Density-dependent regulation of cell growth by contactinhibin and the contactinhibin receptor

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Background: The number of cells within mammalian tissues is maintained by growth-stimulating and growth-inhibiting mechanisms, with inhibitory signals being superimposed over growth stimuli. This is reflected, in the culture of normal adherent cells, by the phenomenon of density-dependent inhibition of growth: cells cease proliferation after becoming a confluent monolayer. We have shown previously that a plasma membrane glycoprotein, contactinhibin, is a major effector of negative growth regulation. Although transformed cells express contactinhibin in a functionally active form, they are not growth-inhibited, suggesting that the defects that lead to their aberrant growth are located 'downstream' of contactinhibin.

Results: Here, we provide evidence that a 92 kD plasma membrane protein, which we call CiR, binds specifically to contactinhibin and acts as a receptor mediating the contact-dependent inhibition of growth of cultured human fibroblasts. When polyclonal antibodies against CiR were introduced into cells using liposomes, confluent cells were released from density-dependent growth control. By contrast, cross-linking CiR that is localized to the plasma membrane, using anti-CiR antibodies, led to growth inhibition, suggesting that CiR is a signalling molecule and implicating CiR oligomerization in signal generation. This conclusion is supported by the finding that binding of contactinhibin by CiR is strongly dependent on the local concentration of both molecules and has a sharp threshold. When CiR was isolated by immunoprecipitation under conditions favouring phosphorylation, it was hyperphosphorylated on serine and threonine residues and had reduced contactinhibin-binding capacity; the binding capacity of CiR was restored after treatment with potato acid phosphatase. Fibroblasts transformed with simian virus 40 had reduced CiR expression, higher CiR phosphorylation levels, and a strongly reduced capacity of CiR to bind to contactinhibin. Phosphatase treatment of the CiR isolated from transformed cells only partially restored its contactinhibin-binding capacity.

Conclusions: Homeostasis is the net result of a highly balanced network of growth-stimulating and growth-inhibitory signals. We have shown that density-dependent inhibition of growth *in vitro* is mediated by the interaction of contactinhibin with a 92 kD plasma membrane glycoprotein, CiR, the contactinhibin-binding capacity of which is regulated by phosphorylation.

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Background

The growth of mammalian cells is under the balanced control of positively and negatively acting factors. Although a vast amount of information about mitogenic factors and their receptors is available, not much is known about their physiological antagonists. In order to maintain tissue and organ size, one must postulate the existence of growth-inhibiting signals reflecting the cellular environment, the action of which would be superimposed over mitogenic signals. As the decision to cease proliferation is determined largely by the cellular environment (for example, in wound healing or the need for replacement of dead cells), negative growth control is probably exerted by mechanisms initiated at the plasma membrane. A good example of such negative growth regulation is the density-dependent inhibition of growth of mammalian cells *in vitro*. The most obvious explanation for this phenomenon would be the interaction of distinct cell membrane molecules that trigger growth-inhibition after cell–cell contact. Although cell–cell contacts attributable to defined classes of cell adhesion molecules have clearly been shown to be involved in processes such as determination and differentiation [1,2], data on the molecular basis of density-dependent growth regulation are scarce. Glycoproteins of the plasma membrane have, however, been implicated in density-dependent growth inhibition in many cell-culture systems [3–7].

We have found that a distinct plasma membrane glycoprotein, referred to as contactinhibin, is responsible for the density-dependent growth inhibition of normal human diploid fibroblasts [8]. Contactinhibin inhibits the growth of cultured cells only in immobilized form, and its activity is mediated exclusively by *N*-glycosidically linked oligosaccharide side chains with terminal β-glycosidically linked galactose residues. Mouse fibroblasts transformed with 3-methylcholanthrene lose contact-dependent inhibition of growth as a result of losing responsiveness to contactinhibin [9], but contactinhibin itself is expressed in biologically active form by these cells.

Because contactinhibin does not interact in a homophilic fashion (our unpublished observations), these findings suggest that there is a break in the signalling pathway of transformed cells, affecting regulatory molecules other than contactinhibin and leading to an escape from growth control. We have therefore focussed on the identification

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of the plasma membrane component that specifically recognizes contactinhibin and triggers a negative growth signal in response to contactinhibin binding. Here, we describe the molecular and functional properties of this candidate contactinhibin receptor isolated from human diploid fibroblasts.

Results and discussion

Contactinhibin binds through its glycans to a 92 kD plasma membrane protein

In a first approach, contactinhibin-binding proteins were isolated by covalent binding of immobilized contactinhibin to cell membrane proteins of cultured human FH109 fibroblasts, using the cleavable cross-linker 3,3′-dithiobis (propionic acid)-*N*-succinimidester. The bound material was separated by two-dimensional polyacrylamide gel electrophoresis (Fig. 1) and was shown to contain a 92 kD component that had remarkable charge heterogeneity, which is probably due to variable degrees of modification with the cross-linking agent. Other major components that precipitated in the acidic region of the isoelectric focusing gel resembled cytoskeletal proteins (on the basis of their apparent molecular weights and their relative cellular abundances) that could have been co-isolated with the membrane proteins cross-linked to contactinhibin (note that the sample was applied to the basic end of the gel).

In order to detect contactinhibin-binding proteins in cell extracts, a novel binding assay was developed (Fig. 2a). This aggregation assay, based on the formation of rosettes made up of immobilized contactinhibin and immobilized cell-extract proteins, was used to isolate the contactinhibin receptor (CiR) and for preliminary studies of its binding characteristics (see below). Figure 2b shows the characteristic 'all-or-none' reaction between immobilized contactinhibin and immobilized proteins from an extract made with 8 mM CHAPS; the all-or-none phenomenon was confirmed using an adhesion assay (see below). Similar binding-threshold effects are widespread, for example in the aggregation of sponge cells [10], the adhesion of hepatocytes to saccharide-coated surfaces [11], cell sorting [12] or the regulation of neurite outgrowth [13].

After acetone precipitation, detergent-solubilized proteins from FH109 fibroblasts were washed extensively with distilled water to remove hydrophilic cytosolic proteins, and thereafter separated by preparative SDS gel electrophoresis. Slab gels were cut horizontally into strips and the proteins electroeluted and tested for contactinhibin-binding activity. An 80–90 kD fraction had the highest binding activity (Figs 3a,b). The next purification step, preparative two-dimensional SDS gel electrophoresis, led to the isolation of a 92 kD contactinhibin-binding protein that had charge microheterogeneity (isoelectric point pH 5.9–6.6; Fig. 3c) and resembled the 92 kD component observed in the cross-linking experiment. In addition to this 'p92', a more acidic, 90 kD protein was observed, but this had no contactinhibin-binding activity (data not shown). The microheterogeneity of p92 can be explained by varying degrees of glycan sialylation (Figs 4c,d) and protein phosphorylation (see below; Fig. 7).

