efficiently, the following methods have been employed: mild oxidation of the surface sialic acid moieties of collected cells with metaperiodate, derivatization of generated aldehydes with biotin hydrazide and solubilization of cells followed by

*To whom correspondence should be addressed.

immunoprecipitation of total contactinhibin. The immunoprecipitate was incubated with solid phase avidin to separate biotinylated (i.e. cell membrane localized) from non-biotinylated (i.e. intracellular) contactinhibin. In detail, 5×10^6 cells from sparse (1.5×10^4 cells per cm^2) or confluent (5×10^4 cells per cm^2) cultures were washed three times with phosphate-buffered saline (PBS), trypsinized (3 min, 0.025% trypsin, 0.02% EDTA in PBS) and collected by centrifugation at $110 \times \mathbf{g}$ for 10 min. Cell surface sialic acid moieties were oxidized for 30 min at 4 °C in the dark with 1 mM $NaIO_4$ in a sodium acetate buffer (1 ml; 0.1 M, pH 5.5). After washing cells three times with 3 ml PBS the generated sialic acid aldehydes were reacted with 1 ml biotin-hydrazide (30 $\mu g\,ml^{-1}$ sodium acetate buffer, 0.1 M, pH 5.5) for 30 min at room temperature or radiolabelled by incubation with 1 ml [^{3}H]$NaBH_4$ (1 $mCi\,ml^{-1}$ PBS, NEN, Dreieich, FRG) for 15 min at 4 °C. After several wash steps in PBS cells were solubilized in 1 ml Tris-NaCl (TN)-buffer (10 mM Tris/HCl, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.5% Triton X-100, 1% CTAB (cetyltrimethylammonium bromide) at pH 8.0) for 30 min at 4 °C and cleared by centrifugation for 10 min at $13\,000 \times \mathbf{g}$. The supernatant was incubated for 1 h at room temperature with 40 μg *anti*-contactinhibin antibodies (see below) covalently bound to 125 μl magnetic beads (Dynabeads, Dynal, FRG) according to [11]. The immunoprecipitate containing both intracellular and plasma membrane localized contactinhibin was washed sequentially with 0.5 ml Tris-NaCl-NP-40 (TNN)-buffer (50 mM Tris/HCl, 0.15 M NaCl, 1% NP-40, pH 7.6), TNN-buffer with 0.1% sodium dodecylsulfate (SDS), TNN-buffer, TNN-buffer containing 0.5 M LiCl, and after changing the cap, with 0.1 M Tris/HCl, pH 7.6 and 10 mM Tris/HCl pH 7.6 followed by a wash step with distilled water. Bound proteins were released by incubation in 100 μl 0.1 M diethylamine pH 11.5 for 20 min. After neutralization of released proteins with 1 N HCl, biotinylated compounds were isolated by incubation with 100 μl streptavidin-beads (Dynal). After 30 min the supernatant containing intracellular contactinhibin was collected and precipitated with chloroform/methanol [12]. Biotinylated (i.e. plasma membrane localized) contactinhibin bound to solid phase streptavidin was released by boiling in an SDS-sample buffer [13]. After precipitation with chloroform/methanol, proteins were redissolved in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) sample buffer and equal amounts separated by 2D-PAGE as described [14]. Proteins were visualized by silver staining.

Flow cytometry

Surface localized and intracellular contactinhibin content was determined by using a FACSStar Plus flow cytometer according to [15]. Briefly, 1×10^6 trypsinized cells were incubated with *anti*-contactinhibin antibodies followed by FITC-conjugated *anti*-rabbit IgG antibodies. After several wash steps cells were fixed by the addition of ice-cold 70% ethanol. After 30 min on ice cells were collected by centrifugation and resuspended in PBS. For staining of total contactinhibin, cells were fixed with ethanol prior to incubation with antibodies.

Determination of newly synthesized contactinhibin-forms

Semiconfluent human fibroblasts were metabolically labelled for 170 h with [^{3}H]-leucine and extracted in 4 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate)/PBS for 30 min at 4 °C. Contactinhibin was purified as described [16] with an additional purification step employing preparative 2D-PAGE. Electroeluted contactinhibin was re-electrophoresed on 2D-PAGE followed by Coomassie blue staining. The single contactinhibin-spots were excised and the gel pieces dissolved according to [17]. The radioactivity of each gel slice was measured by liquid scintillation counting.

