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A 50 kDa protein present in conditioned medium of COLO-16 cells stimulates cell spreading and motility, and activates tyrosine phosphorylation of Neu/HER-2, in human SK-BR-3 mammary cancer cells

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SUMMARY

A factor present in conditioned medium of COLO-16 human cancer cells causes fast spreading, fast plasma membrane ruffling, cell shape change, net translocation, stimulation of chemotaxis and growth arrest in human SK-BR-3 mammary cancer cells. Based on the spreading effect, the factor was purified to homogeneity and migrated as a 50 kDa protein in SDS-polyacrylamide gel electrophoresis. Addition of the purified 50 kDa factor to the target cells in culture results in tyrosine phosphorylation of the p185^{erbB2} receptor concomitant with a fast redistribution and clustering of the receptor. The 50 kDa factor is also specifically retained by affinity chromatography on the immobilized extracellular domain of p185^{erbB2}. Antibodies directed against this domain also inhibit the induction of motility. These data suggest that the 50 kDa factor is a putative ligand of p185^{erbB2} in SK-BR-3 cells. Biochemical and immunological evidence further indicate that this factor differs from p185^{erbB2} ligands described so far. Its activity could play a role in the pathogenesis of breast cancer.

Key words: *Neu*/HER-2, tyrosine phosphorylation, spreading factor, motility

INTRODUCTION

Motility factors and their receptors participate in a variety of physiological events such as embryogenesis, wound healing and leukocyte traffic, as well as in pathological events such as tumor invasion and metastasis (Stoker and Gherardi, 1991; Mareel et al., 1990). Some polypeptide ligands function as motility factors for one type of cells and as growth factors for other cells or as both for the same type of cell, in line with the multifunctional character of peptide ligands (Sporn and Roberts, 1988; Stoker and Gherardi, 1991).

In a search for new motility factors secreted in various culture media, we detected growth-inhibitory and motility-stimulatory activities in conditioned medium of COLO-16 cells. These effects were seen with some but not all types of cultured human breast cancer cells and SK-BR-3 cells were found to be preferred target cells.

The human squamous carcinoma cell line COLO-16 secretes a broad spectrum of cytokines (Baumann et al., 1984), but none of them displays this unusual combination of motility events on SK-BR-3 cells. On the basis of the cell spreading activity, we have purified a 50 kDa factor. Here we present biochemical and immunological evidence that the spreading factor is a putative ligand of the p185^{erbB2} receptor, activating tyrosine phosphorylation in the latter.

The existence of a candidate ligand for p185^{erbB2} was mentioned for the first time by Yarden and Weinberg (1989). This putative ligand was found in medium conditioned by cultured *ras*-transformed fibroblasts and it activated the tyrosine kinase activity, showed Neu protein dimerization and receptor internalization and effected the growth of cells that express the Neu protein. This ligand was later identified as a 35 kDa glycoprotein (Yarden and Peles, 1991). A similar activity was ascribed to a factor present in medium conditioned by the transformed human T-cell line ATL-2 (Dobashi et al., 1991). Other and more recent studies demonstrated that the p185^{erbB2} ligand family consisted of a number of different polypeptides that either stimulate mitogenesis or inhibit growth in a variety of target cells. gp30 specifically inhibits growth of cells that overexpress p185^{erbB2} (Lupu et al., 1990) while activated macrophages secrete a 25 kDa polypeptide that induces p185^{erbB2} downregulation in Neu-expressing NIH 3T3 cells (Tarakhovsky et al., 1991). A 75 kDa protein, secreted by SK-BR-3 cells was also considered as an activator for p185^{erbB2} (Lupu et al., 1992). Peles et al. (1992) described in more detail the purification of a 44 kDa protein called Neu

differentiation factor (NDF) or heregulin that was able to induce a phenotypic differentiation of cultured human breast cancer cells. Later, molecular cloning revealed that the ligand was derived from a larger transmembrane precursor protein (Wen et al., 1992). This protein elevated tyrosine phosphorylation of Neu in human tumor cells of breast, colon and neuronal origin, but not in ovarian cells although the latter also overexpress the receptor, suggesting that an additional factor is necessary for binding of NDF to cells (Peles et al., 1993). Marchionni et al. (1993) showed that several ligands for the p185$^{erb\text{B2}}$ receptor and glial growth factors are products of the same gene and that they are mitogenic for Schwann cells. Another member of the Neu ligand family is ARIA, a protein that stimulates the synthesis of muscle acetylcholine receptors. This suggests that members of the Neu ligand family can also act on the organization of the synapse in neuromuscular junctions (Falls et al., 1993).

The c-*erb*B2 gene is frequently amplified in human breast cancer (King et al., 1985; Slamon et al., 1987) and overexpression of p185$^{erb\text{B2}}$ results in growth inhibition of human breast cancer cells in vitro and in mouse mammary cells in vivo (Dati et al., 1990). A similar overexpression was also associated with differentiation of human skin cells in vivo (Maguire et al., 1989). Noguchi et al. (1993) demonstrated that overexpression of p185$^{erb\text{B2}}$ could contribute to the increase in the tumorigenicity of transfected human bronchial epithelial cells. Our finding, that a putative Neu ligand could also induce cell motility in cells that show a high expression of the p185$^{erb\text{B2}}$, could shed new light on the involvement of p185$^{erb\text{B2}}$ and some of its ligands in breast cancer development and progression.

MATERIALS AND METHODS

Cells

SK-BR-3 (ATCC HTB 30; provided by Dr M. Van de Vijver, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and ZR-75-1 (ATCC CRC 1500; provided by Dr K. Roozendaal, Daniel den Hoed Hospital, Rotterdam, The Netherlands) cell cultures were used (Kraus et al., 1987; Corbett et al., 1990). COLO-16 cell cultures (obtained from Prof. W. Fiers, University, Gent) were derived from a human skin squamous cell carcinoma (Moore et al., 1975). Media used for the maintenance of stock cultures were: Rega 3 minimal essential medium (MEM), containing non-essential amino acids (GIBCO Europe, Gent, Belgium) supplemented with 10% fetal bovine serum (FBS) (PAA Labor- und Forschungsges.MBH, Linz, Austria), 20 mM Hepes, 14.3 mM $NaHCO_3$, 50 units penicillin/ml, 50 units streptomycin/ml and 2 mM L-glutamine for SK-BR-3 cells; Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Europe) supplemented with 10% FBS, 10 mM $NaHCO_3$, 100 units penicillin/ml, 100 units streptomycin/ml, 42 μg gentamycin/ml, 3.7 mM L-glutamine and 1 nM estradiol for ZR-75-1 cells and RPMI-1640 supplemented with 10% FBS, 0.05% L-glutamine, 250 units penicillin/ml and 100 μg streptomycin/ml for COLO-16 cells.