Further studies revealed that the growth-inhibitory activity of contactinhibin and its interaction with p92 — the putative CiR — share common structural features (Table 1), supporting the conclusion that p92 is the contactinhibin-receptor; we refer to it henceforth as CiR. Both are dependent upon the presence of terminal β-glycosidically linked galactose residues on contactinhibin, both are mediated by the *N*-linked glycans of contactinhibin; all other glycoproteins tested, such as asialofetuin or asialo-α_1-acid glycoprotein, were without effect on proliferation

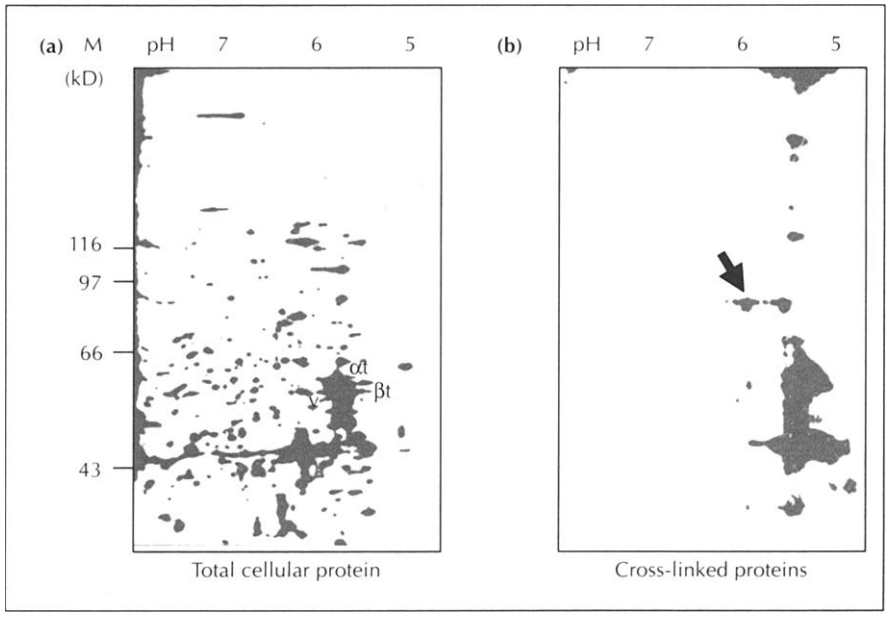


Fig. 1. Covalent cross-linking of contactinhibin to fibroblasts yields a 92 kD protein. **(a)** Two-dimensional gel-electrophoretic analysis (fluorography) of total fibroblast proteins solubilized with 8 mM CHAPS, compared to **(b)** cross-linked proteins. The migration of proteins with known molecular weights is indicated on the left, and the upper scale shows the pH gradient after isoelectric focusing. In addition to the putative contactinhibin receptor, CiR (arrow), several other proteins, probably representing cytoskeletal elements, were found in the cross-linked material: v, vimentin; αt, α tubulin; βt, β tubulin.

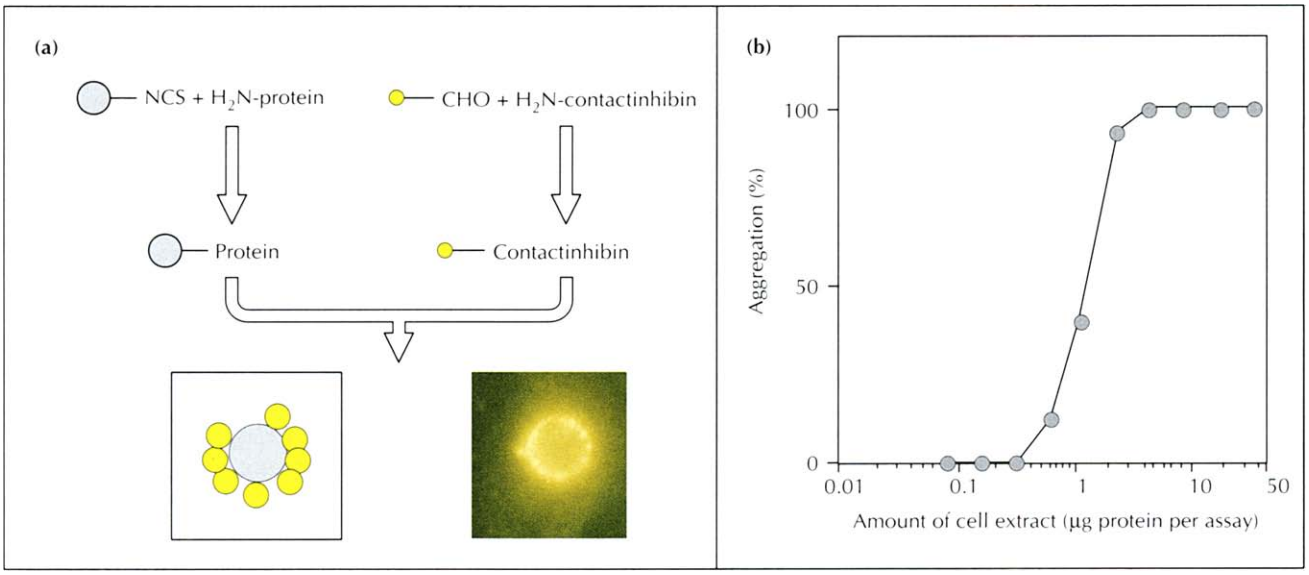


Fig. 2. Aggregation assay. **(a)** Contactinhibin was coupled covalently to 'Fluoresbrite' beads (1 µm in diameter), and CiR- bearing fractions ('protein') were linked to silica beads (10 µm in diameter). Co-incubation of the beads leads to rosette formation, which can be visualized by fluorescence microscopy (insert). **(b)** Immobilized contactinhibin and immobilized proteins of an extract of human fibroblasts made in 8 mM CHAPS were mixed and gently shaken for 30 min, and aggregation was then examined by microscopy. Aggregation was scored as positive when a silica bead was covered with at least eight contactinhibin-coated microspheres and is indicated as the percentage of total beads that were positive.

and did not bind to CiR. Furthermore, in agreement with the finding that fluid-phase contactinhibin lacks growth-inhibitory activity, we observed that detergent-solubilized contactinhibin was not bound by CiR .

Growth inhibition by cross-linking cell-surface CiR using antibodies

To test the hypothesis that CiR generates a growth-inhibitory signal after interaction with contactinhibin, we examined the effects on human fibroblast cultures of polyclonal anti-CiR immunoglobulin G antibodies. The antibodies, raised in rabbits, specifically recognized p92 on western immunoblots of detergent-extracted FH109 and SV40-transformed Wi38 fibroblasts (Fig. 4, lanes e and f), and in immunoprecipitates of similar material (Fig. 4, lane g). In the aggregation assay, these antibodies fully inhibited the interaction between contactinhibin-coated microspheres and silica beads coated with crude