Anti-contactinhibin antibodies

Contactinhibin purified by 2D-PAGE was adsorbed onto powdered nitrocellulose. Briefly, nitrocellulose was cut into small pieces and pulverized through a metal mesh. The resulting particles were resuspended in PBS and sonicated until a fine powder was obtained. Purified contactinhibin (12 μg in 200 μl elution buffer) was added to 100 μl of settled powder and incubated overnight at room temperature. Nitrocellulose-bound contactinhibin was washed three times with PBS and the final volume adjusted to 300 μl. New Zealand White rabbits were immunized subcutaneously on the back with 2 μg of contactinhibin per injection site (six sites per animal). After six boosts at 2 week intervals and two final boosts at a 3 day interval rabbits were bled and the antiserum tested for titre and specificity. The titre was determined by ELISA using plasma membranes as antigen source (3 μg per microtitre well). Usually, the titre ranged between 15 000 and 25 000. Specificity was evaluated by Western blotting using a crude cell extract obtained by incubating confluently grown fibroblasts with 4 mM CHAPS in PBS for 30 min at 4 °C.

Lectin blots

Lectin blots were performed using digoxigenin-labelled lectins and alkaline phosphatase conjugated anti-digoxigenin antibodies (Boehringer Mannheim) according to the manufacturer's procedures.

Isolation of cell membranes

Cultured cells were washed three times with PBS and released by trypsin-treatment. After addition of soybean trypsin inhibitor, cells were washed two times with PBS. Cell number was adjusted to 5×10^5 cells per ml of PBS

(diluted 1:5 with distilled water), followed by Dounce homogenization. The suspension was centrifuged for 20 min at 20000 × **g** followed by centrifugation of the resulting supernatant at 100000 × **g** for 90 min at 4 °C. The pellet was solubilized in TX-buffer (50 mM Tris/HCl, 100 mM NaCl, protease inhibitor cocktail (Boehringer Mannheim), 0.1% Triton X-100, pH 5.5) for 30 min at 4 °C and used immediately for determination of sialidase activity.

Measurement of sialidase activity

Ten μg of solubilized membrane proteins in 500 μl TX-buffer were incubated for 4 h at 37 °C after the addition of freshly prepared 4-methylumbelliferyl-α-D-*N*-acetyl-neuraminic acid (in sodium acetate buffer, 0.25 M, pH 5.5) at a final concentration of 5 μM. The release of 4-methylumbelliferone was determined by measurement of the fluorescence intensity at 450 nm (excitation wavelength 365 nm) after mixing of 900 μl glycine (0.25 M, pH 10.4) with 100 μl of the sample. Measurements were correlated with a 4-methylumbelliferone standard.

Determination of sialic acid release from contactinhibin

Contactinhibin was slot blotted onto PVFD-membranes (Millipore) followed by incubation with blocking solution (20 mM Tris/HCl, 500 mM NaCl, pH 7.4, 1% polyvinylpyrrolidone-40 [18]) overnight at room temperature. After washing the slots with 50 mM Tris/HCl, 500 mM NaCl, pH 7.4, 0.05% Tween-20, Triton X-100 solubilized plasma membranes or Arthrobacter sialidase (50 μU) as a positive control were added and incubated for a further 4 h. After thoroughly washing the membranes with 50 mM Tris/HCl, 500 mM NaCl, pH 7.4, 0.05% Tween-20, sialic acids were visualized by incubation with digoxigenin-conjugated lectin from Sambucus nigra (SNA) as described above.

Protein content

Protein content was determined according to [19] using bovine serum albumin as a standard.

Results

Experimental design

Experiments were first undertaken to separate intracellular from surface contactinhibin of sparse and confluent human fibroblasts. One major problem arose with the observation that approximately 90% of total contactinhibin was stored in the form of an intracellular pool, as measured by flow cytometry (Fig. 1). No significant changes between sparse and confluent cells with respect to the total content of contactinhibin and the amount expressed on the cell surface were observed in these studies (data not shown). The following procedure has

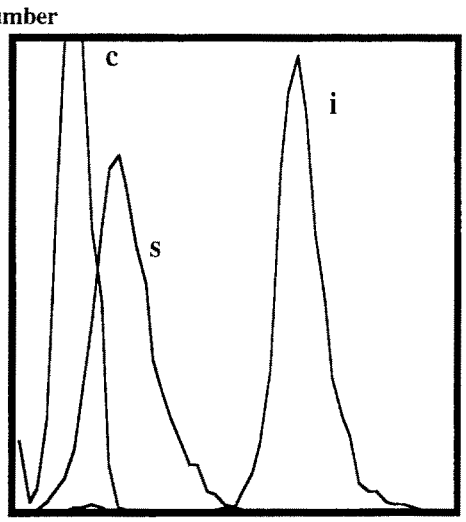

Figure 1. Determination of contactinhibin content by flow cytometry. Cells were incubated with *anti*-contactinhibin antibodies, followed by incubation with FITC-conjugated *anti*-rabbit IgG antibodies, before (s, staining of membrane localized contactinhibin) or after (i, staining of total contactinhibin) permeabilization. c, control staining with preimmune serum. Abscissa: fluorescence intensity (log scale).

