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Head activator acts as an autocrine growth factor for NH15-CA2 cells in the G_2/mitosis transition

H.Chica Schaller, Silke Druffel-Augustin and Stefan Dübel

Zentrum für Molekulare Biologie, Im Neuenheimer Feld 282, 6900 Heidelberg, FRG

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The neuropeptide head activator (HA) acts as an autocrine growth factor for the neural cell line NH15-CA2. Cell proliferation is increased in the presence of HA and inhibited by HA peptide-specific antisera. Stimulation of cellular proliferation is visible 2 h after HA application as an increase in cells in mitosis. HA has no direct effect on stimulating DNA synthesis. HA thus functions as a control signal in the G_2/mitosis transition and not in the G_1/S transition. Receptors for HA are present on small round cells in clusters of foci and not on cells with differentiated morphology, suggesting cell-cycle-dependent HA receptor expression.

Key words: autocrine growth factor/cell cycle control/G_2 signal/head activator/mitogen

Introduction

Cell proliferation can be regulated at two control points in the cell cycle, before DNA synthesis and before mitosis. Most of the growth factors described so far act on cells arrested in G_0 stimulating them to re-enter the cell cycle, start a new round of DNA replication and finally divide. Recently, a number of publications on cell cycle mutants and on proteins involved in cell-cycle control, like cyclin, have made it very likely that in addition to factors acting at the G_1/S border, others may exist which control the G_2/mitosis transition (Fantes, 1988; Lee and Nurse, 1988; Murray, 1988). For the blood cell cycle multiple points of control were also described (Melchers and Lernhardt, 1985). In lower organisms like hydra and early in development, where the G_2 phase very often represents the only resting or variable phase of the cell cycle, this second type of control in the G_2/mitosis transition is predominant (David and Campbell, 1972; Edgar and O'Farrell, 1989).

Several factors have been characterized from hydra that influence cell proliferation. Head activator (HA) and foot activator stimulate cell proliferation; head inhibitor inhibits cell proliferation. All three factors act in the G_2/mitosis transition (Schaller and Bodenmüller, 1985; Hoffmeister and Schaller, 1987; Hoffmeister, 1989). HA was originally discovered in hydra, but was later found to occur in identical sequence in other animals, including mammals (Bodenmüller and Schaller, 1981). Its presence early in the development of mammalian brain and intestine and the high levels found in brain and endocrine tumours (Schaller *et al.*, 1988) hinted at a possible growth control function of HA for mammalian cells. We show in this paper that HA not only acts in hydra as a mitogen in the G_2/mitosis transition but also in mammals, as evidenced by its effect on the neural cell line NH15-CA2.

Results

Production and release of HA from NH15-CA2 cells

Several neural cell lines were screened for their content of HA. Table I shows that the neuroblastoma-glioma double hybrid cell line NH15-CA2 (Heumann *et al.*, 1979) is especially rich in HA. Immunocytochemical preparations of NH15-CA2 cells react with monoclonal antibodies specific for the HA peptide (Figure 1a) and with monoclonal antibodies specific for the HA precursor (Figure 1b), which indicates that HA is produced by the cells.

NH15-CA2 cells were routinely grown in medium containing 10% fetal calf serum. Since fetal calf serum is rich in HA we tried to culture the cells in serum-free, defined medium in which the serum was replaced by insulin, transferrin and some other minor components (Bottenstein and Sato, 1979). NH15-CA2 cells could grow in such medium, but growth was dependent on cell density. Table II shows that cell proliferation increased with cell density hinting at release of autocrine growth promoting factor(s) from NH15-CA2 cells into the medium.

Such conditioned media were assayed for the content of HA. We found that NH15-CA2 cells release HA into the medium. Medium conditioned for 2 days by cells at sub-confluency density contained up to nanomolar concentrations of HA. This HA was, as in hydra, released bound to a carrier complex (Roberge, 1985; Schaller *et al.*, 1986) from which the HA peptide could be liberated by methanol or salt extraction. This HA peptide eluted from HPLC C_{18} columns at the same position as tritiated synthetic HA (Figure 2), reacted with all the HA antibodies that HA did, and had the same amino acid composition as HA. We take this as evidence that HA released from NH15-CA2 cells and synthetic HA are identical.

Action of HA as mitogen in the G_2/mitosis transition of NH15-CA2 cells

To study a possible effect of HA as growth factor, NH15-CA2 cells were cultured at low density in defined medium in the presence and absence of 10^{-8} M HA.

Table I. Content of HA in neural cell lines

Cell line	Cell type	Animal	Content of HA (fmol/mg protein)
$C_6$12	glial	rat	16
N_2A	neuronal	mouse	20
108 CC5	neural	rat/mouse hybrid	250
108 CC15	neural	rat/mouse hybrid	200
NH15-CA2	neural	rat/mouse hybrid	800