Preparation of conditioned medium

Confluent COLO-16 cultures were maintained in 175 cm^2 Falcon no. 3028 vessels, 850 cm^2 Falcon no. 3027 roller flasks (Becton Dickinson Benelux, Erembodegem, Belgium) and in 6,000 cm^2 Nunc Cell Factories (A/S Nunc, Roskilde, Denmark) during 1 to 6 months without subcultivation. To produce COLO-16 conditioned medium (CCM), the culture medium was replaced during 1 to 3 days by MEM Rega 3 without serum but supplemented with Na_2SeO_3 (5 μg/l), transferrin (5 mg/l) and insulin (5 mg/l). CCM was produced about once a week in accordance with the status of the COLO-16 cultures. After harvesting, CCM was centrifuged at 1,800 ***g*** for 20 minutes and stored at −20˚C.

Assay for spreading

Cells from stock cultures were detached with trypsin (O.O5%) and EDTA (0.2%) in Ca^{2+}- and Mg^{2+}-free balanced salt solution for most experiments or with EDTA alone for experiments with antibodies. Samples (1 ml) of 1.5×10^4 cells in stock culture medium were seeded in 24-well plates (A/S Nunc) and incubated for 24 hours at 37˚C in an atmosphere of 5% CO_2 in air and 100% humidity. Then, CCM or purified factor were added at various dilutions as indicated in Results. Cultures were examined by phase-contrast microscopy after different times of incubation; they were fixed and stained with crystal violet after 24 hours. Spread and round cells were counted in at least 3 microscopic fields (objective ×25; at least 200 cells) and the result was expressed as percentage of spread cells.

Several cytokines were tested for comparison with the Spreading Factor (SpF). EGF (Sigma, St Louis, Mo), Scatter Factor (kindly provided by M. Weidner, Essen, Germany), TNF-α (obtained from W. Fiers, University, Gent, Belgium), TGF-β (British Biotechnology, Ltd, GB), IL-1 and IL-6 (Boehringer, Mannheim, Germany).

Antibodies

The monoclonal antibodies (mAbs) 9G6 and 3B5 (both from Oncogene Science, Inc, Manhasset, NY) were shown to recognize the extracellular and the cytoplasmic domains of p185$^{erb\text{B2}}$, respectively (Van de Vijver et al., 1988). They were used for functional inactivation and for immunodetection of the p185$^{erb\text{B2}}$ receptor. For functional inactivation we also used five ICR (Institute of Cancer Research) rat monoclonal antibodies (kindly provided by Dr S. Eccles, ICR, Sutton, UK) binding to different epitope clusters on the extracellular domain of p185$^{erb\text{B2}}$ (Dean et al., 1992; Styles et al., 1992).

Possible functional and structural similarity or identity between our spreading factor and the Neu differentiation factor (NDF) was studied by trying to inhibit the SpF activity with antibodies raised against NDF. NDF antiserum was kindly provided by Dr Yarden (The Weizmann Institute of Science, Rehovot, Israel). These antibodies were raised against a part of the EGF-like domain that is assumed to recognize the receptor (Wen et al., 1992). Dilutions of antiserum ranging from 1/5 to 1/2000 were preincubated for 30 minutes and 5 hours with dilutions of SpF (ranging from 1/100 to 1/1000). Inhibition of spreading was measured in three independent experiments.

For immunocytochemistry, subconfluent SK-BR-3 cell cultures on glass coverslips were fixed and permeabilized in 96% ethanol at −20˚C for 30 minutes followed by two 5-minute washes in PBS (pH 7.4) at room temperature. The primary antibody was applied for 30 minutes at a dilution of 1:10 in PBS followed by two 5-minute washes in PBS. The secondary FITC-conjugated rabbit anti-mouse antibody (F232, Dakopatts, Glostrup, Denmark) was applied for 30 minutes followed by 2 washes in PBS and mounted with glycerol (Merck, Darmstadt, FRG). Controls were carried out by omitting the primary antibody in the staining procedure. Immunostaining on unpermeabilized cells was done without ethanol treatment. For flow cytometry, cells were detached from stock cultures with 0.2% EDTA in Ca^{2+}- and Mg^{2+}-free balanced salt solution, and samples of 2×10^5 cells were prepared in 200 μl PBS containing 0.1% NaN_3 at 4˚C. They were washed and incubated at 4˚C for 30 minutes with mAb 9G6 at 10% in PBS with NaN_3, followed by washing in the same buffer, incubation with goat anti-mouse FITC conjugated at 10% in buffer for 30 minutes and 2 final washings in buffer. Fluorescence intensity was measured with a FACScan III (Becton Dickinson, Mountain view, CA). For western blotting, the procedure based on that of Towbin et al. (1979) as modified by De Potter et al.(1989a) was used.

Protein purification procedures

A C_4-reversed-phase HPLC column (4.6 mm × 250 mm, 5 μm particle size, 300 Å pore size; Vydac, Separations Group, Hesperia, CA) was equilibrated with 0.1% trifluoroacetic acid (TFA) and adsorbed proteins were subsequently eluted with a linear gradient of 0 to 70% acetonitrile in 0.1% TFA. The gradient was started 5 minutes after sample loading and finished after 70 minutes. The flow rate was 1 ml/minute and the eluted proteins were detected by absorbance at 214 nm. The HPLC equipment consisted of two Waters-Millipore (Milford, CT) pumps (model 510), a 481 UV detector, and an automated gradient controller (model 680). SDS-polyacrylamide gel electrophoresis was carried out on mini gels prepared and run according to Matsudaira and Burgess (1984). Proteins were detected either by Coomassie blue staining or by silver staining.

Protein iodination

A 50 μl protein solution was mixed with 20 μl of a 2 mCi $Na^{125}I$ solution. A 10 μl sample of a 0.5% chloramine T solution was added and allowed to react for 30 seconds at 25˚C. Iodination was terminated by adding sequentially 100 μl of $Na_2S_2O_3$ (0.4%), KI (1%) and BSA (5 mg/ml) (all solutions were in PBS). The iodination mixture was then passed over a PD-10 column (Pharmacia, Uppsala, Sweden) to remove free [^{125}I]iodide and samples of 1 ml were collected manually. Fractions containing iodinated protein were combined.

In vivo phosphorylation

Subconfluent cultures of cells were grown in 50 mm dishes. Cells were rinsed and incubated with phosphate-free Dulbecco's modified Eagle's medium (DMEM; GIBCO, Europe, Gent, Belgium) supplemented with 2% dialyzed fetal calf serum. After 1 hour at 37˚C, cells were labelled for 90 minutes at 37˚C with 1.0 mCi/ml [^{32}P]orthophosphate (Amersham, Brussels, Belgium) and then treated with the purified 50 kDa factor for 5 and 15 minutes, respectively. Afterwards cells were washed three times with ice-cold PBS and once with water. The cells were solubilized on ice with 1.5 ml lysis buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 5 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 1% aprotinin, 5 μg/ml leupeptin and 2 mM PMSF). Undissolved material was removed by centrifugation (1,400 ***g*** for 10 minutes at 4˚C). For immunoprecipitation the mAb 3B5 was used. Following adsorption to protein G-Sepharose (by continuous mixing for 2 hours at 4˚C), the beads were pelleted and washed six times with 1 ml of buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM Na_3VO_4, 5 mM EDTA and 10 mM NaF) and once with 20 mM Hepes-NaOH, pH 7.5. The immuno-complexes were eluted by boiling in Laemmli sample buffer and the material was loaded onto a 7.5% SDS polyacrylamide mini-slab gel and analysed by autoradiography and densitometry.