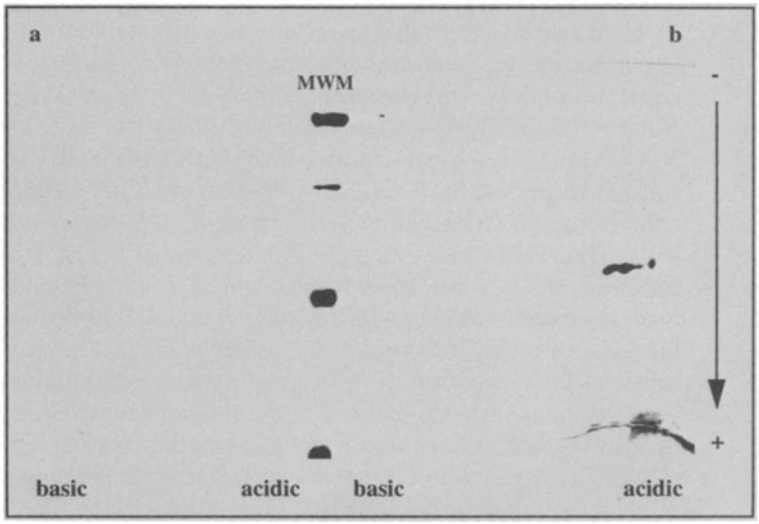

Figure 2. Two-dimensional gel electrophoresis of plasma membrane localized contactinhibin. Sialic acids of confluent cells were oxidized with sodium periodate and the generated aldehydes reacted with biotin hydrazide either directly (b) or after a preincubation step with sodium borohydride (a). Thereafter, contactinhibin was isolated according to the procedure described in the Materials and methods section and analysed by two-dimensional gel electrophoresis. First dimension: isoelectric focusing (right: acidic; left: basic end); second dimension: SDS-PAGE (top to bottom); MWM: molecular weight markers (top to bottom): β-galactosidase, 116 kDa, phosphorylase b, 97.4 kDa, bovine serum albumin, 66 kDa, ovalbumin, 43 kDa. Note that for better comparison two rods of the isoelectric focusing step were separated on one SDS-PAGE slab gel.

been found suitable for the selective isolation of plasma membrane localized contactinhibin. (1) Sialic acid moieties of trypsinized human fibroblasts were mildly oxidized by periodate [20, 21] and the generated aldehydes reacted with biotin hydrazide. In this context it has to be mentioned that contactinhibin is trypsin-resistant. (2) Contactinhibin was immunoprecipitated by immobilized *anti*-contactinhibin antibodies after detergent solubilization of the biotinylated cells. (3) The immunoprecipitate was redissolved and biotinylated contactinhibin isolated by incubation with immobilized streptavidin. The resulting supernatant contained intracellular contactinhibin as well as unsialylated surface contactinhibin. Specificity of modification has been proved by (1) reduction of periodate treated cells with borohydride prior to modification with biotin hydrazide, in which case no contactinhibin binding to streptavidin was found (Fig. 2); (2) by treatment of periodate oxidized cells with (^{3}H)-borohydride followed by solubilization and immunoprecipitation. Upon digestion of immunoprecipitated contactinhibin with sialidase, radioactivity was reduced to background levels (Table 1), indicating that $NaIO_4$ treatment selectively oxidized sialic acids. The effectiveness and specificity of the enzymatic release of sialic acids were monitored by probing of SDS-PAGE separated and electroblotted contactinhibin by Western blotting with the lectins from Maackia amurensis (MAA) and from Sambucus nigra (SNA), specific for α-2,3 and α-2,6 linked sialic acids, respectively (Fig. 3). It is shown that after sialidase-treatment both lectins failed to bind to contactinhibin, indicating complete removal of sialic acids. In addition, in the presence during digestion of the potent inhibitor of sialidases, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, contactinhibin was still detected, indicating that loss of reactivity after sialidase-treatment was due to the release of sialic acids only.

Table 1. Sparse cells express higher sialylated surface contactinhibin, compared with confluent cells. After labelling of periodate-oxidized cell surface sialic acids with [^{3}H]$NaBH_4$ cells were solubilized, contactinhibin was immunoprecipitated with *anti*-contactinhibin antibodies and immunoprecipitated radioactivity measured by liquid scintillation counting. Results are expressed as cpm per 5×10^5 cells and represent the mean of two values. The results of three independent experiments are shown. Values in brackets (Exp. 3) show remaining radioactivity after treatment of parallel immunoprecipitates with sialidase (Arthrobacter ureafaciens, 0.5 U, 16 h, 37 °C).