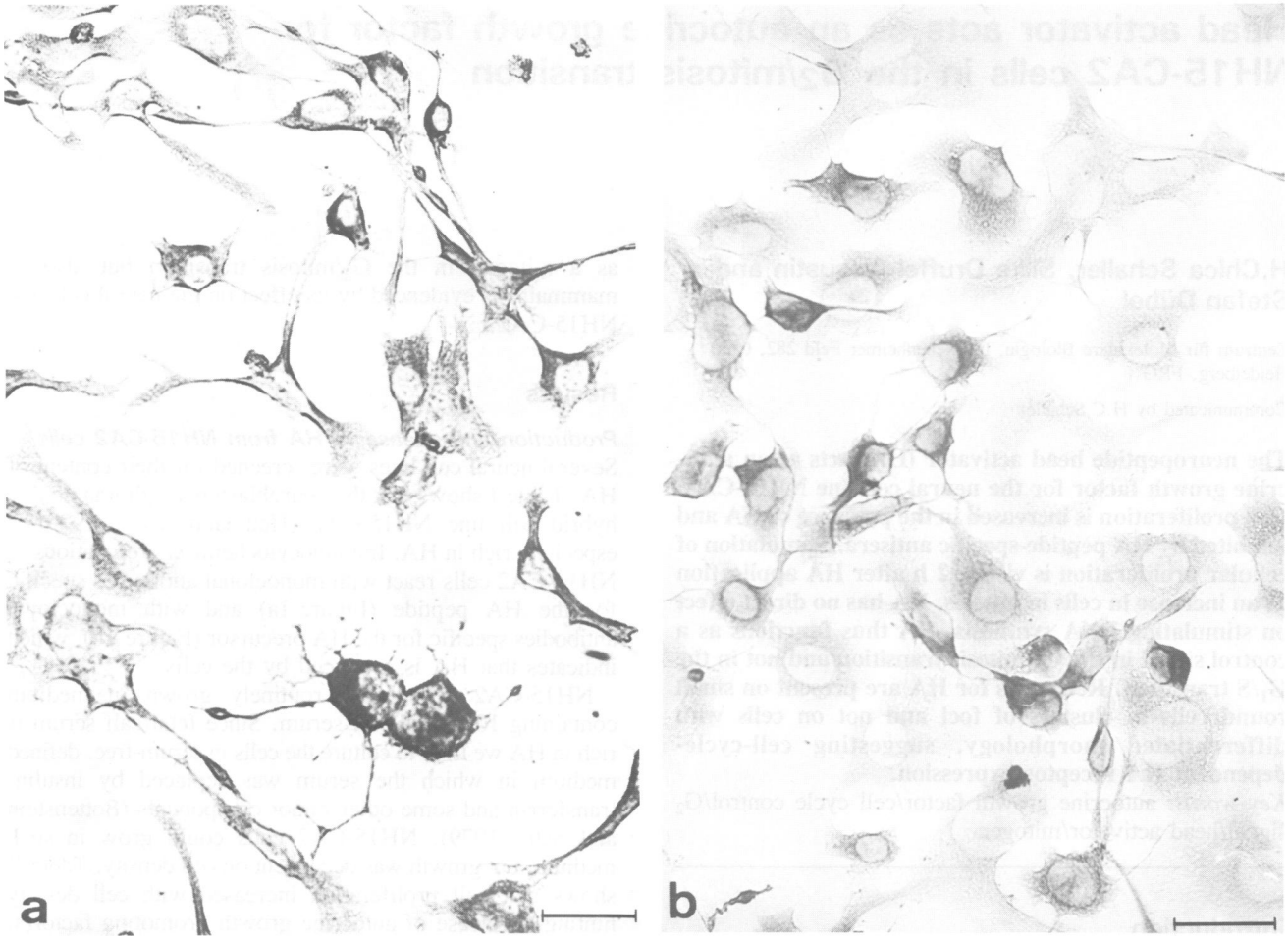


Fig. 1. Localization of HA peptide and HA precursor immunoreactivity in NH15-CA2 cells. (**a**) Cells were fixed with 1% ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 80% methanol and reacted with the monoclonal HA peptide specific antibody F5 (1:10). (**b**) Cells were fixed with 4% formaldehyde and reacted with the monoclonal HA precursor specific antibody B74 (1:10). The HA peptide remains freely diffusible under such fixation conditions (Schawaller *et al.*, 1988). The second antibody was labelled with peroxidase. Scale bars, 50 μm.

Figure 3a shows that after a lag phase of 2−3 days, cells in the control cultures gradually started to proliferate. Cells treated with HA started to proliferate earlier and at a faster rate leading to a higher density of cells in HA-treated (Figure 3b) over control cultures (Figure 3c). Small clusters of cells and foci formed more frequently in HA treated than in control cultures (Figure 4).

Proliferation of cells in control cultures could be inhibited by incubation with HA peptide-specific antisera. Antisera like 189/6 (Figure 5) which contain antibodies recognizing internal and carboxy-terminal HA epitopes were efficient as growth inhibitors. Antisera (like 12/5) requiring a freely accessible amino terminus, which is hidden in the carrier-bound HA (Schaller *et al.*, 1986), were less efficient (data not shown). Incubation of cells with pre-immune serum had no measurable effect on cell proliferation. We take this as evidence that HA released from NH15-CA2 cells is inactivated by binding to HA antibodies and is no longer available as autocrine growth factor.

To investigate where in the cell cycle HA acts, cells were incubated for variable periods of time in HA and separately assayed for mitotic activity and for DNA synthesis. Figure 6 shows that 2 h after adding 10^{-8} M HA to NH15-CA2 cells a significant increase in the number of cells in mitosis was observed. These cells had been plated onto slides in defined medium the previous day. Analysis of the DNA content before HA addition showed that ~20% of all the cells were in G_2 (Figure 7). Treatment of cells with HA led to an increase of cells in mitosis from 1% in the control to 3% in the presence of HA and this over a 3-h period. Since only cells in metaphase plate stage were counted, and under the assumption that this phase does not last longer than half an hour, almost all cells in G_2 were triggered by HA to enter mitosis.

HA had no direct effect on stimulating DNA synthesis, since no increase in [^{3}H]thymidine incorporation was measurable during the first 20 h after HA application. The

Table II. Dependence of cell proliferation on cell density

Starting cell density ($\times 10^3$)	Cell density 2 days later ($\times 10^3$)	Increase (fold)
49 ± 4	123 ± 27	2.5
153 ± 14	528 ± 46	3.5
305 ± 26	1340 ± 225	4.3
882 ± 123	4680 ± 844	5.3

The starting cell density was determined 1 day after transfer of cells from FCS containing medium into defined medium. Three flasks each were harvested and the total cell number determined in a Neubauer cell chamber.