Proteins resolved by SDS-polyacrylamide gel electrophoresis, were also transferred onto a nitrocellulose paper (Amersham, Brussels, Belgium). The blot was saturated for 3 hours at room temperature with PBS and 0.05% Tween-20 and incubated overnight at 4˚C with an anti-phosphotyrosine antibody (5 μg/ml) (kindly provided by Prof. M. Joniau, KULAK, Belgium). The blot was rinsed several times with PBS and 0.05% Tween-20 and subsequently incubated for 3 hours at room temperature with goat anti-rabbit IgG conjugated with alkaline phosphatase (Fluka AG Biochem, Buchs, Switzerland). The membrane was rinsed and developed with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride (Bio-Rad, Richmond, USA).

Cloning expression and purification of the p185 extracellular domain HER2EX-6HIS

The mammalian expression vector (pCMVHER2EX-6His) was constructed by cloning a 2.1 kb fragment from pCVNHER2 (Hudziak et al., 1987) encoding the entire extracellular domain of p185^{erbB2}, fused to an oligonucleotide 5′-CCCACCATCACCATCACCATTGATA-AGC-3′ (encoding the His6 tag) into the cytomegalovirus promotor/enhancer-driven vector. This vector also carries the DHFR (dihydrofolate reductase)-selectable marker, encoding methotrexate resistance (Eaton et al., 1986).

BHK21 cells were cultured in DMEM/F12 supplemented with glutamine (2 mM) and 2% FBS. The extracellular domain construct was stably introduced into BHK21 cells by calcium phosphate precipitation (Wigler et al., 1978) along with pSV2PAC (Vara et al., 1986). Expression of pSV2PAC confers resistance to puromycin. A stable subclone was obtained by puromycin selection and amplification by methotrexate. Secretion of the HER2/p185^{erbB2} extracellular domain was assayed by in vitro labelling followed by radio-immunoprecipitation.

Ten liters of BKH21 conditioned medium were concentrated by ammonium sulfate precipitation to 45% saturation. Precipitated proteins were collected by centrifugation and dialysed overnight against buffer A (20 mM phosphate buffer, pH 7.5, containing 0.5 M NaCl). Dialysed protein solution was applied to a chelating Sepharose column (Pharmacia) preloaded with aqueous solution of $CuSO_4$ (10 mg/ml) and pre-equilibrated with buffer A. The column was washed first with equilibration buffer and then washed with buffer A containing 2 M ammonium chloride. Bound proteins were eluted with 50 mM EDTA in buffer A. Eluted proteins were dialysed overnight against buffer B (50 mM HEPES, pH 7.5, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.1% Triton X-100).

Dialysed protein solution was applied to lentil-lectin Sepharose (Sigma) pre-equilibrated in buffer B. The column was extensively washed with equilibration buffer and bound proteins were eluted with 0.5 M mannose (Sigma) in buffer C (50 mM HEPES, pH 7.5, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$). The eluate was dialysed against buffer C for 4 hours and concentrated on Centricon-30. The activity of the purified protein was checked by immunoprecipitation and stored at –20˚C with 10% glycerol. All purification steps were performed at 4˚C.

Receptor binding assay

Pieces of Immobilon membranes (Millipore, Bedford, USA) (4 mm × 4 mm), were pretreated in methanol (10 minutes) and hydrated for 10 minutes. A 10 μg sample of the extracellular domain of p185^{erbB2} HER2EX-6His was incubated with the Immobilon membranes for 4 hours at room temperature and blocked overnight at 4˚C with 5% quenching buffer (5% skimmed milk in PBS, 0.1% BSA, 0.05% Tween-20, 0.02% NaN_3). Positive fractions from the heparin-Sepharose column purification were collected and subjected to protein iodination as described above. One ml of ^{125}I-labeled proteins was added to the p185^{erbB2}-bound membranes for 4 hours at room temperature, followed by washings in 5% quenching buffer, PBS and water. Elution of adsorbed proteins was achieved by boiling the Immobilon membranes in Laemmli sample buffer (25 μl), water (75 μl) and BSA (10 μg). The eluted material was Speed Vac dried and taken up in a minimal volume of 20% glycerol followed by SDS-PAGE using the mini-slab gel system. Iodinated proteins were detected by autoradiography.

Assays for motility

Fast plasma membrane movements from cultures on solid substrata were quantitated as motile area in μm^2 per cell as described by Van Larebeke et al. (1992a,b) using a Kontron Vidas image analysis computer. This method scored mostly for ruffling and has previously been applied to breast cancer cells (Bracke et al., 1991).

To quantitate cell shape changes corrected for translocation, d_{shape} was measured from time lapse video films over a period of 30 minutes (Verschueren and Van Larebeke, 1984). The parameter d_{shape} is an index of on-spot motility in populations of lymphoid, fibroblastic and epithelioid cells (Verschueren and Van Larebeke, 1984; Verhasselt et al., 1990).

Translocation of individual cells was measured from time lapse video films of cultures in 25 cm^2 flasks. Translocation was observed over a period of 30 minutes and speed was expressed in μm per 30 minutes.

Chemotaxis was analysed in 24-well plates with 6.5 mm polycarbonate microporous (pore size = 8 μm) cell culture inserts (Transwell™, Costar Cambridge, MA; cat. no. 3422). In this modified Boyden chamber assay, the lower chamber contained 600 μl culture medium and the upper chamber 100 μl culture medium plus 2×10^5 cells. The 50 kDa factor was added to the upper and lower chambers as described for a checker-board assay (Zigmond and Hirsch, 1973). Plates were incubated for 24 hours with 5% CO_2 and 100% humidity, followed by methanol fixation of the filter and staining with 4′,6-diamidino-2-phenylindole 2-HCl as described by Verhasselt et al. (1992). Transmigrated cells were counted in at least 20 high-power fields and expressed as number per field.

Assay for growth

To study the influence of CCM or the 50 kDa factor on the growth of SK-BR-3 cells we used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983) as modified by Tada et al. (1986). This assay reflects metabolic activity and is measured as the reduction of a tetrazolium bromide derivative, which is converted to a blue insoluble formazan salt by the active mitochondrial enzymes. In the present experiments, 100 μl of culture medium containing 1×10^3 SK-BR-3 cells or 2×10^3 ZR-75-1 cells was transferred to each well of three microtiter plates. One day later, 100 μl of culture medium was added to the wells of one plate. To the wells 100 μl of CCM appropriately diluted in culture medium was added, using eight duplicates for each dilution. The plates were further incubated during 1 day and 4 day for MTT test analysis. For analysis of reversibility, cultures were incubated with CCM for 12, 24 and 48 hours, followed by washing and further incubation with fresh culture medium. Plating efficiency was evaluated by seeding 3×10^2 to 1×10^5 cells on 75 cm^2 tissue culture substratum and counting colonies after 3 weeks.