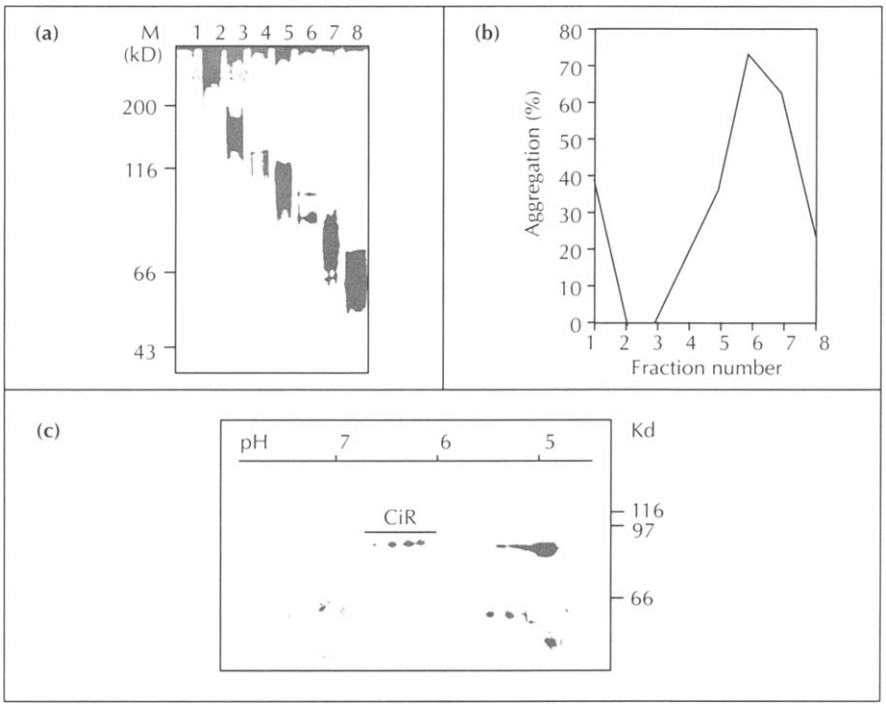


Fig. 3. Purification of CiR. **(a)** SDS polyacrylamide gel electrophoresis of individual fractions of FH109 proteins obtained after preparative electrophoresis, and **(b)** the corresponding contactinhibin-binding activity as monitored using the aggregation assay shown in Fig. 2. **(c)** Two-dimensional SDS gel electrophoresis of fraction 6 obtained after preparative electrophoresis. The position of CiR is indicated; purified CiR was obtained by electroelution of the relevant spots. The migration of proteins with known molecular weights is indicated on the right, and the upper scale shows the pH gradient after isoelectric focusing.

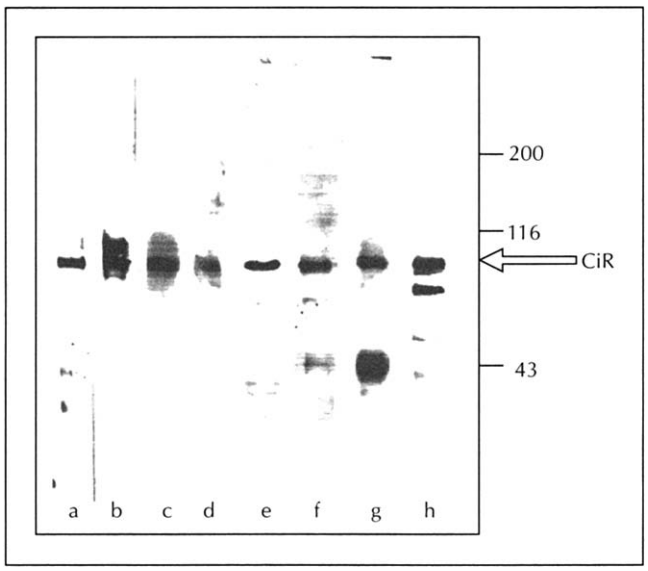


Fig. 4. Characterization of CiR and anti-CiR antibodies. Lanes are as follows. **(a)** 200 ng of CiR purified by two-dimensional SDS-gel electrophoresis and silver stained. Detection of **(b)** glycan moieties, **(c)** α-2,3- and **(d)** α-2,6-linked sialic acid residues on 1 μg CiR. Western immunoblot using anti-CiR antibodies and 20 μg protein in each of **(e)** FH109 fibroblasts and **(f)** SV40-transformed Wi38 fibroblasts solubilized with CHAPS; **(g)** silver stain of immunoprecipitated CiR (note that the protein migrating with an approximate molecular weight of 43 kD is co-precipitated actin, as assessed by immunoreactivity (data not shown). **(h)** Western blot analysis of immunoprecipitated CiR . The faster-migrating compound is a CiR degradation product, which is observed variably both in extracts and in purified CiR preparations; compare also with Fig. 8a. All analyses were performed on SDS polyacrylamide slab gels (7.5 %).

detergent-extracted fibroblast proteins (Table 1). The addition of anti-CiR antibodies to sparsely seeded fibroblasts had only marginal growth-inhibitory effects (Fig. 5b), which could, however, be increased by cross-linking the primary antibodies with secondary antibodies (Fig. 5b). A similar growth-inhibitory activity of anti-CiR antibodies on sparsely seeded cells was observed when the antibodies were coupled covalently to silica beads before addition (Fig. 5a). This indicates that oligomerization of CiR is necessary, and sufficient, for the induction of negative growth signals.

In agreement with this suggestion, cell growth was not influenced by the addition of anti-CiR Fab antibody fragments to sparsely seeded fibroblasts, suggesting that, in this case, no signal is generated and excluding the possibility that the signal after antibody addition results from an antibody-induced conformational change in CiR, as has been observed in the case of the epidermal growth factor (EGF) receptor and anti-EGF-receptor antibodies [14]. Anti-CiR antibodies inhibited proliferation of sparsely seeded fibroblasts by up to 40 % when coated onto microtiter wells (Fig. 5c), suggesting that clustering of CiR by surface-immobilized antibodies is more effective than cross-linking with secondary antibodies or with antibodies immobilized onto bead surfaces. In summary, the results described above indicate that CiR is the signal-transducing receptor for contactinhibin in mediating density-dependent inhibition of growth.

Intracellular capturing of CiR with anti-CiR-antibodies releases cells from density-dependent growth inhibition