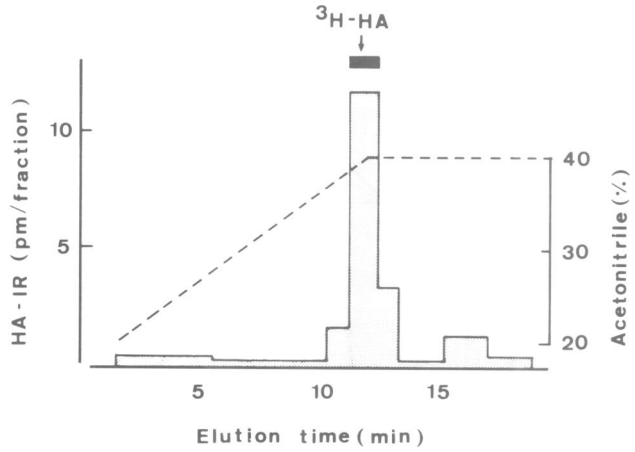


Fig. 2. HPLC analysis of HA extracted from NH15-CA2 cell conditioned medium. HA was extracted by addition of 9 vol of methanol and purified over Seppak C_{18} cartridges. The 80% methanol eluate was applied to a LiChrosorb C_{18} column (250 × 4 mm) and eluted using a 10-mm linear gradient of 20–40% acetonitrile in 0.1% trifluoracetic acid at a flow rate of 1 ml/min. HA content was determined by RIA. Tritiated HA was applied in a separate run to determine the position of synthetic HA (bar).

fast effect on mitosis suggests that HA acts as signal in the G_2/mitosis transition. The lack of effect on DNA synthesis indicates that HA has no effect on the G_1/S transition.

Receptors for HA on NH15-CA2 cells

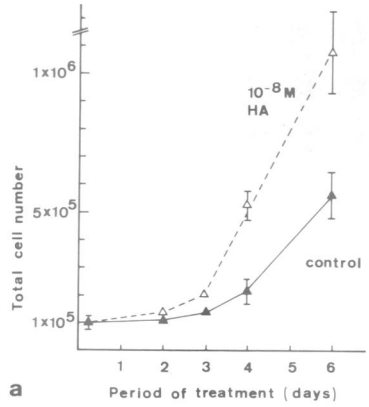
HA does not contain an iodinatable tyrosine. For binding studies we constructed a HA bipeptide ($N^{\epsilon 7}$-suberato-$n^{\epsilon 7}$[Tyr^{11}]HAyl-HA) which consisted of one normal HA cross-linked over the ε-amino group of lysine at position 7 by a C_8 spacer to a second HA in which the phenylalanine at position 11 was exchanged by tyrosine:

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe

C_8

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Tyr

This HA bipeptide was biologically active and was used to monitor binding to NH15-CA2 cells. The iodine-labelled HA bipeptide preferentially bound to small round cells and less efficiently to differentiated cells (Figure 8a and b). This binding was specific and did not occur in the presence of excess unlabelled HA bipeptide (Figure 8c and d). Some



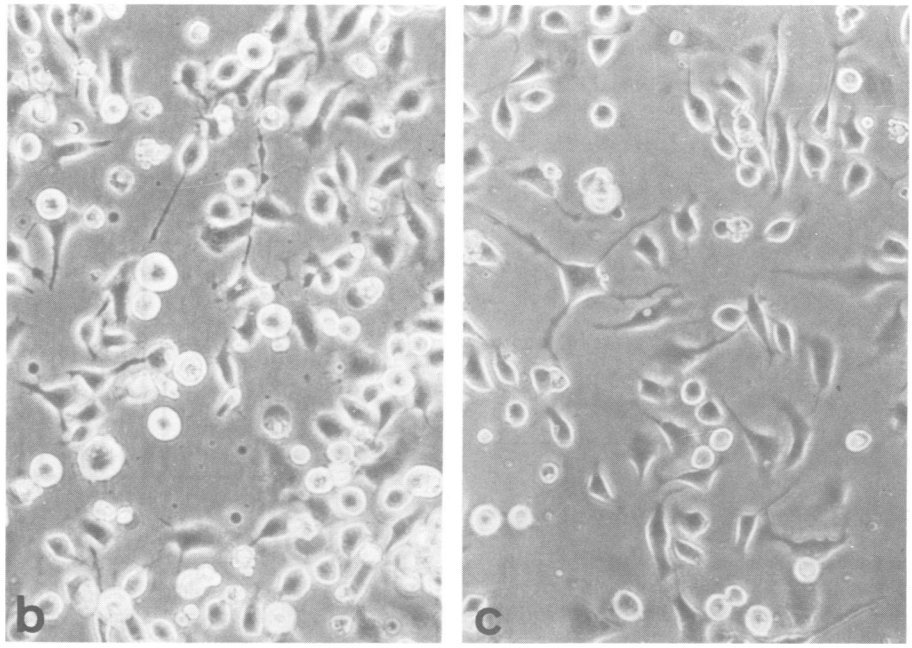


Fig. 3. Effect of HA on the proliferation of NH15-CA2 cells. (**a**) Cells were transferred from serum-containing medium to defined medium. 10^5 cells were seeded per flask containing 7 ml of medium with or without 10^{-8} M HA. HA was monomerized by mixing a 10^{-3} M HA solution with an equal volume of 2 M $(NH_4)_2SO_4$ prior to use to ensure biological activity (Bodenmüller *et al.*, 1986). The medium was renewed every second day. For each time point cells from three flasks each were harvested and counted. (**b**) Cell density in HA treated flask and (**c**) in control flask at day 4.