Source of immunoprecipitates	*Immunoprecipitated radioactivity*		
	Exp. 1	*Exp. 2*	*Exp. 3*
Sparse cells	302	1327	1009 (50)
Confluent cells	88	288	489 (27)

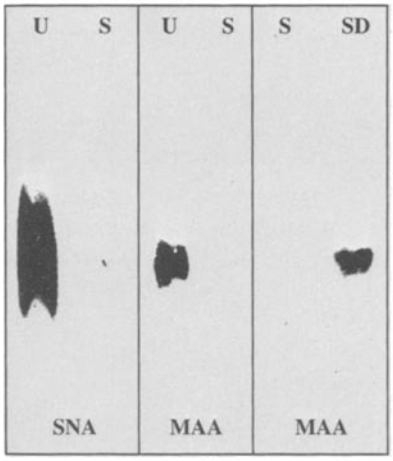

Figure 3. Blot analysis of sialylation of contactinhibin. Contactinhibin was electroblotted after separation by SDS-PAGE and probed with digoxigenin-conjugated lectins from Maackia amurensis (MAA) and Sambucus nigra (SNA), respectively. Lectin binding was visualized after incubation of blots with alkaline-phosphatase conjugated *anti*-digoxigenin-antibodies. U, untreated samples; S, contactinhibin treated with 0.5 U of sialidase from Arthrobacter ureafaciens for 16 h at 37 °C; SD, treatment of contactinhibin with sialidase in the presence of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (1 mM). (Note that different experiments are shown with differing amounts of contactinhibin employed.)

Surface-contactinhibin from confluent cells is undersialylated

In the course of these studies, it was found that in the case of sparse cells 2.3-fold higher radioactivity was immunoprecipitated from cells labelled in their sialic acids with [^{3}H]-borohydride as described above than in the case of confluent cells, giving the first hint of a higher degree of sialylation of contactinhibin in sparse cells (Table 1).

The immunoprecipitates and the streptavidin-bound fractions from sparse and confluent cells were analysed by two-dimensional gel electrophoresis (2D-PAGE) followed by silver staining. Intracellular contactinhibin from both sparse and confluent fibroblasts consistently showed up to eight distinct spots differing in their isoelectric points (Fig. 4). Earlier studies have shown that sialylation was the only posttranslational modification responsible for the observed microheterogeneity [16]. In contrast, when plasma membrane localized contactinhibin was analysed by 2D-PAGE, the highly sialylated forms were observed only when it was isolated from sparse cells, while contactinhibin from confluent cells consistently gave only four spots (Figs 2 and 4). This clearly indicates that in confluent cells plasma membrane localized contactinhibin was expressed in a less sialylated form. It has to be mentioned that unsialylated forms of plasma membrane localized contactinhibin are not

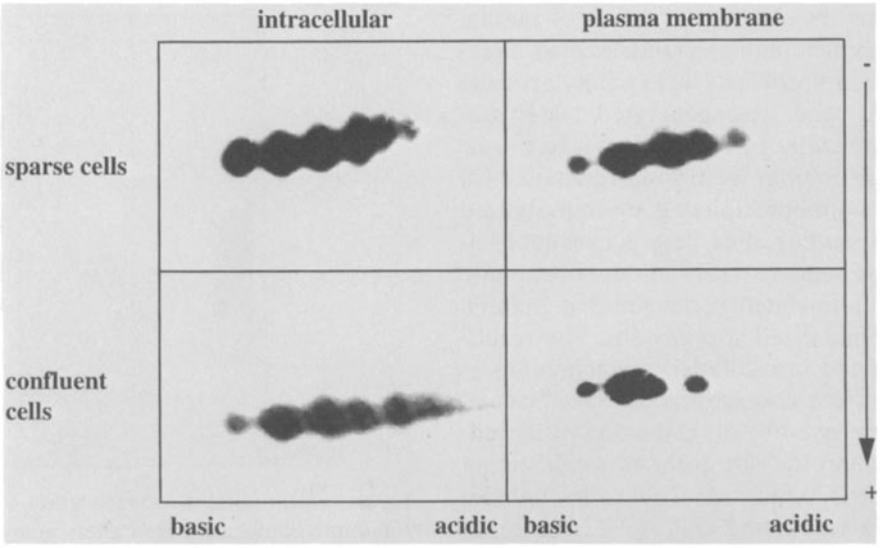

Figure 4. Two-dimensional gel electrophoresis of intracellular and plasma membrane localized contactinhibin. Intracellular and plasma membrane localized contactinhibin from sparse and confluent fibroblasts were isolated as described in the Materials and methods section and analysed by two-dimensional gel electrophoresis. First dimension: isoelectric focusing (left: basic; right: acidic end); second dimension: SDS-PAGE (top to bottom). Only the gel region containing contactinhibin is shown (compare with Fig. 2).

detected since biotinylation is confined to sialic acid moieties.