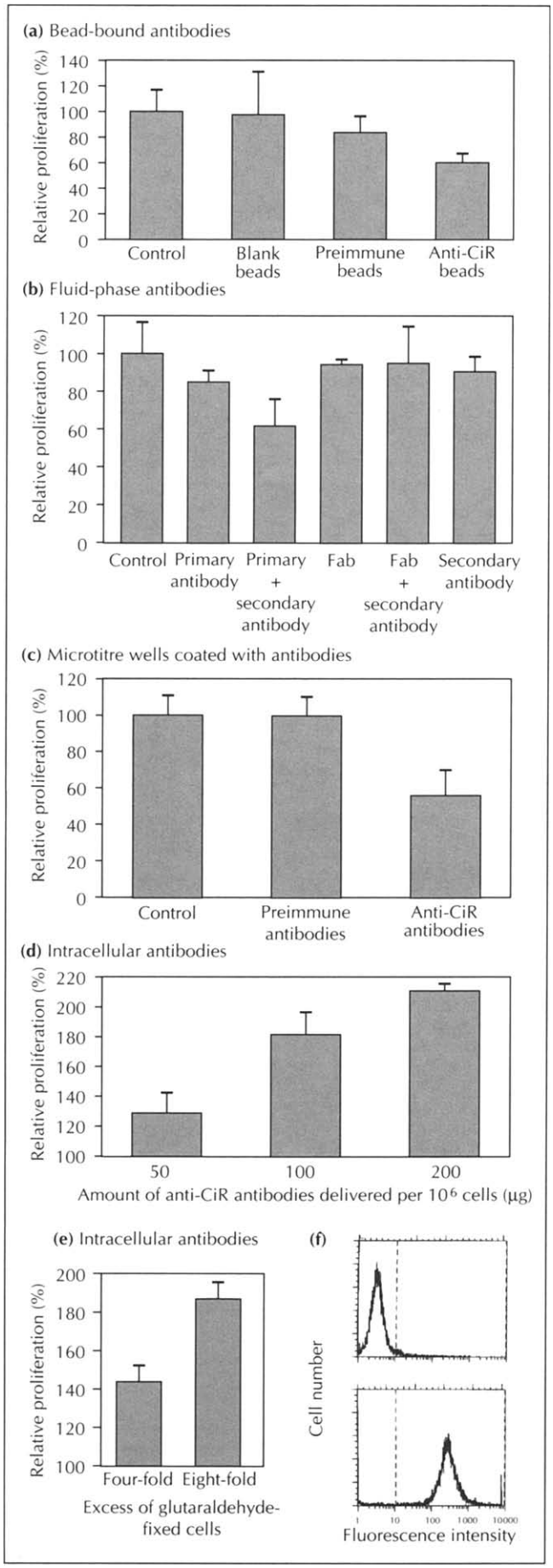
We have suggested that a growth-inhibitory signal is transduced either by a direct action of CiR, or by the action of proteins associated with CiR, after its interaction with contactinhibin (as suggested above). Both effects should be prevented by intracellular masking of CiR with anti-CiR-antibodies, which should thus lead to growth stimulation in confluent cell cultures. We therefore introduced anti-CiR-antibodies into confluent FH109 fibroblasts using cationic liposomes (made up of the lipid DOTAP; see [15]). The efficiency of antibody-loading was monitored by flow cytometry (Fig. 5f). A remarkable increase in proliferation (up to 100 %) could be induced with these antibodies in confluent cultures (Fig. 5d). This effect was probably due to a lack of responsiveness towards the contactinhibin present on neighboring cells, as was confirmed by the fact that growth was never affected when cells loaded with anti-CiR antibodies were seeded subconfluently (data not shown).

A further approach for studying the density-dependent inhibition of growth involves the addition of glutaraldehyde-fixed cells to sparsely seeded cells [16]. Experiments with sparsely seeded cells that received either a four-fold or an eight-fold excess of glutaraldehyde-fixed cells showed that cells loaded with anti-CiR antibodies had strongly reduced responsiveness to the growth-inhibitory signal provided by glutaraldehyde-fixed cells

Table 1. Structural requirements for the biological activity of contactinhibin and for its recognition by CiR.

Sample	Aggregation (% positive beads)	Proliferation rate (% control)
Fluid-phase contactinhibin	No binding	69 ± 13
Immobilized contactinhibin (iCi)	100	19 ± 9
iCi, treated with β-galactosidase (0.5 U)	0	87 ± 7
iCi, treated with glycopeptidase F	n.t.	78 ± 11
iCi, pronase-digested	100	29 ± 10
iCi + anti-Ci antibodies	0	n.t.
iCi + anti-CiR antibodies	0	n.t.
Immobilized BSA, asialofetuin or asialo-α_1 acid glycoprotein	0	100 ± 5

For proliferation assays, 0.1 μg ml^{-1} immobilized and 1 μg ml^{-1} of fluid-phase contactinhibin were used, respectively. The aggregation assay was performed as described in Fig. 2. Ci, contactinhibin; iCi, immobilized contactinhibin; CiR, contactinhibin-receptor; n.t., not tested.



(Fig. 5e). Together, these results provide evidence that it is the contactinhibin-induced growth-inhibitory pathway that is bypassed by the introduction of anti-CiR antibodies into cultured cells.

The CiR–contactinhibin interaction occurs with a threshold

In order to measure the contactinhibin-binding capacity of CiR, we established a novel adhesion assay that involved the adsorption of immunoprecipitated CiR onto microtiter wells and the addition of contactinhibin bound to microspheres bearing an alkaline phosphatase reporter enzyme. A threshold of contactinhibin binding was observed (Fig. 6a, solid line). Together with the data obtained using the aggregation assay, these results indicate that the interaction between contactinhibin and CiR is highly specific, but of low affinity — characteristics that are common to receptor interactions with ligands which are insoluble under physiological conditions, such as the extracellular matrix component, fibronectin [17,18]. The low affinity of contactinhibin–CiR binding is also demonstrated in the adhesion assay by the decreased binding of contactinhibin-coated spheres to adsorbed CiR at above-optimal local concentrations (Fig. 6b). This effect might result from reduced binding valencies per contactinhibin-coated sphere when CiR is adsorbed at high concentration, in which case the beads would removed during washing (Fig. 6b). Affinity was also low when too little CiR was adsorbed.

CiR isolated in the presence of the protein-tyrosine phosphatase inhibitor, vanadate, had enhanced phosphorylation (Fig. 7) and strongly reduced contactinhibin-binding capacity (Fig. 6a, blue line), pointing to an inverse correlation between CiR's phosphorylation and its contactinhibin-binding activity. This was confirmed by treatment of immunoprecipitated CiR that had been isolated in the presence of vanadate with potato acid phosphatase; this led to a recovery of maximal binding (Fig. 6a, broken line). Despite these results with orthovanadate, phosphoamino-acid analysis revealed that CiR is phosphorylated on serine and threonine residues only, with no detectable phosphorylation on tyrosine (Fig. 8b). The finding that vanadate enhanced CiR phosphorylation, despite not

Fig. 5. Antibody-mediated CiR cross-linking results in growth-inhibition. Relative proliferation rates of FH109 fibroblasts cultured for 24 h in the presence of **(a)** anti-CiR antibodies coupled to silica beads; **(b)** fluid-phase antibodies in the presence of anti-rabbit antibodies; and **(c)** antibodies coated onto the microtiter wells, as described in Materials and methods. Values are given as percentages ± SD, compared to control cells not treated with antibodies. Liposome-mediated loading of anti-CiR antibodies into fibroblasts; the relative proliferation rates of FH109 fibroblasts after introduction of anti-CiR antibodies as described in Materials and methods: **(d)** 2×10^4 cells per well, (cells seeded confluently), or **(e)** 5×10^3 cells per well, in the presence of a four-fold or eight-fold excess of glutaraldehyde-fixed cells. Values are given as percentages ± SD, compared to control cells treated with anti-epoxide hydrolase antibodies. **(f)** Flow cytometric analysis, as described in Materials and methods, of liposome-mediated antibody-loading of human fibroblasts; upper panel, control cells; lower panel, antibody-loaded cells.