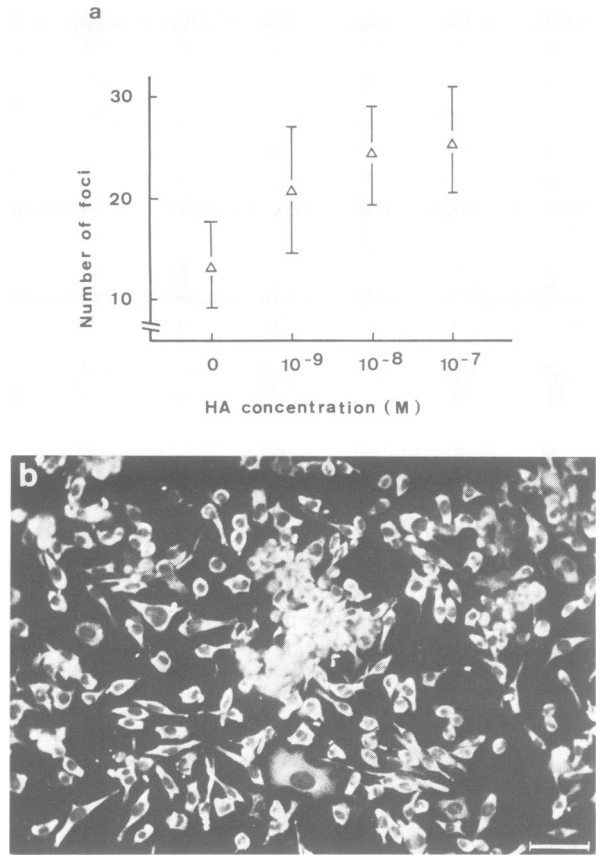


Fig. 4. Effect of HA on foci formation in NH15-CA2 cells. (**a**) For the different HA concentrations three flasks each were screened 3 days after the initiation of the experiment for occurrence of foci per 10 cm^2. (**b**) Demonstration of foci formation in cells after fixation with 1% carbodiimide in 80% methanol and reaction with the monoclonal HA antibody B74 (1:100). The second antibody was FITC labelled. Scale bar, 100 μm.

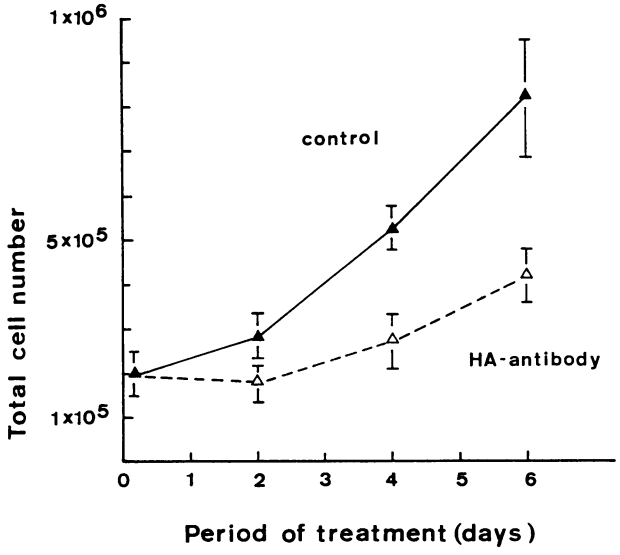


Fig. 5. Effect of HA antiserum on proliferation of NH15-CA2 cells. Cells were seeded at a density of 2 × 10^5/flask containing 7 ml of defined medium with and without the HA antiserum 189/6 at a dilution of $1:10^3$. For each time point cells from three flasks each were harvested and counted.

cells seemed to be in cell division (Figure 8e and f). To confirm this, cells were incubated for 1 h with HA (10^{-8} M), fixed and reacted with HA antiserum, and the

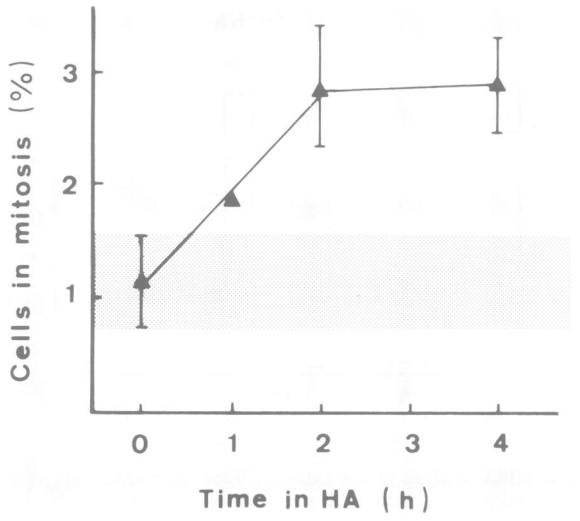


Fig. 6. Action of HA as mitogen in the G_2/mitosis transition of NH15-CA2 cells. Cells were grown overnight in defined medium, and incubated for various periods of time with and without monomerized HA (10^{-8} M). At the times indicated control cells and HA treated cells were fixed wtih 4% formaldehyde, stained with Hoechst 33258, and the mitotic index was determined. For each time point, 3 × 2000 cells were counted.