RESULTS

Induction of cell spreading activity by a factor present in conditioned medium of COLO-16 cells

When epithelial SK-BR-3 cells are seeded on a plastic tissue culture substratum in fresh medium they attach but remain spherical during at least two days. However, within less than 1 hour after addition of the CCM, the cells start to form multiple filopodia and lamellipodia, leading to highly spread cells after 24 hours (Fig. 1A). The number of spread cells recorded is proportional to the amount of added CCM (Fig. 1B). A similar concentration-dependent spreading is also observed after 1 hour of incubation when a purified 50 kDa factor, referred below as the spreading factor (SpF), is added (Fig. 1B). When SK-BR-3 cultures are seeded at 2,000 cells per 75 cm^2 and allowed to form colonies, all of these react to CCM, indicating clonal homogeneity for fast spreading (data not shown). ZR-75-1 cells are also sensitive to the spreading factor present in CCM but react only at a much higher concentration of CCM than is necessary for SK-BR-3 cells (data not shown).

A 50 kDa protein induces spreading of SK-BR-3 cells

It is known that the motility of some cell types is provoked or stimulated by various cytokines such as TGF-β, EGF, TNF-α, IL-1 and IL-6 (Stoker and Gherardi, 1991; Verhasselt et al., 1992). It is therefore important to know in the first instance if the observed spreading effect is due to one of these factors.

TGF-β (at 10 ng/ml) and scatter factor (at 100 to 400 units/ml) have no effect. Similarly, human TNF-α (at 10 to 1000 units/ml) fails to induce fast spreading of SK-BR-3 cells. IL-1, IL-6 and EGF (at 100 ng/ml) induce a slow spreading, which is only visible 24 hours after cytokine addition. The CCM-promoted spreading cannot be inhibited by IL-1 and IL-6 antibodies, which desensitize the cytokine effect in other target cells (data not shown). In addition, slower cell spreading promoted by EGF, IL-1 and IL-6 is not sensitive to 9G6 antibodies (see below) although the latter shows an inhibitory

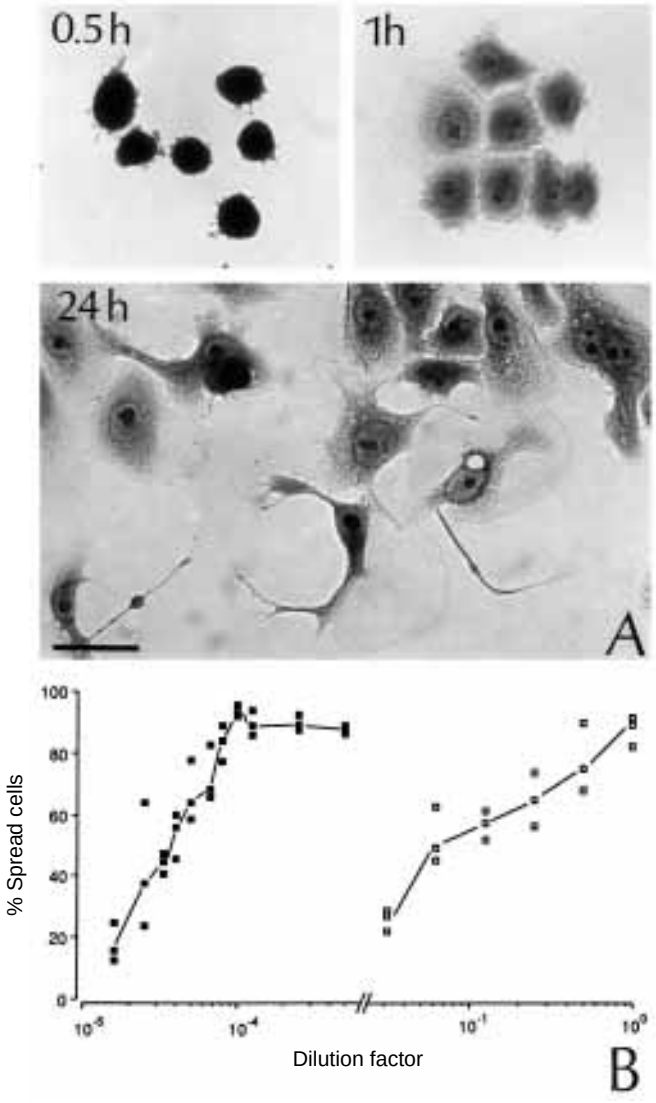

Fig. 1. Spreading effect of COLO-16 conditioned medium (CCM) on SK-BR-3 cells. (A) The cells were grown on tissue culture plastic substratum. At the indicated times after addition of CCM, cells were fixed and stained with crystal violet. Bar, 50 μm. (B) Dose-response curve of SK-BR-3 cell spreading activity. Ordinate: percentage of total cells that spread 1 hour after addition of CCM (open symbols) or of the 50 kDa SpF (filled symbols). Each symbol represents one microscopic field. Abscissa: dilution factor of CCM or of the 50 kDa factor.

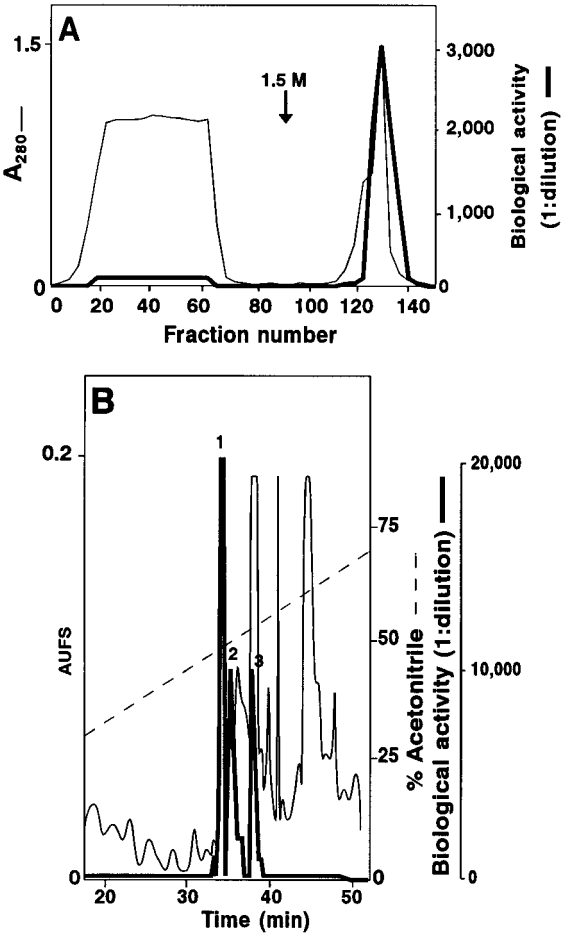

Fig. 2. Purification of the 50 kDa spreading factor. (A) Heparin-Sepharose chromatography. The protein profile measured by absorbance at 280 nm is shown with a narrow line. Thick lines indicate the spreading activity (i.e. maximal dilution allowing spreading of 90% of cells within 1 hour). The column was washed with 0.4 M NaCl and bound proteins were eluted with 1.5 M NaCl (arrow). (B) HPLC traces of the purification of the spreading factor. The absorbance at 214 nm (AUFS; absorption unit full scale) and spreading activities are represented as in A. The percentage acetonitrile is shown by the broken line and indicated as the right ordinate. The three peaks with spreading activity are numbered 1, 2 and 3.

effect on the CCM activity. On the basis of these results, we can exclude all the factors mentioned above.