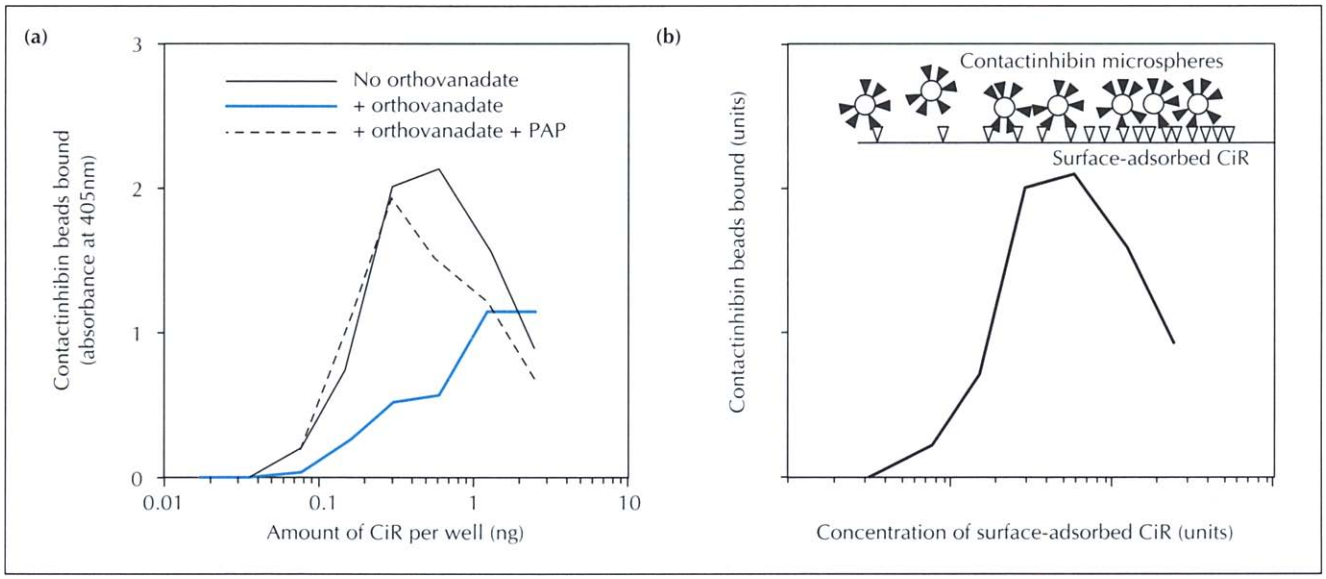


Fig. 6. The contactinhibin–CiR interaction. **(a)** The indicated amounts of CiR immunoprecipitated from detergent extracts of FH109 fibroblasts was adsorbed onto the surface of microtiter wells and incubated with contactinhibin immobilized onto microspheres bearing alkaline phosphatase as reporter enzyme; immunoprecipitation was in the presence (blue line) or absence (solid black line) of orthovanadate, and one fraction of CiR immunoprecipitated in the presence of orthovanadate was then treated with potato acid phosphatase (broken line). Values are given as absorbance units at 405 nm as an indication of coloured product produced by the alkaline phosphatase reporter. **(b)** Mechanisms of adhesion of contactinhibin bound to microspheres to adsorbed CiR. At low CiR concentrations, multiple interactions between contactinhibin-coated spheres and CiR are not possible, and as a result of low binding avidity, the beads are washed away. At optimal ratios of contactinhibin:CiR, multiple interactions are favoured and thus increased adhesion strength is observed. At higher concentrations of CiR, as a result of the geometry of the assay, multiple interactions are hindered again, and beads are again washed away.

being able to detect tyrosine phosphorylation of CiR, suggests an as yet unknown, indirect, effect of vanadate.

It remains to be seen whether CiR phosphorylation at a distinct serine and/or threonine residue results in a conformational change followed by loss of affinity for the ligand, or whether there is an additional impairment of signal generation by CiR following phosphorylation. In this context, it should be noted that treatment of human diploid fibroblasts with tetradecanoyl phorbol acetate, which is known to activate the threonine/serine protein kinase, PKC [19], leads to a loss of contact-dependent inhibition of growth [20]. Most intriguing is the working hypothesis that CiR could be a substrate for certain oncogene products, such as the serine kinase Raf [21], activation of which would lead to CiR over-phosphorylation and a loss of responsiveness of cells to contactinhibin. In this context, the higher phosphorylation level and reduced binding activity of CiR in SV40-transformed fibroblasts is particularly noteworthy.

SV40-transformed fibroblasts have reduced CiR expression and increased CiR phosphorylation

Western blot analysis of SV40-transformed human Wi38 lung fibroblasts showed that they contain less CiR than non-transformed FH109 fibroblasts (Fig. 8a). The phosphorylation level of CiR was higher in the transformed fibroblasts (Fig. 8a), but phosphoamino-acid analysis yielded the same phosphorylation pattern of serine and threonine residues as in the non-transformed fibroblasts (Fig. 8b). In addition, the phosphorylation level of CiR in SV40-transformed Wi38 fibroblasts was only marginally influenced by vanadate (Fig. 8c). As well as a higher

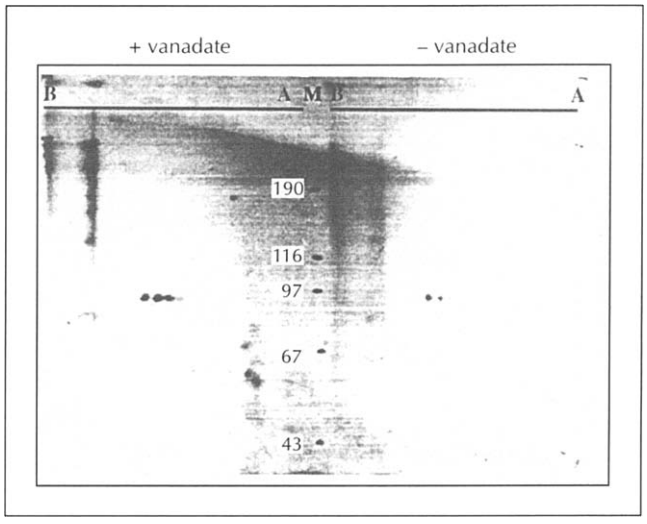


Fig. 7. CiR is a phosphoprotein. CiR was immunoprecipitated, in the presence (+ vanadate) or absence (– vanadate) of orthovanadate, from lysates of cells metabolically labelled with ^{32}P-orthophosphate; it was then analyzed by two-dimensional gel electrophoresis and visualized by autoradiography. Basic (B) and acidic (A) ends of the isoelectric focusing gels, and the migration in one dimension of proteins with known molecular weights (M), are indicated. Note that for improved comparison, both gel rods of the isoelectric focusing step were separated on a single slab gel.