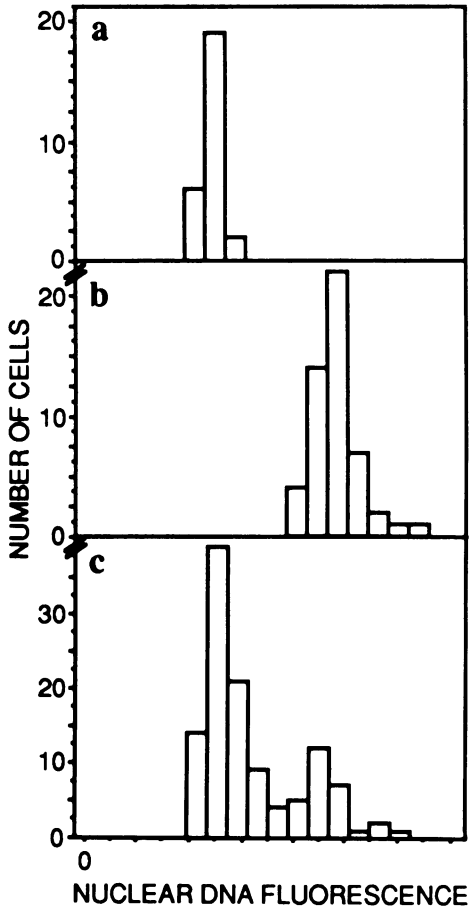


Fig. 7. Quantitative microfluorometric determination of nuclear DNA of NH15-CA2 cells grown overnight in defined medium. The nuclear DNA content was determined after staining the cells with Hoechst 33258 (Cowell and Franks, 1980). Cells in telophase (**a**) were used as standard for a G_1 DNA content and cells in metaphase (**b**) for a G_2 DNA content. The content of randomly chosen interphase cells is shown in (**c**). A few cells (<2%) had larger than G_2 DNA contents and were neglected.

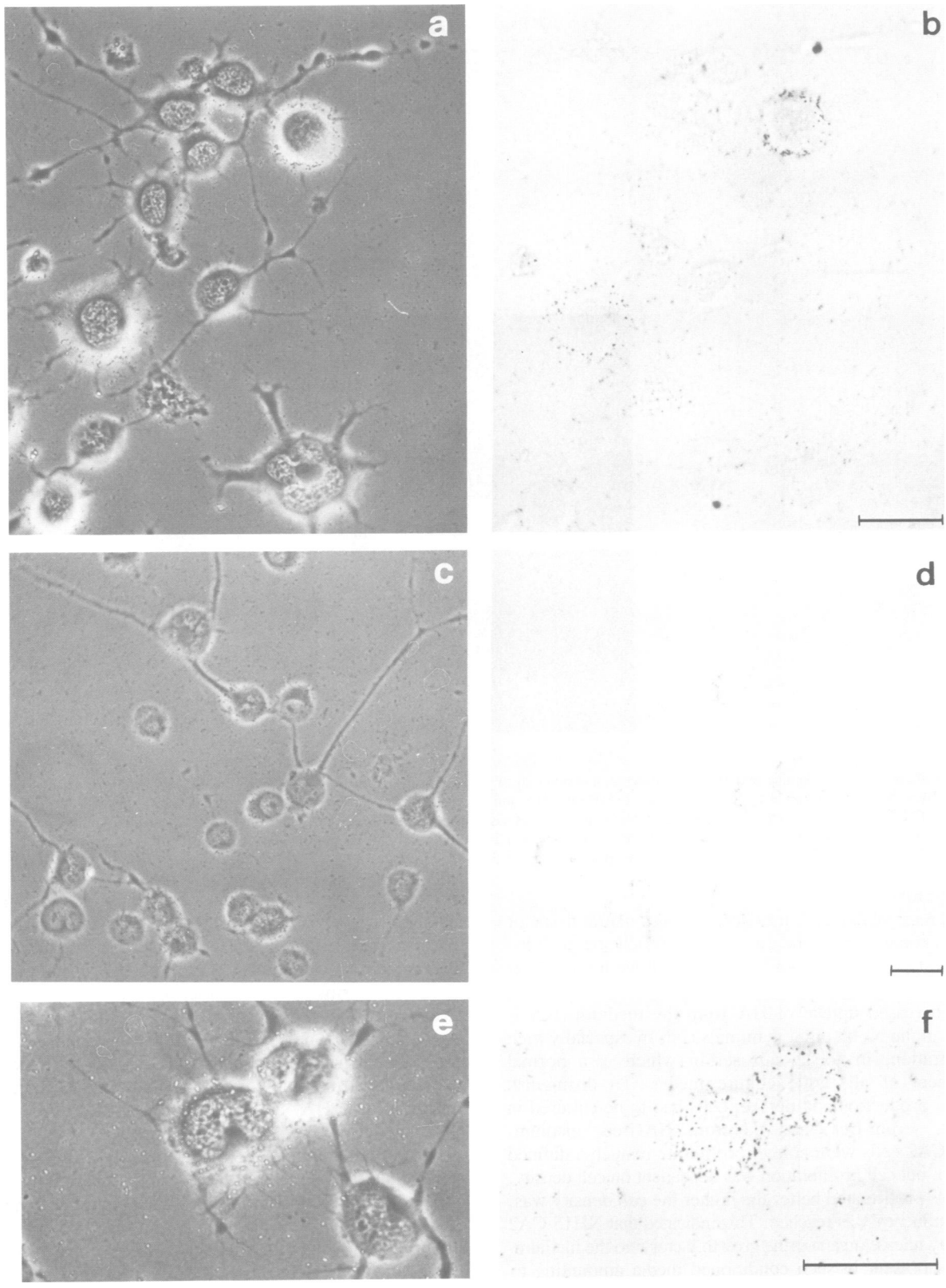


Fig. 8. Localization of HA receptors on NH15-CA2 cells. Cells were incubated for 1 h at room temperature with a ^{125}I-labelled HA bipeptide (10^{-9} M) in the absence (**a,b,e,f**) and as control in the presence of a 1000-fold excess of unlabelled HA bipeptide (**c,d**). Left column, phase-contrast optics; right-column, bright field optics; scale bars, 50 μm.

DNA stained with Hoechst 33258. Cells in clusters were more frequently in mitosis and more intensely stained with the HA antiserum (Figure 9a and b) than single, differentiated cells. HA-responsive cells could be visualized directly by using, instead of HA, a HA anti-idiotypic antiserum as ligand. Binding of such HA anti-idiotypic antibodies to cells was specific as evidenced by inhibition of binding in the presence of excess HA bipeptide (Figure 10). HA anti-iodiotypic antibodies bound like HA preferentially to cells in foci (Figure 10a) and to cells in mitosis (Figure 9c–e).