Contactinhibin is synthesized as a highly sialylated form

Several mechanisms could account for the observed differences of contactinhibin-sialylation: i) replacement in the cell membrane of highly sialylated contactinhibin by less sialylated contactinhibin with increasing cell densities; ii) growth-phase related differences of sialyltransferase activities; iii) generation of the low sialylated membrane form of contactinhibin by the action of a surface sialidase in a cell contact dependent fashion. The major question was therefore in which form contactinhibin was synthesized. To answer this, cells were labelled metabolically for 7 days with [^{3}H]-leucine followed by isolation of contactinhibin. After the final purification step, preparative 2D-PAGE, purified contactinhibin was separated by analytical 2D-PAGE and stained with Coommassie blue. Each of the individual spots was cut out and the incorporated radioactivity measured by liquid scintillation counting. With increasing degrees of sialylation, increased radioactivity was observed leading to an eight-fold higher value of the most acidic spot (highest sialylated form) compared with the most basic (less sialylated) spot (Fig. 5). It has to be noted that this value has not been corrected for protein content and would thus be much higher, as the highest sialylated form contained the lowest protein content, as judged from Coommassie blue staining intensity. This result indicates that contactinhibin was synthesized as a highly (maximum) sialylated form. In agreement with this, in the course of our studies on the biosynthesis of contactinhibin it was observed that upon 2D-PAGE of immunoprecipitated contactinhibin from extracts of metabolically labelled cells, followed by fluorography, consistently only the three most acidic spots were detected (data not shown).

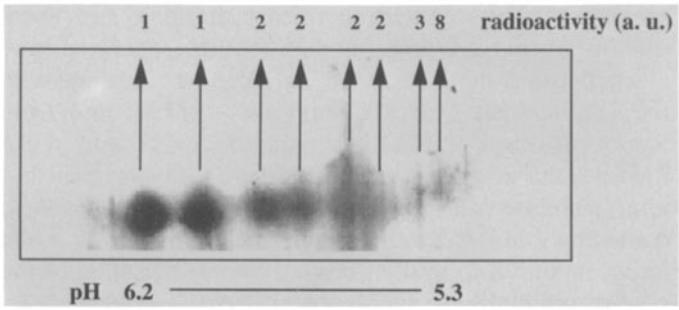

Figure 5. Evaluation of newly synthesized contactinhibin-forms. After metabolic labelling of cultured human fibroblasts contactinhibin was isolated [16] and separated on 2D-PAGE followed by Coomassie blue staining. The individual spots were excised, the gel pieces solubilized and the radioactivity measured by liquid scintillation counting. Only the gel region containing the contactinhibin spots is shown, the isoelectric points of which range from pH 5.3–6.2, as indicated. Arbitrary units (a.u.) of incorporated radioactivity are shown (not corrected for protein content).

Human fibroblasts express surface sialidase-activity

The results on contactinhibin-synthesis led to our working hypothesis, that for the observed differences in contactinhibin-sialylation in confluent cells a surface sialidase might be responsible. We therefore isolated

plasma membranes from sparse and confluent human fibroblasts and determined sialidase activity with 4-methylumbelliferyl-α-D-*N*-acetyl-neuraminic acid as a substrate. Initial experiments revealed that the enzyme showed a sharp pH-optimum at pH 5.5 (Fig. 6) and was activated by Triton X-100 (0.1%) and by Ca^{2+}-ions (10 mM). Under these conditions specific activities of $188 \pm 68\ \mu U\,mg^{-1}$ (pmol mg^{-1} min, $n = 4$) in plasma membranes from confluent cells, and of $219 \pm 49\ \mu U\,mg^{-1}$ ($n = 3$) in plasma membranes of sparse cells have been determined. The sialidase activity was inhibited by Cu^{2+}-ions (approximately by 50% by 1 mM $CuSO_4$), but not by 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (data not shown). In the culture supernatant of sparse cells a markedly higher sialidase activity ($27.5\ \mu U\,mg^{-1}$) compared with culture supernatants of confluent cells ($8.8\ \mu U\,mg^{-1}$) was found, although the overall activity was strongly reduced compared with the activity obtained in the isolated cell membranes. In contrast, sialidase activity was below detection limits in plasma membranes and culture supernatants of the human keratinocyte cell line HaCat [37].