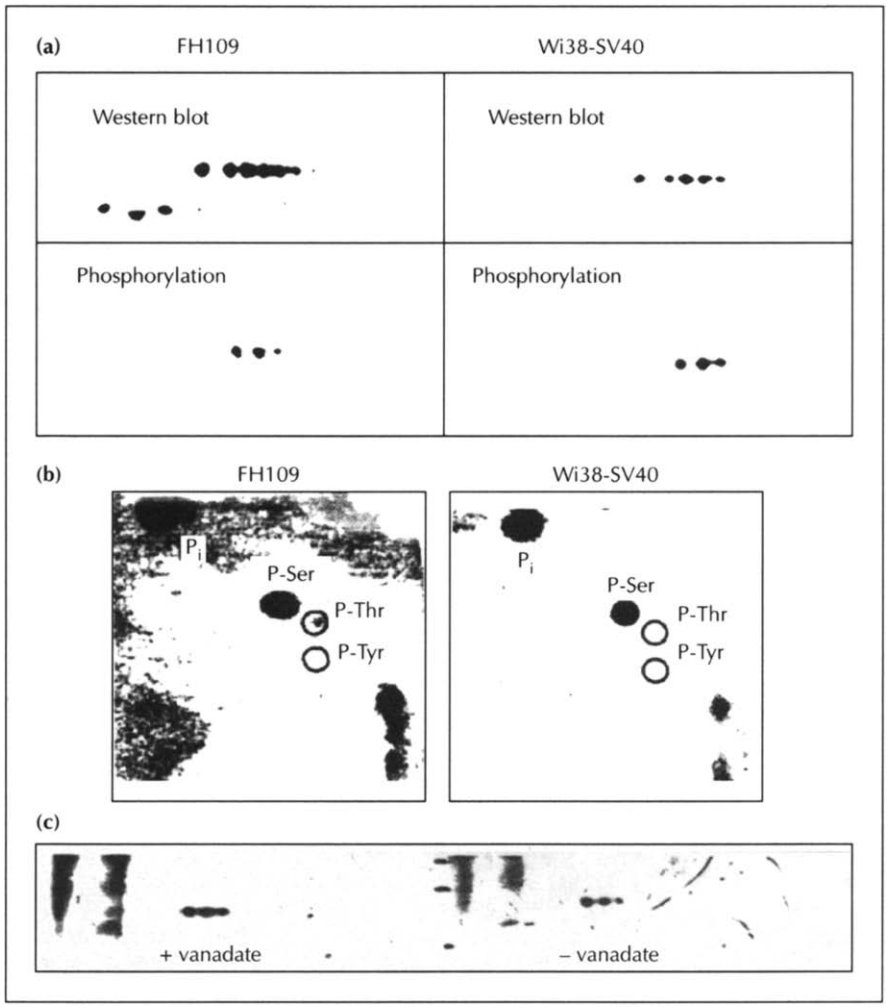


Fig. 8. **(a)** SV40-transformed human fibroblasts contain reduced amounts of CiR but it is highly phosphorylated. Equal numbers of ^{32}P-labelled cells were lysed, CiR was immunoprecipitated and separated by two-dimensional gel electrophoresis and either processed for western blotting with anti-CiR antibodies (upper panels) or for autoradiography (lower panels). **(b)** CiR contains both phosphoserine and phosphothreonine residues. Phosphoamino-acid analyses of CiR from FH109 and Wi38 SV40 fibroblasts. Migration of the phosphoamino-acid standards is indicated by circles (P_i, inorganic phosphate). **(c)** CiR phosphorylation in SV40-transformed fibroblasts is only marginally influenced by vanadate. Autoradiography of CiR immunoprecipitated from ^{32}P-labelled cells followed by two-dimensional gel electrophoresis.

phosphorylation level, strongly reduced contactinhibin-binding activity was observed in these cells, and binding was only partially restored after phosphatase treatment (Fig. 9). This makes it likely that there are additional alterations to the CiR from transformed cells.

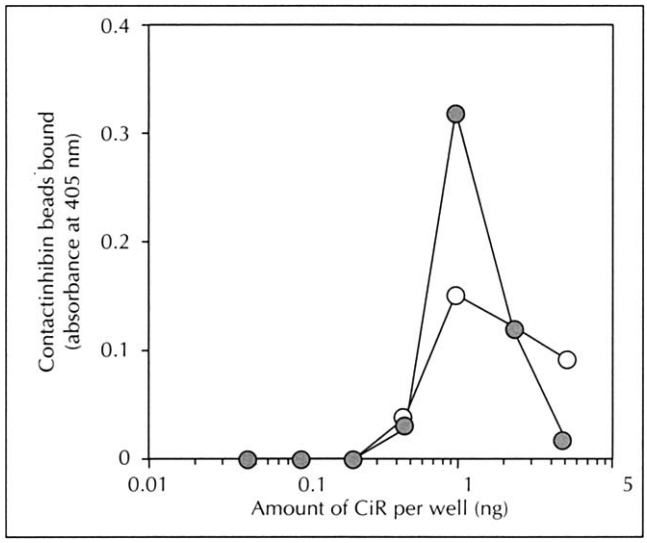


Fig. 9. CiR from transformed cells has strongly reduced contactinhibin-binding affinity, even after treatment with potato acid phosphatase (PAP). Experiments are analogous to those shown in Fig. 6. White dots, before PAP treatment; grey dots, after PAP treatment.

Conclusions

We suggest that contactinhibin signalling through the contactinhibin receptor is essential for the maintenance of controlled cell growth. The function of the contactinhibin receptor, and in particular its binding to contactinhibin, is modulated by phosphorylation. As CiR from SV40-transformed fibroblasts has drastically reduced contactinhibin-binding ability, impaired CiR function may result in loss of growth control, which may eventually lead to tumorigenesis.

Materials and methods

All buffer chemicals and salts were purchased from Roth (Karlsruhe, Germany); other chemicals and reagents were obtained from Sigma (Deisenhofen, Germany) unless otherwise specified.

Cell culture

FH109 human diploid fibroblasts and Wi38 SV40-transformed fibroblasts were cultured as described [16], except that CG

medium (Vitromex, Vilshofen, Germany) supplemented with 0.5 % fetal calf serum was used instead of DMEM with 10 % fetal calf serum.

Cross-linking experiments

FH109 fibroblasts were seeded onto 15 cm culture dishes at a density of 1.5×10^4 cells cm^{-2}. The next day, medium was exchanged for methionine-free DMEM (Biochrom/Seromed, Berlin, Germany), and after 1 h, 925 kBq [^{35}S]methionine (53 TBq $mmol^{-1}$; Amersham, Braunschweig, Germany) was added for 24 h. Contactinhibin was coupled to derivatized silica beads as described [22] and added to the cells. After another 24 h in the incubator, the cells were washed three times with phosphate buffered saline (PBS) and 10 ml 1 mM 3,3′-dithiobis(propionic acid)-*N*-succinimidester (Pierce, Rockford, USA) in PBS + 10 % DMSO was poured onto the plate. The dish was gently shaken for 30 min and washed with PBS, cells were scraped from the dish with a 'rubber policeman' and lysed with non-reducing SDS sample buffer (50 mM Tris–HCl, pH 6.8, 5 % SDS, 10 % saccharose). Silica beads were collected by centrifugation and washed with non-reducing SDS sample buffer, then covalently bound material was eluted by cleavage of the disulfide bond in the cross-linker with 50 mM dithiothreitol in sample buffer. Proteins in the eluate were precipitated with chloroform/methanol and separated by two-dimensional electrophoresis.

Membrane protein solubilization

The detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was used to solubilize membrane proteins for CiR isolation and aggregation studies; 8 mM CHAPS was found to be the most effective concentration for solubilization of CiR in a binding-competition study using the aggregation assay (data not shown). Cells cultured to confluence were washed three times with PBS, and membrane proteins were solubilized on the culture dish with 8 mM CHAPS in PBS containing protease inhibitors (0.3 μM aprotinin, 0.1 μM soybean trypsin inhibitor, 0.4 mM iodoacetamide, 5 μM pepstatin, 60 μM phenylmethylsulfonylfluoride (PMSF) and 4 μM leupeptin; final concentrations). After incubation at 4 °C for 30 min, the supernatant was centrifuged at 4 °C for 10 min at 3 000 × *g*, to remove cell debris.