The purification of a factor was carried out using fast spreading (spreading of 90% of the cells after 1 hour) as a bioassay. A typical purification protocol started from 16 liters of serum-free CCM. This was cleared from cell debris by centrifugation at 1,800 ***g*** for 20 minutes and by filtration over Whatman 3MM filter paper. The CCM was further concentrated to one fourtieth of its original volume by passage over Minitan Ultrafiltration plates with a cut-off of 10 kDa (Millipore, Bedford, MA). Ammonium sulphate was added to saturation and the precipitated proteins were collected by centrifugation for 30 minutes at 5,800 ***g***. The pellet was resuspended in 500 ml of buffer D (20 mM Tris-HCl, pH 7.4, 4% glycerol, 0.05% CHAPS, 0.02% NaN_3). Insoluble material was removed by centrifugation at 5,800 ***g*** for 30 minutes and the supernatant was loaded onto a heparin-Sepharose column (50 ml bed volume, flow rate 25 ml/hour), which was equilibrated in buffer D. The column was then washed with buffer D supplemented with 400 mM NaCl until the absorbance (measured at 280 nm) of the eluate reached baseline values. Proteins were then eluted in one step with buffer D containing 1.5 M NaCl (Fig. 2A). Fractions of 2 ml were collected and were assayed for spreading activity. Positive fractions were pooled and loaded onto a C_4-reverse-phase HPLC column. Proteins were eluted with a linear gradient of acetonitrile in 0.1% TFA and fractions of 1 ml were collected, while the eluate was continuously monitored at 214 nm with a flow-rate of 1 ml/minute. Biological activity was found in a tailing peak eluting between 30 minutes and 50 minutes. These fractions were combined, dried in a Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY), redissolved in 0.9 ml of 0.1% TFA in 30% acetonitrile and passed over the same C_4 column under the same conditions. Now, fractions of 500 μl were collected and assayed for activity. Fig. 2B shows the profiles of the absorbance at 214 nm and the biological activities. Three distinct peaks with biological activity were found, eluting with maximal values at 34 minutes, 35 minutes and 38 minutes, respectively. Most of the activity was measured in the peak eluting at 34 minutes and coeluted with a high UV-absorbing peak. This absorbance was not derived from protein but from a component (probably CHAPS) of buffer D used in the previous steps. The fractions eluting between 30 minutes and 40 minutes were analysed individually for their protein content on a 15% SDS-polyacrylamide gel. A weak Coomassie-stained 50 kDa band was visible in the most active fractions (Fig. 3A). The upper doublet (64 kDa and 66 kDa) does not represent an active component because it was also present, even more prominently, in the preceding inactive fractions.

This preparation could be diluted 1:15,000 to 1:20,000 and still promote cell spreading. In contrast, CCM could only be diluted 1:10 to 1:20 before reaching blank values. Thus the 50 kDa preparation corresponds with a 1000 times enriched cell spreading activity. On the basis of a semi-quantitative estimation of the Coomassie-stained protein band, one can calculate that the spreading effect in SK-BR-3 cells is still obtained at factor concentrations of approximately 1 ng/ml.

A new preparation of the spreading factor was iodinated after HPLC purification. This preparation still showed dose-dependent spreading of SK-BR-3 cells in a way similar to the non-iodinated sample (not shown). The autoradiogram showing the protein separation pattern revealed a single major band at 50 kDa, in agreement with the Coomassie Brilliant Blue staining (Fig. 3B). The contaminating doublet seen in the previous purification was not observed here. These data suggest that SK-BR-3 cell spreading is due to a 50 kDa factor present in CCM, because no other major proteins were detected.

The two remaining activity peaks (eluting at 35 minutes and 38 minutes) were combined, iodinated and analysed by SDS-PAGE. The autoradiogram now revealed bands at 23 kDa, 27 kDa, 30 kDa and 50 kDa (Fig. 3C). At this stage it was not clear if the lower molecular mass proteins represent degradation products of the active 50 kDa protein that may have been formed during consecutive purification steps or if they

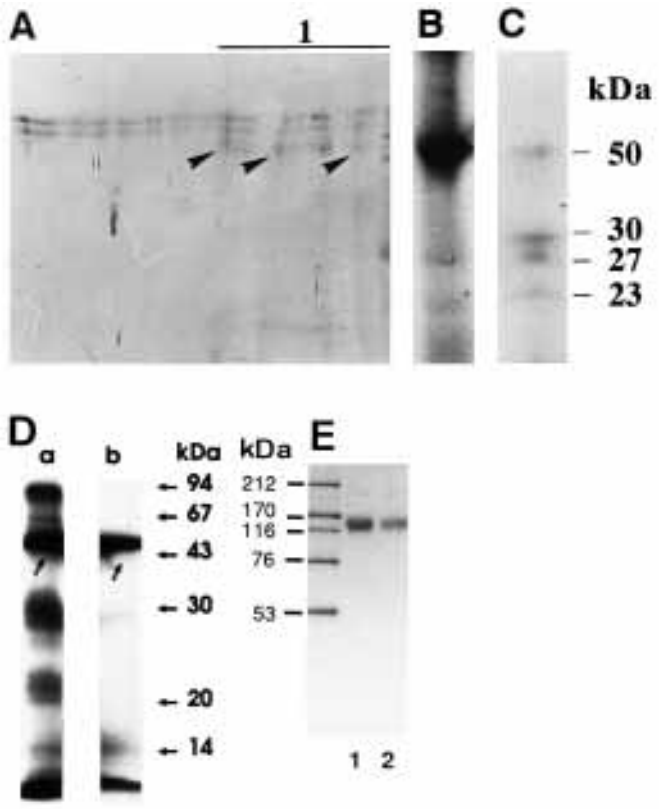

Fig. 3. SDS-polyacrylamide gel analysis of the purified spreading factor. (A) Biologically active material from peak 1 in Fig. 2 was collected in 3 fractions. They were analysed on their protein composition together with four inactive fractions eluting in front of peak 1. The 50 kDa band exclusively found in the active fractions is indicated by arrowheads. The gel was stained with Coommassie Blue. The doublet of 64 and 66 kDa noticed in all fractions, non-active and active, is most likely due to contamination in the sample buffer or in the HPLC elution system. (B) Material of a new HPLC run corresponding with peak fraction 1 after iodination. This fraction contains one major band of 50 kDa. (C) Combined peaks 2 and 3 (see Fig. 2B), after iodination. The 50 kDa, 30 kDa, 27 kDa and 23 kDa protein bands are indicated. (D) Lane a, mixture of iodinated proteins containing biological activity eluting from the heparin-Sepharose column (Fig. 2A). Lane b, proteins that are specifically retained by the PVDF-bound extracellular domain of p185^{erbB2}. Notice a specific enrichment for a 50 kDa protein. (E) SDS-polyacrylamide analysis of the purified HER2EX used in the ligand binding assay. Lane 1, protein after Cu^{2+} chelate chromatography. Lane 2, protein purified from a lentil-lectin column (± 2 μg was loaded). Molecular mass markers are indicated on the left side. Detection was done by Coomassie Blue staining.

represent variants of it. Alternatively, some of them could just be protein contaminants unrelated to the spreading activity. It is interesting to notice that the amount of secondary activity material, recovered in the later eluting peaks, was recovered in yields that varied from one batch of CCM to another. These additional peaks could represent variants of the SK-BR-3 spreading factor but their amounts were too small for further analysis. We therefore carried out further work with the 50 kDa factor present in the first peak of the HPLC chromatogram.