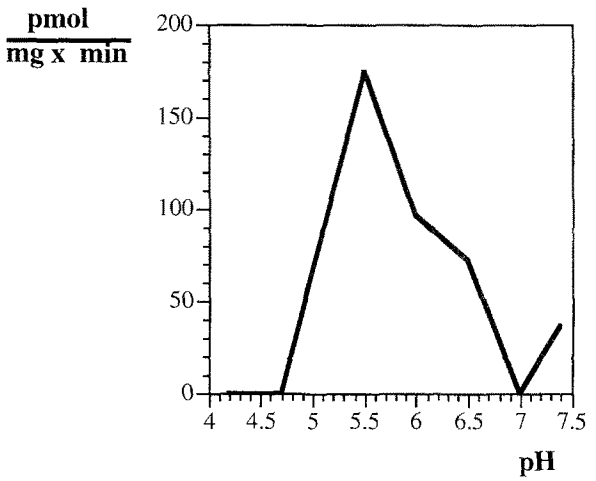

Figure 6. Cell surface sialidase activity dependence on pH. Assays were carried out with isolated plasma membranes (10 μg) using 4-MU-Neu5Ac as a substrate as described in the Materials and methods section.

Contactinhibin is desialylated by plasma membrane extracts

One essential property of the identified sialidase should be its ability to be active on contactinhibin. We immobilized highly sialylated contactinhibin onto PVFD-membranes in a slot blot device and incubated it with Triton X-100 solubilized plasma membranes at pH 5.5 for 4 h at room temperature in the presence of a protease inhibitor cocktail. Detection of sialic acids was performed with the lectin from Sambucus nigra (SNA), specific for α-2,6-linked sialic acids, as described in the Materials and methods section. As shown in Fig. 7, a decrease in SNA reactivity with increasing concentrations of solubilized plasma membranes was observed. In preliminary experiments with fetuin as a sialidase model substrate it was shown that concomitantly with a reduced SNA reactivity an increased signal with the lectin from Ricinus communis, specific for terminal galactose residues, was obtained, thus confirming that the reduced SNA-signal was due to the removal of sialic acids only and not due to degradation of the oligosaccharide chains (data not shown). By repeating these experiments with plasma membrane extracts obtained from the human keratinocyte cell line HaCat the reactivity of SNA remained unchanged compared with the controls, confirming that these cells were without cell surface sialidase. These results indicate that the membranes of human fibroblasts contained surface sialidase activity capable of desialylating contactinhibin. Parallel incubations carried out with comparable activities of Arthrobacter ureafaciens sialidase led to similar results (Fig. 7).

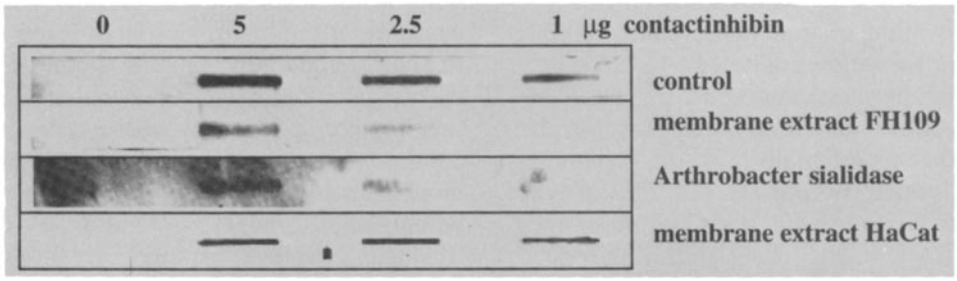

Figure 7. Slot-blot analysis of sialidase activity towards contactinhibin. Varying amounts (1–5 μg as indicated) of purified contactinhibin were slot-blotted onto PVFD-membranes and incubated with 10 μg of solubilized plasma membranes. Thereafter, sialic acid content of contactinhibin was determined by incubation with digoxigenin-conjugated lectin from Sambucus nigra (SNA), specific for α-2,6-linked sialic acids, followed by incubation with alkaline phosphatase-conjugated secondary antibodies. As a positive control, contactinhibin was incubated with Arthrobacter ureafaciens sialidase instead of plasma membranes. (0, control for unspecific staining of blocked membranes.) FH109, human dipolid fibroblasts, HaCat, human keratinocytes.