Isolation of CiR

Membrane proteins from 100 confluent FH109 fibroblast culture dishes (150 mm diameter) were solubilized with 8 mM CHAPS as described, with PMSF as the protease inhibitor. Proteins were precipitated at 4 °C overnight with four volumes of ice-cold acetone, followed by centrifugation at 1 500 × *g* for 20 min at 4 °C. The pellet was extensively washed with distilled water (40 ml), dissolved in Laemmli sample buffer [23] and heated for 10 min to 96 °C. Proteins were separated by preparative SDS-gel electrophoresis (7.5 % acrylamide gels) and stained with Coomassie blue. The slab gels were sectioned horizontally into eight slab strips and the proteins were electro-eluted using a device from Biometra (Cologne, Germany); 100 μg of each fraction was precipitated by chloroform/methanol according to [24], and the pellet redissolved in 50 μl denaturation buffer (6 M urea, 0.01 M β-mercaptoethanol in 0.01 M Tris–acetate, pH 7.8). The solution was diluted with 10 volumes of renaturation buffer (8 mM CHAPS in PBS, 0.02 % sodium azide) and dialyzed three times against 1 000 volumes of renaturation buffer at 4 °C overnight.

Each fraction was tested for contactinhibin-binding in the aggregation assay. The 80–90 kD fraction was subjected to preparative two-dimensional electrophoresis and resolved into two fractions, which were then rescreened for contactinhibin-binding in the aggregation assay. Only the basic fraction (pI 5.9–6.6) had contactinhibin-binding activity. Purified CiR was obtained by electroelution of the relevant spots. Using the described purification scheme, approximately 2 μg purified CiR was obtained from 100 mg fibroblast proteins solubilized with 8 mM CHAPS, corresponding to approximately 1×10^8 cells.

Native CiR was isolated by immunoaffinity chromatography; 1 mg anti-CiR IgG antibody was coupled to 2 ml cyanogen bromide-activated sepharose (Sigma) in a total volume of 6 ml buffer, according to the manufacturer's instructions. Cells (5×10^6) were extracted with 4 ml CHMN (8 mM CHAPS in 20 mM HEPES, 1 mM $MgCl_2$, 0.5 M NaCl) for 15 min on ice and precleared by 10 min centrifugation at 3 000 × *g*; the resulting supernatant was incubated with the gel slurry at 4 °C overnight. The gel was then packed into a small column and washed with CHMN until no proteins could be traced in the eluate by photometric detection at 280 nm. CiR was eluted with 6 ml 0.1 M diethylamine, pH 11.6; 0.9 ml fractions were collected and immediately neutralized with 0.1 ml 1 M Tris–HCl, pH 7.0. Approximately 4 ng CiR was obtained from 1×10^6 FH109 fibroblasts.

Aggregation assay

Contactinhibin (10 μg) dissolved in 15 μl coupling buffer (0.1 M sodium carbonate, pH 9.0) containing 0.1 % SDS was added to 500 μl suspension of 'Fluoresbrite' polyacrolein microspheres (Polysciences, Eppelheim, Germany; 2.5 % solids; 1 μm diameter) that had been prewashed three times with coupling buffer; 50 μl 0.1 M sodium cyanogen borohydride (Merck, Darmstadt, Germany) solution was added immediately and the suspension was shaken gently overnight. Afterwards, the remaining reactive aldehyde groups were blocked with 0.2 M glycine, pH 8.0, and imino groups were again reduced with sodium cyanogen borohydride overnight. After washing with PBS, microspheres were stored at 4 °C in PBS containing 0.02 % sodium azide. The CiR-containing fractions were coupled covalently to isothiocyanatopropyltriethoxysilane derivatized silica beads (50 μg protein coupled to 100 μl of 1.5 % (w/v) suspension of silica beads) as described [22].

Aggregation was carried out as follows: 50 μl beads attached to fractions to be tested for contactinhibin-binding activity was added to 2 μl contactinhibin microspheres in 400 μl aggregation buffer (8 mM CHAPS in PBS containing 0.1 mg ml^{-1} bovine serum albumin (BSA) and 0.02 % sodium azide). The beads were gently shaken on a rocker plate at room temperature for 30 min and aggregation was assessed by microscopy. Aggregation was scored positive when a silica bead was covered with at least eight microspheres, and was defined as the percentage of positive beads out of 100 total beads. Under control conditions, binding of blank microspheres to silica beads was < 2 %.

Adhesion assay

The contactinhibin-coated microspheres were prepared as described, except that a second coupling step with bovine intestine alkaline phosphatase (2.57 U mg^{-1}) was introduced using the glutaraldehyde method. The microspheres were washed three times with 50 mM sodium phosphate, pH 6.8, and resuspended in 5 μl of this buffer containing 20 μg bovine intestine alkaline phosphatase; 50 μl 1 % glutaraldehyde solution in PBS was added under vigorous stirring. Coupling was stopped after 20 h at 4 °C with 100 μl 1 M ethanolamine,

pH 7.0. After 2 h blocking, microspheres were washed three times with PBS; 100 µl native CiR fractions, isolated with the anti-CiR IgG column, were added to wells of a high-binding-capacity ELISA plate (Nunc, Dreiech, Germany) in triplicate, at several dilutions. After overnight incubation at 4 °C, wells were washed three times and blocked with 3 % BSA in PBS containing 0.02 % sodium azide for 4 h. Each well received 2 µl contactinhibin-coated microsphere suspension. Microspheres were allowed to settle and bind overnight at 4 °C, and non-bound microspheres were removed by carefully washing with blocking buffer four or five times. The wells were then washed with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and filled with 200 µl 10 mM para-nitrophenylphosphate in 1 M diethanolamine–HCl, pH 9.5. Color development was evaluated in an ELISA reader (InterMed, Wiesbaden, Germany) at 405 nm after 24 h.

Anti-CiR antibodies

CiR separated by preparative two-dimensional gel electrophoresis as described was electroblotted onto nitrocellulose, briefly stained with Coomassie brilliant blue, and the spots corresponding to CiR cut out. The nitrocellulose was dissolved in DMSO and antibodies were generated by immunization of white New Zealand rabbits. A total of eight boosts were performed with incomplete Freund's adjuvant at three-week intervals. The titer was determined to be > 20 000 by an ELISA assay with purified CiR adsorbed onto the well surface.