The 50 kDa spreading factor stimulates p185^{erbB2} receptor tyrosine phosphorylation in vivo

When the 50 kDa protein fraction was added to ^{32}P-labelled SK-BR-3 cells, an increase in phosphorylation of a 185 kDa protein was noticed. This protein could be precipitated with the p185^{erbB2}-specific monoclonal antibody 3B5 (mAb 3B5). With equal amounts of immunoprecipitated protein loaded on the gel (Fig. 4A), we recorded a 4-fold increase of ^{32}P incorporation into p185 between 5 minutes and 15 minutes, while the blank value did not increase (Fig. 4B). Antibodies, specific for phosphotyrosine showed a similar increase in western blot staining in the same period, suggesting that the major portion of increase of ^{32}P label was due to phosphotyrosine incorporation (Fig. 4C). These data show that the 50 kDa protein stimulates p185^{erbB2} receptor tyrosine phosphorylation in SK-BR-3 cells in vivo.

In another experiment, proteins eluting in the positive fractions of the heparin-Sepharose column (see Fig. 2A) were iodinated and incubated with PVDF-bound HER2EX6His (the recombinant extracellular domain of p185^{erbB2}) (Fig. 3E). A 50 kDa protein was specifically retained and recovered under SDS-denaturing conditions (Fig. 3D).

The putative 50 kDa p185^{erbB2} ligand differs from previously characterized Neu/HER-2 ligands

The family of Neu/HER-2 ligands comprises a large number of proteins with molecular masses ranging from 25 kDa to 75 kDa (see Discussion). On the basis of their molecular masses we can exclude most of them. Only the 44/45 kDa NDF could still be similar. Two points of evidence indicate that the SpF is different from the 45 kDa NDF: (1) our 50 kDa factor did not bind to a Cu^{2+}-chelating Sepharose column as did the 44/45 kDa heregulin; and (2) the spreading activity of the factor was not inhibited by NDF antibodies added in various ratios. In all experiments 87-90% of the cells remained spread.

Inhibition of cell spreading by antibodies raised against p185^{erbB2}

In order to define a more direct correlation between p185 receptor phosphorylation and cell spreading, we studied the effect of several p185-specific antibodies on spreading of SK-BR-3 cells. A commercially available monoclonal antibody (mAb 9G6) was found to inhibit CCM-induced cell spreading when added prior to CCM addition. This inhibition was proportional to the degree of antibody dilution (Fig. 5A). Other antibodies (kindly provided by Dr S. Eccles, The Royal Cancer Hospital, Surrey, UK) that have recently been shown to cross-react with p185^{erbB2} (referred to as ICR antibodies; Styles et al., 1990; Dean et al., 1992) were also used in this inhibition assay. Only ICR 12 was able to inhibit cell spreading. Other antibodies (ICR 27, ICR 52, ICR 53 and ICR 54) that recognize different epitopes on the external domain of p185^{erbB2} gave no effect. The same results were seen with mAb 3B5, which is specific for the intracellular domain of p185^{erbB2} (Van de Vijver et al., 1988). The results for ICR 12 and the negative controls with ICR 54 and 3B5 are shown in Fig. 5B.

The effect caused by the purified SpF was only inhibited during 2 hours by 9G6 antibodies but was fully inhibited by ICR 12 antibodies. Only full inhibition by 9G6 over a period of 24 hours (similar to that of CCM) was obtained when the 50 kDa preparation was diluted 1,000-fold. This suggests that

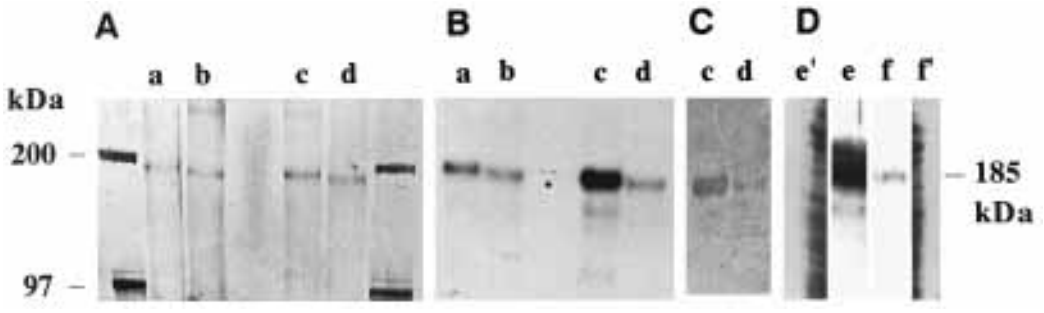

Fig. 4. In vivo phosphorylation of p185^{erbB2} in SK-BR-3 cells. SDS-polyacrylamide gel analysis of total lysates of ^{32}P-labelled SK-BR-3 cells from which the p185^{erbB2} receptor was immuno-precipitated. (A) Silver staining; (B) autoradiogram of the gel shown in (A); (C) western blot of a parallel gel analysis of the same sample as in (A) and (B) using specific phosphotyrosine antibodies. Lanes a and c, the immunoprecipitated p185 after 5 minutes and 15 minutes of stimulation, respectively. Lanes b and d, the p185 protein of the non-stimulated cells after 5 minutes and 15 minutes, respectively. (D) Immunodetection of p185^{erbB2} in total lysates of SK-BR-3 (e) and ZR-75-1 cells (f). (e,f) Western blotting with mAb 3B5, (e′,f′) Coomassie Blue staining of the same total cell extracts showing that equal amounts of total protein extract were loaded. Only the 250 to 100 kDa range is shown.