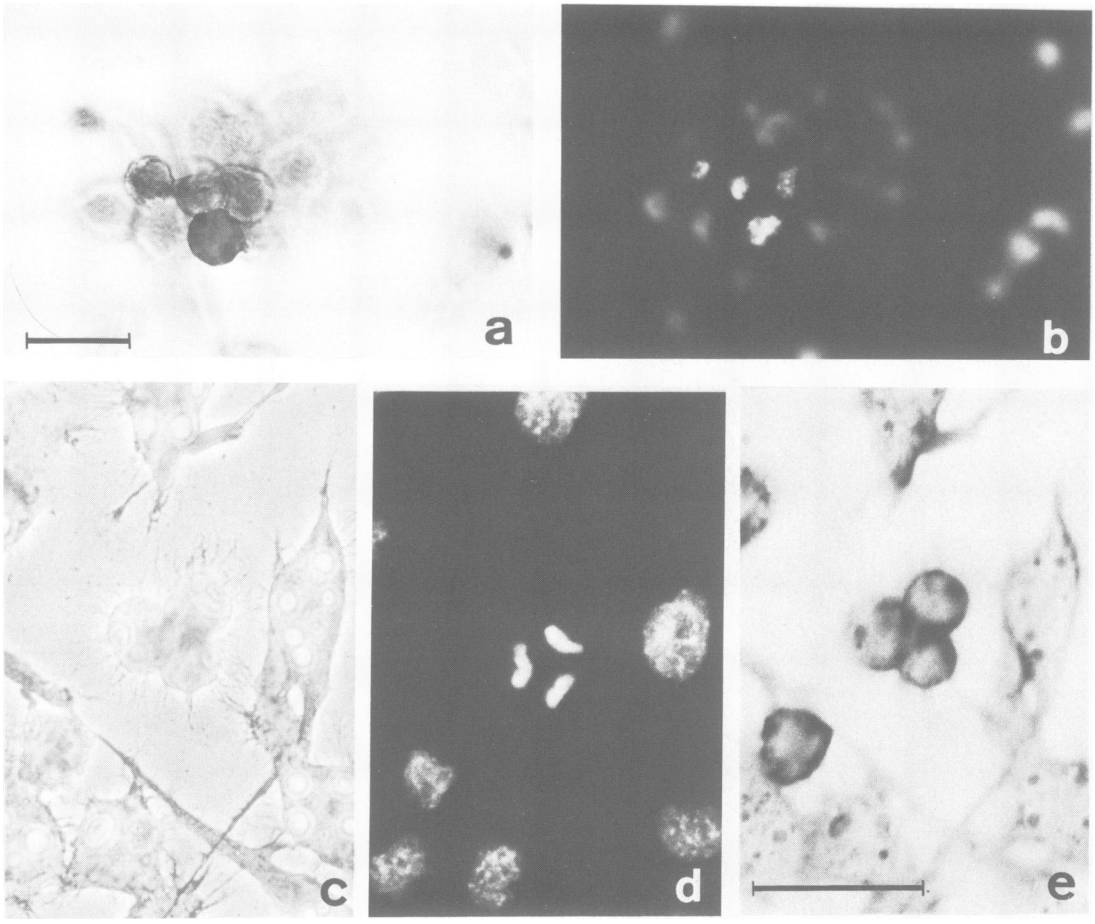


Fig. 9. Localization of HA peptide and HA anti-idiotypic antibodies in or on NH15-CA2 cells in mitosis. NH15-CA2 cells were incubated for 60 min (**a,b**) with monomerized HA (10^{-8} M) or (**c–e**) with the HA anti-idiotypic antibody 64/10 (1:100), washed 3 × 10 min with medium containing FCS, and fixed with 4% formaldehyde for 60 min. HA was visualized in (**a**) by reaction with the HA peptide specific antibody 12/5 (1:500) by incubation overnight at 4°C. The second antibody was labelled in each case with alkaline phosphatase. (**c**) Phase-contrast optics, (**a,e**) bright-field optics, (**b,d**) staining of DNA with the Hoechst reagent 33258. Scale bars, 50 μm.

Discussion

Several mammalian cell lines derived from neural tissue or tumours contain HA. One especially HA-rich source is the neuroblastoma–glioma double hybrid cell line NH15-CA2. A high HA content may be caused by increased synthesis or by increased uptake of HA from the medium. HA is present in the blood of all mammals and, in especially high concentration, in foetal calf serum which is a normal component of all tissue culture media. To distinguish between production and uptake, cells had to be cultured in defined, serum-free, and therefore HA-free, medium. NH15-CA2 cells were able to proliferate in such a defined medium, but cell proliferation was dependent on cell density. The cells proliferated better the higher the cell density was, until confluency was reached. This indicated that NH15-CA2 cells may release an autocrine growth factor into the medium. HA was present in such conditioned media amounting to 10^{-9} M at high cell density. NH15-CA2 cells from defined media were immunoreactive both with HA-peptide and HA-precursor-specific monoclonal antibodies, confirming the notion that they are indeed able to produce and release HA. Chemical and immunological analysis showed identity of the released HA with synthetic HA.

The increase in concentration of HA in media of cells with high proliferation rates hinted at a possible function of HA as autocrine growth factor. This was supported by the finding that addition of HA (10^{-8} M) to cells plated at low density led to a stimulation of cell proliferation detectable as an increase in total cell number, faster increase in cell density, and earlier formation of cell foci. Cell proliferation could be inhibited by antisera against HA. Since this was observed in cultures, where no HA was added, the HA antibodies must have reacted with endogenous, NH15-CA2 cell-produced HA leading to inhibition of cell proliferation. From this we conclude that HA is an autocrine growth factor for NH15-CA2 cells.