Immunoprecipitation

Cells (5×10^6) were trypsinized, washed with PBS and extracted with 500 µl solubilization buffer (50 mM HEPES–NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM EDTA, 0.4 mM EGTA, 0.5 mM dithiothreitol (DTT), 2.5 mM NaF and 1 % Nonidet P-40 (NP-40), containing the protease inhibitors listed above) for 20 min on ice. Lysates were cleared by centrifugation (12 000 × *g*, 10 min). CiR was immunoprecipitated with rabbit polyclonal anti-CiR antibodies coupled covalently to protein A sepharose, as described [25], for 2 h at room temperature. Immunoprecipitates were washed three times with 500 µl solubilization buffer, once with 500 µl TNL buffer (50 mM Tris–HCl, 0.15 M NaCl, 10 mM EDTA, 0.5 mM LiCl, 0.5 % NP-40, pH 7.4), followed by high and low salt buffer (0.1 or 0.01 M Tris–HCl, pH 7.4) and bound material was eluted with 100 µl 0.1 M diethylamine, pH 11.6. Eluates were precipitated with chloroform/methanol and aliquots loaded onto SDS–polyacrylamide slab gels (7.5 %) or onto isoelectric focusing rod gels.

Treatment of CiR with potato acid phosphatase

CiR immunoprecipitated from 5×10^6 cells in the presence of orthovanadate was resuspended in 20 µl 40 mM PIPES, 1 mM dithiotreitol, 20 µg ml^{-1} aprotinin, 20 µM leupetin, pH 6.0, and treated with 1.2 U potato acid phosphatase at 37 °C for 1 h [26]. Controls were mock-treated without enzyme. After digestion, CiR was assessed in the adhesion assay for contactinhibin-binding activity, or, in the case of ^{32}P-labelled CiR, analyzed by two-dimensional gel electrophoresis, followed by autoradiographic visualization of CiR.

Western immunoblotting

Proteins separated by SDS-gel electrophoresis were electroblotted overnight onto PVDF membranes (Millipore, Germany) and blocked with 3 % BSA in TBS (50 mM Tris–HCl, 0.15 M NaCl, pH 7.5) containing 0.1 % Tween-20 (TTBS) for 1 h. The membrane was incubated with anti-CiR polyclonal antiserum in 1:5 000 dilution in TTBS plus 3 % BSA for 1.5 h, and after three quick and three 10 min washes with TTBS, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG in 1:30 000 dilution for 1.5 h. After a final short washing step in TBS, color was developed with 4.6 mM 4-nitroblue tetrazolium chloride and 4.3 mM 5-bromo-4-chloro-3-indolylphosphate in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$.

The glycan chains of CiR electroblotted onto PVDF after SDS-gel electrophoresis were detected with the Glycan Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions; α-2,3- and α-2,6-linked neuraminic acid was detected with *Maackia amurensis* (MAA) digoxigenated lectin and *Sambucus nigra* (SNA) digoxigenated lectin (both from Boehringer Mannheim, Mannheim, Germany), respectively, with the same kit. Fetuin was used as a positive control in all three cases.

Proliferation assays

The basic assay was as follows: 5×10^3 FH109 fibroblasts were seeded per microtiter well in DMEM containing 0.5 % FCS. After 24 h, reagents were added according to the schemes listed below, and after a further 24 h culture period, cells were given 0.25 µCi [^3H]thymidine (NEN, Dreieich, Germany) and cultured for an additional 4 h. Cultures were processed for measurement of incorporated radioactivity as described [27].

The following schemes were used:

1. Anti-CiR IgG antibody was coupled to silica beads as described [22]; 100 µl bead suspension in DMEM containing 5 % FCS was added to wells.
2. Anti-CiR IgG was added to a final concentration of 25 µg ml^{-1}, and after 30 min, goat anti-rabbit IgG was added to a final concentration of 80 µg ml^{-1} in DMEM plus 5 % FCS.
3. Microtiter wells were precoated overnight at 4 °C with 50 µl per well 1 mg ml^{-1} anti-CiR IgG before seeding fibroblasts in CG medium containing 5 % FCS. After 24 h culture, cells were given 0.25 µCi [^3H]thymidine (NEN, Dreieich, Germany) and cultured for an additional 4 h.
4. Anti-CiR IgG was introduced into cells with N-1-(2,3-dioleoyloxy)propyl-N,N,N-trimethyl-ammonium methylsulfate (DOTAP, Boehringer Mannheim, Germany) as follows. FH109 fibroblasts were trypsinized and washed as described [28]; 15 µl DOTAP (1 mg ml^{-1} in distilled water) and 35 µl 20 mM HEPES, pH 7.4, were mixed with 200 µl IgG solution (1 mg ml^{-1} in PBS) for 15 min at room temperature and added to 1×10^6 fibroblasts in 1 ml CG medium containing 0.5 % FCS. After 15 min in a 37 °C water bath, 9 ml CG medium containing 5 % FCS was added and cells were collected by centrifugation for 10 min at 800 × *g*. Cells were resuspended in 1 ml CG medium (0.5 % FCS), seeded into microtiter wells and proliferation determined as described.
5. Glutaraldehyde-fixed cells were prepared as described in [9] and added in CG plus 5 % FCS to the cells 24 h after seeding. Proliferation was determined as described.

Metabolic phosphate labelling and phosphoamino-acid analysis

Fibroblasts (1×10^6) were seeded onto 9 cm petri dishes in CG medium containing 5 % FCS and cultured for 3 days. Cells were washed three times with phosphate-free medium (110 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 30 mM $NaHCO_3$, 23 mM D-glucose, 15 mM HEPES, 1 mg ml^{-1} BSA) and starved for 3 h; 300 µCi [^{32}P]orthophosphate (338 TBq $mmol^{-1}$, NEN) was added per ml medium. After 2 h, cells were washed four times with phosphate-free

medium and lysed in 1 ml solubilization buffer, followed by CiR immunoprecipitation as described above; the last two washing steps were omitted. Probes were separated by two-dimensional gel electrophoresis, the gels dried and autoradiographed for 1 week. The relevant spots were cut out, subjected to protein hydrolysis and analyzed with a Hunter Thin Layer Electrophoresis System (C.B.S. Scientific Company, Del Mar, USA) according to the manufacturer's instructions. Fluorographs were developed after 20 days.

Preparation of contactinhibin glycopeptides

Contactinhibin (5 μg) dissolved in 50 μl TC buffer (0.1 M Tris–HCl, pH 8.0, 1 mM $CaCl_2$) was digested with 1 mg pronase that had previously been heated for 30 min to 65 °C. Digestion was performed for 72 h at 37 °C with addition of fresh pronase (1 mg) every 24 h. The enzyme was precipitated by addition of ethanol (9:1, v/v) overnight at –20 °C, followed by centrifugation. The supernatant was vacuum-dried, dissolved in 50 μl coupling buffer and, after buffer exchange against coupling buffer over a P2 column (Biorad, Munich, Germany) to remove residual Tris molecules, glycopeptides were coupled to microspheres as described.

Treatment of contactinhibin-coated microspheres with β-galactosidase

Contactinhibin-coated microspheres (5 μl) were washed twice with β-GI-buffer (0.1 M citric acid, 0.1 M NaHPO, 10 % glycerol, 0.1 % Triton X-100, pH 4.3) and incubated in 100 μl β-GI-buffer containing 0.5 U β-galactosidase from bovine testis (Boehringer Mannheim, Mannheim, Germany) at 37 °C for 16 h. Control beads were treated the same way but without enzyme.

Anti-contactinhibin-antibodies

Antibodies were generated as described [8].

Protein quantification

Protein quantification was performed according to [29], with bovine serum albumin as a standard.

Gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was performed as described [30].

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