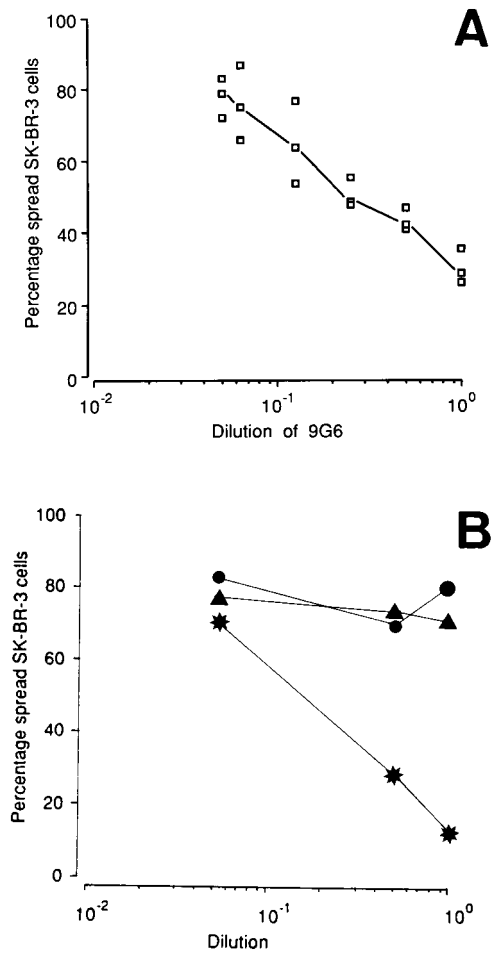

Fig. 5. (A) Inhibition of CCM-promoted SK-BR-3 cell spreading by mAb 9G6. Ordinate: percentage of spread cells after 24 hours. Abscissa: dilution of antibodies. Each symbol represents one microscopic field. (B) Inhibition of CCM-promoted SK-BR-3 cell spreading by mAbs 3B5(▲), ICR 12(★) and ICR 54(●). Ordinate: percentage of spread cells after 24 hours. Abscissa: concentration of antibodies in μg/ml. The values shown are means of three independent experiments.

either the concentration of active antibodies was different in both samples or they showed different affinities.

When SK-BR-3 cells were first induced to spread, addition of either mAb 9G6 or ICR 12 did not alter the spread morphotype any more.

Addition of CCM to SK-BR-3 cells kept in suspension for 5 minutes to 60 minutes at 4˚C or at 37˚C did not alter the level of p185^{erbB2} as revealed by flow cytometry (data not shown). This argues against receptor-ligand internalization.

Expression of p185^{erbB2} in the target cells

Immunofluorescence microscopy, using mAb 9G6 as primary antibody, revealed a strong but diffuse staining pattern in SK-BR-3 cells (Fig. 6A). This staining is much weaker in ZR-75-1 cells (not shown). Western blotting performed on total cell extracts, using an antibody that scores positively for the COOH tail of p185 on western blots (mAb 3B5), confirmed that p185 was more abundantly expressed in SK-BR-3 cells than in ZR-75-1 cells (Fig. 4D). These data are in line with the higher sensitivity of SK-BR-3 cells for the putative p185^{erbB2} ligand.

Immediately (5 minutes) after stimulation of SK-BR-3 cells with the 50 kDa factor, we noticed aggregation of the p185 antigen into intensively stained villus-like structures (Fig. 6B). After longer incubation times (e.g. 60 minutes), long filopodia were formed, containing most of the p185 antigen present in the cell (Fig. 6C,D).

These experiments show that stimulation by the 50 kDa factor provokes immediate aggregation of the p185^{erbB2} receptor and a preferential segregation into extended filopodia formed in a later stage of the activation process.

The 50 kDa factor stimulates cell motility in SK-BR-3 cells

At concentrations of SpF active in promoting spreading, an increase in the fast plasma membrane motility was registered both in round as well as in spread SK-BR-3 cells. This phenomenon could be inhibited with the 9G6 antibody (Fig. 7A).

Change in shape and translocation, two other aspects of SK-BR-3 cell motility, were also stimulated by CCM and SpF (Fig. 7B).

CCM and SpF stimulated migration of SK-BR-3 cells in a modified Boyden chamber assay and this effect could be neutralized in the presence of mAb 9G6. These observations were