Most of the mammalian growth factors, be they proteins or peptides, act on cells in the G_0 or G_1 phase of the cell cycle before transition into the S phase (Figure 11). Recent discovery of cell cycle mutants and the isolation of genes and proteins involved in cell cycle control (Lee and Nurse, 1988; Murray and Kirschner, 1989) and earlier experiments with blood cells (Melchers and Lenhardt, 1985) suggest that mammalian cell cycles may be under multiple control, one control point being located in G_0 or at the G_1/S border, the other at the G_2/M border. In hydra, HA acts as a growth factor at the G_2/M border. It was, therefore, not surprising for us that application of HA to NH15-CA2 cells after 2 h led to a significant increase in cells in mitosis. This means that HA must have acted on cells in G_2 inducing them to

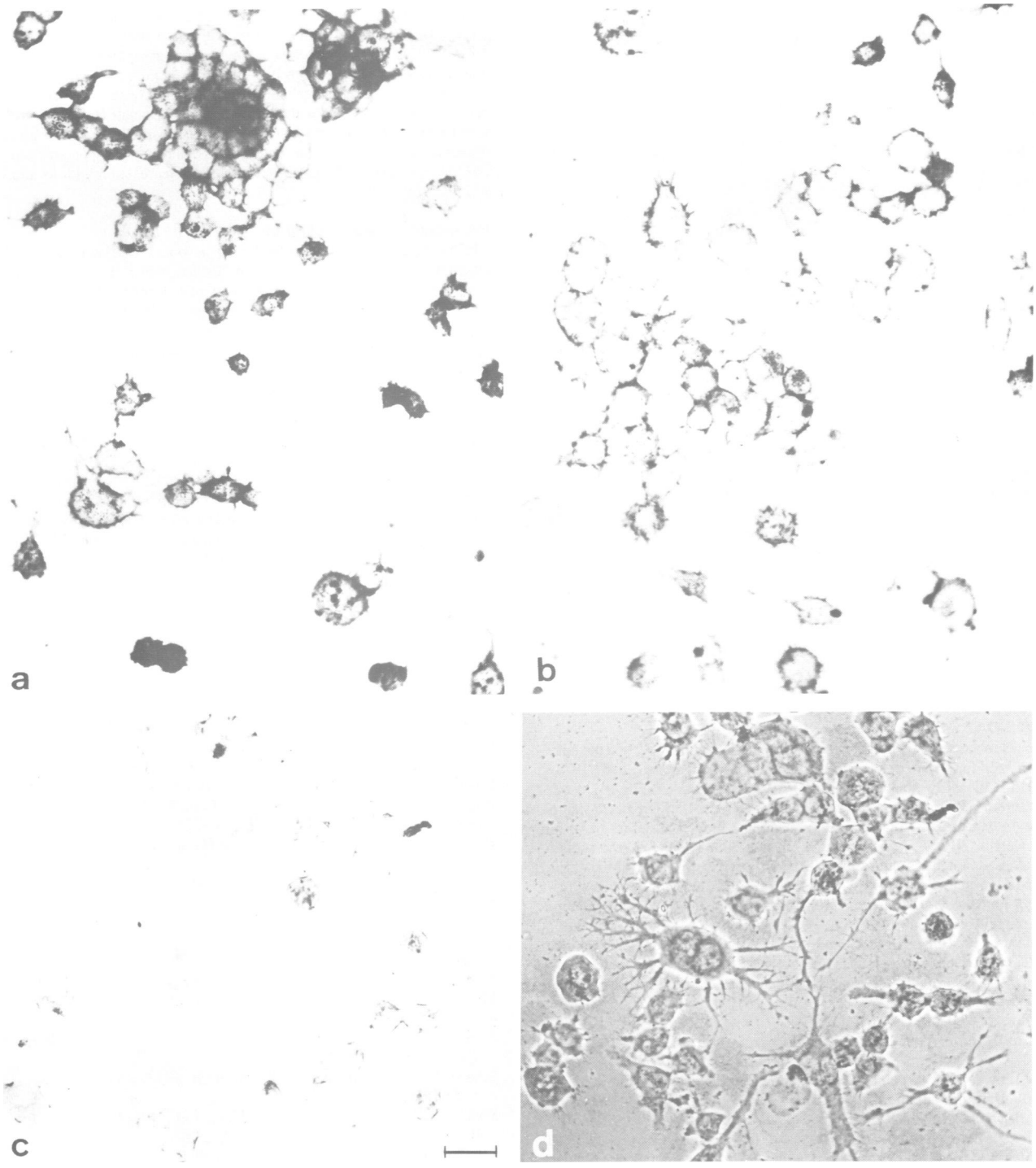


Fig. 10. Specificity of binding of HA anti-idiotypic antibodies to NH15-CA2 cells. NH15-CA2 cells were incubated for 30 min at room temperature with the HA anti-idiotypic antiserum 64/10 (1:100) in the absence of competing HA bipeptide (**a**), or in the presence of 10^{-7} M (**b**) and 10^{-6} M (**c**) HA bipeptide. (**a**–**c**) Bright-field optics, (**d**) phase-contrast to (c); scale bar, 50 μm.

undergo mitosis. HA did not stimulate incorporation of [^{3}H]thymidine into DNA, at least not during the first 20 h after HA application, suggesting that it did not act at the G_1/S boundary. This may be supported by the finding that receptors for HA were abundant on small round cells, in particular on those in foci, suggesting that HA receptor expression is cell cycle regulated and probably restricted to cells ready for cell division.