Discussion

Our results provide evidence that contactinhibin, a glycoprotein involved in contact-dependent inhibition of growth of non-transformed cells, undergoes cell density-dependent desialylation in a cell contact-dependent fashion. Several lines of evidence support this conclusion. 1) In the cell membrane of confluent cells four distinct forms of contactinhibin are present with different degrees of sialylation, as judged from the migration behaviour in 2D-PAGE. In this context it has to be mentioned that in earlier studies, sialylation has been confirmed to be the only modification contributing to the observed micro-heterogeneity of contactinhibin in 2D-PAGE [16]. 2) Intracellular contactinhibin shows, both in sparse and confluent cells, additional forms with higher degrees of sialylation, resulting in eight distinct spots in 2D-PAGE. The two proliferative states of the cells (i.e. sparse vs. confluent) differ greatly in their growth phases: while approximately 38% of sparse cells are found in S/G2-phase of the cell cycle, as measured by flow cytometry, only approximately 15% of confluent cells have been observed in these cell cycle compartments (unpublished results). These findings argue against a cell cycle dependent regulation of contactinhibin-sialylation. 3) The higher sialylated forms of contactinhibin were also found in the cell membrane of sparse cells. This argues against a preferential insertion of low sialylated contactinhibin-forms into the cell membrane. 4) The finding that only in the case of confluent cells was surface contactinhibin composed of lower sialylated forms points to the action of a cell surface sialidase acting on sialoconjugates of neighbouring cells or a sialidase secreted into the culture medium. The studies presented here confirm the presence of a surface sialidase which desialylated contactinhibin and fetuin. In addition, a secreted sialidase which shows a three-fold higher specific activity in culture supernatants of sparse cells compared with those of confluent cells was identified. However, the finding that in sparse cells membrane contactinhibin contained the highly sialylated forms as well makes a causal relationship between desialylation of membrane contactinhibin and the secreted sialidase rather unlikely.

While most of the plasma membrane sialidases described until now utilize gangliosides as a substrate, including the sialidases of rat brain [22], rat hepatomas [23] and cultured human fibroblasts [24, 25], activity towards glycoprotein bound sialic acids has also been shown in a few cases [26, 27]. The sialidase described here takes glycoproteins (contactinhibin and fetuin) and 4MU-NeuAc as a substrate, has a pH optimum of 5.5 and is inhibited by Cu^{2+}-ions, but not by NeuAc2en. In addition, it is stimulated by Triton X-100 and by Ca^{2+}-ions. Based upon these results, this sialidase is different from the enzymes described so far. The specific activity of the sialidase was found to remain relatively constant with respect to cell density, pointing to a constitutive expression of the sialidase. This is in contrast to a plasma membrane ganglioside sialidase of neuroblastoma cells suggested to be involved in the regulation of growth and differentiation [28]. In this case, sialidase activity increased with increasing cell density.

Our working model of contactinhibin-activation is fairly simple. Contactinhibin is synthesized as a fully sialylated form. This is confirmed by the finding that even after metabolic labelling over a very long period significant radiolabel was found only in the highest sialylated contactinhibin form. In addition, this result shows that contactinhibin turn-over takes place very slowly, and that most of the contactinhibin form with the lowest sialic acid content has been present in the cells before the labelling experiment was set up, as shown by the discrepancy between Coommassie blue staining intensity and incorporated radioactivity. These results are in agreement with previous findings that after metabolic labelling for 24–48 h followed by immunoprecipitation, 2D-PAGE and fluorography only the most acidic spots of contactinhibin were detected (unpublished results). Many cell membrane glycoproteins are inserted into the membrane in fully processed form with respect to glycosylation, i.e. in fully sialylated form [29, 30]. The finding that after prolonged metabolic labelling some radiolabel was also found in contactinhibin forms with reduced sialic acid content suggests that the highest sialylated form undergoes desialylation, most likely by endocytosis and recycling to the cell membrane [31, 32]. It is well known that in the course of endocytosis terminal monosaccharides, especially fucose and sialic acids, are partially removed from plasma membrane glycoproteins [33]. However, at the moment it cannot be excluded that the sialidase secreted into the culture medium may participate in the process of partial desialylation of surface contactinhibin. With progressing cell growth, cell density increases leading to the establishment of cell-cell contacts. At this stage, the cell surface sialidase cleaves off sialic acids on neighbouring cells leading to the disappearance of the highly sialylated forms of surface contactinhibin. In addition, *de novo* synthesis and insertion into the cell membrane of highly sialylated contactinhibin is suggested to be greatly reduced in confluent cells, as deduced from the fact that metabolic labelling of contactinhibin in confluent cells occurs only at very low rates. Desialylation leads to activation of contactinhibin, as terminal galactose residues on N-glycans have been found essential for contact-dependent inhibition of growth [16, 34]. In this context a similar cell density dependent activation of a growth inhibiting compound has been described, which, however, is achieved by removal of terminal N-acetylglucosamine residues instead of sialic acids [35].

The functional significance of contactinhibin-sialylation is not yet understood. However, the high degree of sialylation could prevent the intracellular form of contactinhibin from binding to its receptor prior to its membrane insertion, as binding of contactinhibin to the receptor is inhibited by sialylation of terminal galactose residues (manuscript submitted). In addition, one might speculate that contactinhibin-sialidase acts as a tissue specific protection against cross-inhibition of growth. It is well known that interaction of fibroblasts with epithelial cells results in epithelial growth stimulation rather than in inhibition [36] and according to this hypothesis, contactinhibin-sialidase may be confined to mesenchymal cells, thereby restricting contact-dependent inhibition of growth via contactinhibin to special cell types. The finding that the human keratinocytes HaCat [37] were without detectable surface sialidase points to such a mechanism.