Materials and methods

Peptides

Synthetic HA was from BACHEM, Switzerland. Tyr^{11}-HA was synthesized by R.Pipkorn, ZMBH. The HA bipeptide, $N^{\epsilon 7}$-suberato-$N^{\epsilon 7}$-[Tyr^{11}]HAyl-HA, in which a Tyr^{11}-HA was crosslinked to a normal HA over the ε-amino group of the lysine at position 7 by disuccinimidyl suberate (DSS; Pierce) was synthesized by P.Muhn, ZMBH. This HA bipeptide is biologically active in aqueous solution, whereas HA is not. HA forms a stable, inactive dimer which has to be monomerized for biological

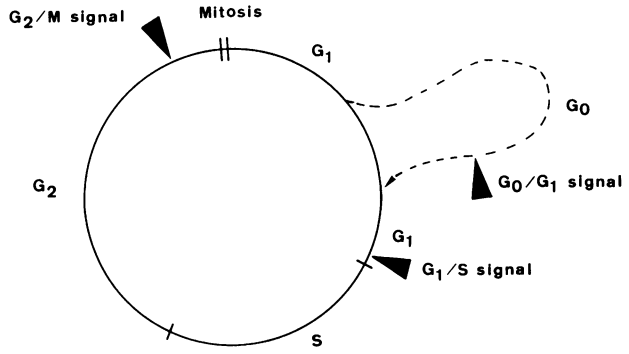


Fig. 11. Control points in the cell cycle. Arrows indicate attack points for factors acting as signals for cell proliferation.

activity. Monomerization is achieved by mixing a 10^{-4}M HA solution with an equal volume of 2 M $(NH_4)_2SO_4$ prior to use (Bodenmüller *et al.*, 1986).

HA antisera

The polyclonal antiserum 12/5 and the monoclonal antibody F5 were produced against a HA conjugate which was coupled over the carboxy terminus to the carrier. The monoclonal antibody E21 was produced against a synthetic HA precursor. The polyclonal antiserum 189/6 was produced against HA coupled over the amino terminus to the carrier. The monoclonal antibody B74 was produced against a mixture of HA conjugates, but was very similar in its properties to E21. The characterization of these antibodies is described in Schaller *et al.* (1984) and in Schawaller *et al.* (1988).

The HA anti-idiotypic antiserum 64 was produced against the monoclonal IgM HA antibody B74. It was purified by absorption to protein A sepharose, and anti IgM-specific antibodies were removed by absorption to IgM sepharose.

NH15-CA2 cell culture

The neuroblastoma–glioma double hybrid cell line NH15-CA2 and the other hybrid cell lines were obtained form B.Hamprecht (Heumann *et al.*, 1979). NH15-CA2 cells were propagated in DMEM medium containing 10% foetal calf serum (FCS). For experiments with HA they were transferred to a serum-free (= HA free), defined medium containing 5 μg/ml insulin, 30 μg/ml transferrin, 20 μM ethanolamine, 30 nM sodium selenite, 1 μM sodium pyruvate, 1% non-essential amino acids, 1 mM glutamine, 10 mM HEPES, 125 IU/ml penicillin, 125 μg/ml streptomycin in DMEM.

For immunocytochemistry, autoradiography and determination of mitotic index, cells were harvested from FCS-containing medium and plated at a density of 10^5 cells/ml onto 8-well multitest slides (Flow Lab.) in defined medium to which, for faster attachment of cells, 1 μM dexamethasone (Yeats *et al.*, 1983) was added. The cells were cultured overnight before starting an experiment.

HA extraction from cells or media

Cells were homogenized by ultrasonication in distilled water. An aliquot was removed for protein determination using the microversion of the Bradford method (Bradford, 1976). HA was extracted from cell homogenates or cell media by adding 9 vol of methanol and centrifuging at 1000 *g* for 10 min. After evaporation of the methanol the supernatants were absorbed to Seppak C_{18} cartridges, washed with water and 20% methanol, and HA eluted with 80% methanol. HA content was determined by RIA using the polyclonal antiserum 12/5 (1:20 000) and [^{125}I]Tyr^{11}-HA (5000 c.p.m./tube) as tracer in 500 μl of 0.1% bovine serum albumin (stripped from contaminating HA by prior treatment with 100% methanol) in phosphate-buffered saline (PBS). After incubation overnight at 4°C separation of free from bound HA was achieved by adding 200 μl of 1% bovine globulin in PBS and 1.5 ml of 20% polyethyleneglycol in 0.03% Triton X-100 in PBS and centrifuging at 2000 *g* at 4°C for 30 min. The pellets were monitored for their content of radioactivity.

Immunostaining

Cells were fixed for 30–60 min at room temperature with 4% paraformaldehyde in PBS or with freshly prepared 1% ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 80% methanol. Superfluous fixative was inactivated with 0.1 M glycine in PBS. Before addition of the first antibody, slides were washed thoroughly with wash buffer (0.05% Triton X-100 in PBS), and incubated with incubation buffer (1% BSA, 0.05% Triton in PBS) at room temperature for at least 1 h. Antisera were diluted with incubation buffer, applied to the slides, and incubated at 4°C overnight. The slides were washed three times for 10 min with wash buffer and saturated for 1 h with incubation buffer before addition of the appropriate second antibody, which was incubated for 4–6 h at room temperature or overnight at 4°C. The slides were washed three times for 10 min with wash buffer, and viewed either directly, if labelled with fluorescein isothiocyanate (FITC), or treated with the respective colour substrate, if labelled with peroxidase or alkaline phosphatase. To inactivate endogenous alkaline phosphatase activity NH15-CA2 cells were treated before the colour reaction with levamisole (0.24 mg/ml).

HA receptor autoradiography

NH15-CA2 cells were incubuated for 1 h at room temperature with the HA bipeptide (10^{-9} M), which had been labelled with ^{125}I by the chloramin T method. As control a 1000-fold excess of unlabelled HA bipeptide was added. The cells were washed three times with defined medium, fixed with 1% carbodiimide in 80% methanol, washed three times with 0.1% Triton X-100 in PBS, once with distilled water, and after drying coated with NTB2 nuclear track emulsion (Kodak). The slides were developed after 5–15 days of exposure at 4°C.

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