Acknowledgements

This work was supported by a grant of the Deutsche Forschungsgemeinschaft Wi 727/2-1 and is part of the PhD thesis of C.E.B.

References

1. Whittenberger B, Glaser L (1977) *Proc Natl Acad Sci USA* **74**: 2251–55.
2. Raben D, Lieberman M, Glaser L (1981) *J Cell Physiol* **108**: 35–45.
3. Heimark RL, Schwartz SM (1985) *J Cell Biol* **100**: 1934–39.
4. Stein G, Atkins L (1986) *Proc Natl Acad Sci USA* **83**: 9030–34.
5. Higgins PJ, Peterson SW (1989) *Experientia* **45**: 370–72.
6. Wieser RJ, Heck R, Oesch F (1985) *Exp Cell Res* **158**: 493–99.
7. Wieser RJ, Oesch F (1987) *J Biochem Biophys Meth* **15**: 13–22.
8. Wieser RJ, Oesch F (1988) In *Sialic Acids* (Schauer R, Yamakawa T, eds) pp. 276–77. Kiel, FRG: Kieler Verlag Wissenschaft + Bildung.
9. Wieser RJ, Oesch F (1986) *J Cell Biol* **103**: 361–67.
10. Maki K, Sagara J, Kawai A (1991) *Biochem Biophys Res Comm* **175**: 768–74.
11. Karlsson GB, Platt FM (1991) *Anal Biochem* **199**: 219–22.
12. Wessel D, Flügge UJ (1984) *Anal Biochem* **138**: 141–43.
13. Laemmli UK (1970) *Nature* **227**: 680–85.
14. Rodemann HP, Bayreuther K (1986) *Proc Natl Acad Sci USA* **83**: 2086–90.
15. Ormerod MG (1990) In *Flow Cytometry* (Ormerod MG, ed.) pp. 75. Oxford: IRL Press.
16. Wieser RJ, Schütz S, Tschank G, Dienes H-P, Thomas H, Oesch F (1990) *J Cell Biol* **111**: 2681–92.
17. Bonner WM, Laskey RA (1974) *Eur J Biochem* **46**: 83–88.
18. Haycock JW (1993) *Anal Biochem* **208**: 397–99.
19. Smith PK, Krohn RJ, Hermanson GT, Malli AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) *Anal Biochem* **150**: 76–85.
20. Van Lenten L, Ashwell GJ (1971) *J Biol Chem* **246**: 1889–94.
21. Blumenfeld OO, Gallop PM, Leo TH (1972) *Biochem Biophys Res Commun* **48**: 242–51.
22. Miyagi T, Sagawa J, Konno K, Handa S, Tsuiki S (1990) *J Biochem* **107**: 787–93.
23. Miyagi T, Hata K, Konno K, Tsuiki S (1992) *Tohoku J Exp Med* **168**: 223–29.
24. Schneider-Jakob HR, Cantz M (1991) *Biol Chem Hoppe-Seyler* **372**: 443–50.
25. Zeigler M, Bach G (1981) *Biochem J* **198**: 505–8.
26. Miyagi T, Sagawa J, Konno K, Tsuiki S (1990) *J Biochem* **107**: 787–93.
27. Cross AS, Wright DG (1991) *J Clin Invest* **88**: 2067–76.
28. Kopitz J, von Reitzenstein C, Mühl C, Cantz M (1994) *Biochem Biophys Res Comm* **199**: 1188–93.
29. Kreisel W, Hildebrandt H, Mössner W, Tauber R, Reutter W (1993) *Biol Chem Hoppe-Seyler* **374**: 255–63.
30. Hayes BK, Varki A (1993) *J Biol Chem* **268**: 16155–69.
31. Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF (1993) *J Biol Chem* **268**: 13756–63.
32. Mayor S, Presley JF, Maxfield FR (1993) *J Cell Biol* **121**: 1257–69.
33. Tauber R, Park CS, Reutter W (1983) *Proc Natl Acad Sci USA* **80**: 4026–29.
34. Wieser RJ, Oesch F (1988) *Exp Cell Res* **176**: 80–86.
35. Natraj CV, Datta P (1978) *Proc Natl Acad Sci USA* **75**: 6115–19.
36. Limat A, Hunziker T, Boillat C, Bayreuther K, Noser F (1989) *J Invest Dermatol* **92**: 758–62.
37. Boukamp P, Petrussevska RT, Breitkreuz D, Hornung J, Markham A, Fusenig NE (1988) *J Cell Biol* **106**: 761–71.