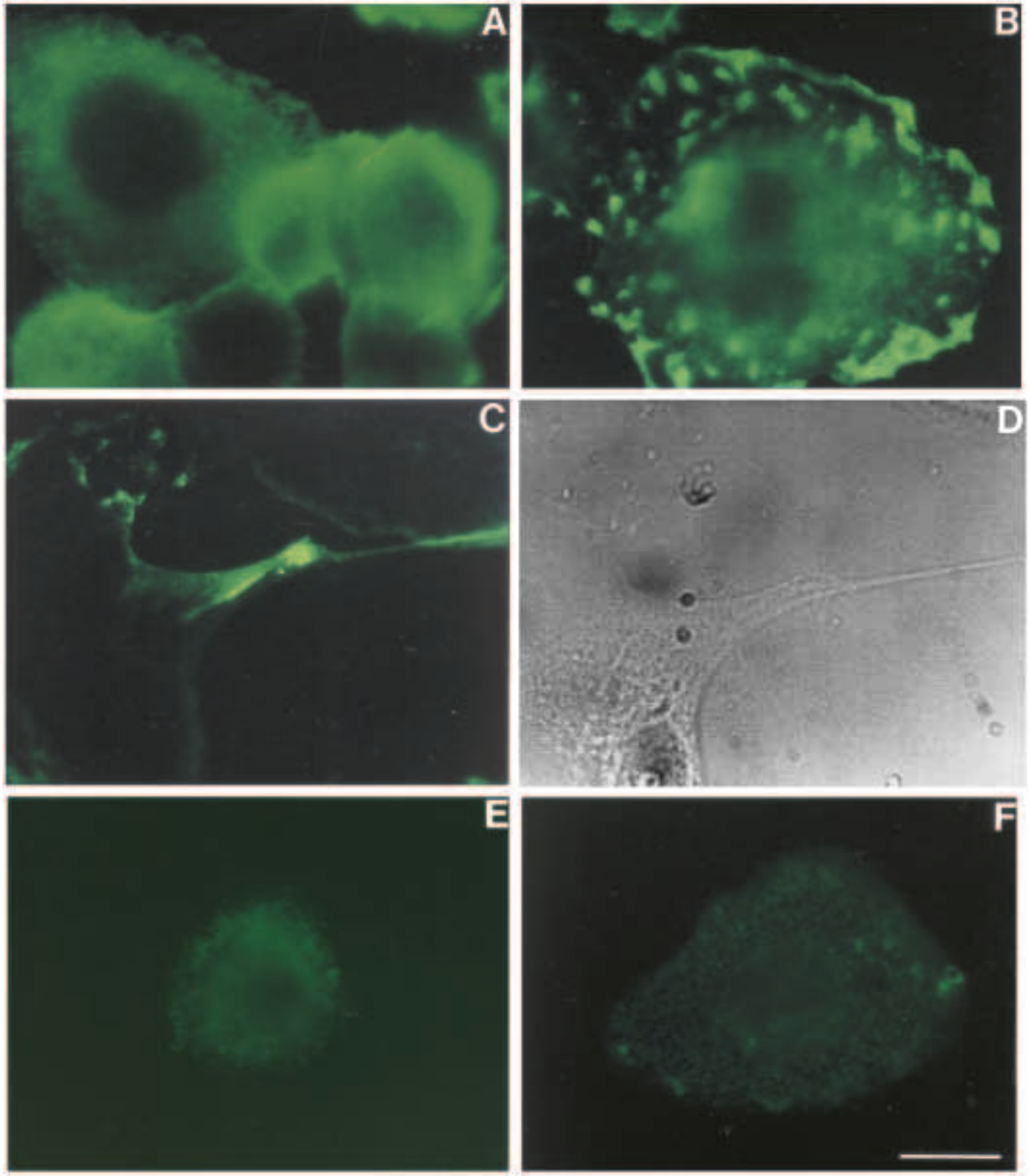

Fig. 6. Neu receptor reorganization upon SK-BR-3 activation. Location of the p185^{erbB2} receptor in ethanol-permeabilized SK-BR-3 cells was done by indirect immunofluorescence staining with a 3B5 antibody. (A) 0 minutes; (B) 5 minutes; and (C) 1 hour after addition of SpF. (D) Phase-contrast microscopy of the same cells as in C. (E) and (F) Controls prepared as (A) and (B), respectively, with the omission of the primary antibody. Bar, 32 μm.

confirmed using a checker board analysis (Fig. 7C). Considering each column of the board, transmigration increased when the concentration of the 50 kDa factor increased in the lower compared with the upper chamber, indicating chemotactic activity. However, when the concentration of SpF was kept identical in both chambers, we noticed an increase in the

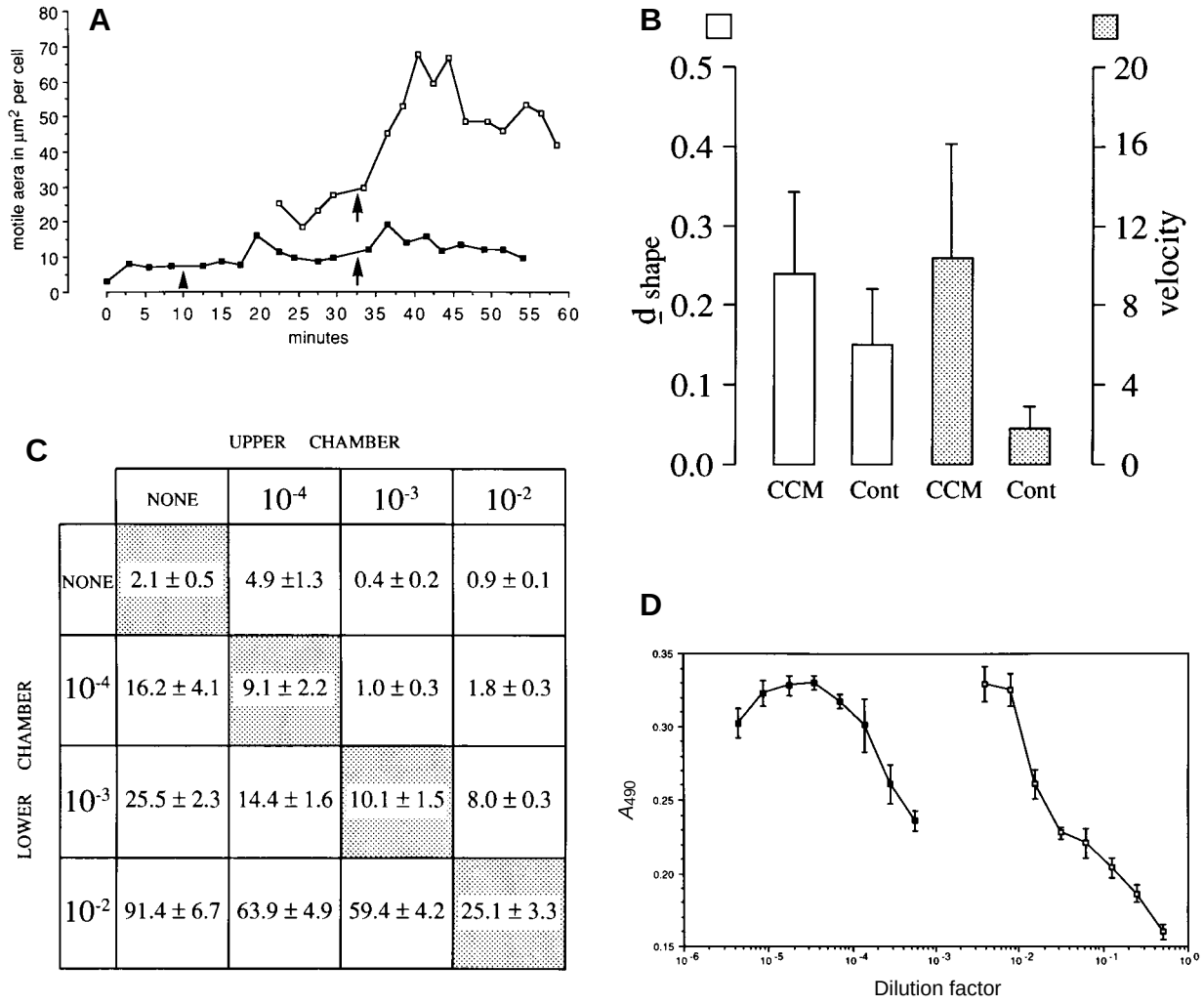

Fig. 7. Stimulation of cell motility in SK-BR-3 cells. (A) Typical curves from fast plasma membrane motility measurements with the 50 kDa SpF (added at a dilution 1:2,000 at the moment indicated by arrows) and antibody 9G6 (added at 3 µg/ml as indicated by the arrowhead). Different symbols indicate different SK-BR-3 cell cultures in experiments with (filled symbols) and without (open symbols) antibody. Ordinate: motile area per cell; each symbol represents one measurement. Abscissa: time of observation. (B) Effect of CCM on shape changes and translocation of SK-BR-3 cells; d_{shape} was calculated as described in Materials and Methods; velocity of translocation is in µm/30 minute. Bars indicate standard deviations. Differences between CCM-treated (CCM) and control (Cont) groups are significant at $P<0.001$ for both d_{shape} and distance. (C) Chemokinetic and chemotactic activity of the 50 kDa factor on SK-BR-3 cells in a modified Boyden chamber with various dilutions of the spreading factor in the upper and lower chambers. Numerical values are numbers of cells (mean ± s.e.m.) counted per high-power field (total number of fields was 30 to 50) on the lower side of the filter. Stippled fields indicate equal dilutions of the 50 kDa factor in upper and lower chambers, decreasing from top left to bottom right. (D) Growth of SK-BR-3 cells in the presence of CCM (open symbols) or the 50 kDa spreading factor (filled symbols). Ordinate: absorbance at 490 nm from MTT-tests performed 5 days after seeding and 4 days after addition of CCM or of the 50 kDa factor. Abscissa: dilution of CCM and of the 50 kDa factor in culture medium.

number of transmigrating cells proportional to the SpF-concentration. This suggests chemokinetic next to chemotactic activity of the 50 kDa factor. The SpF-induced chemotaxis was also inhibited by antibody mAb 9G6. The number of transmigrated cells per high-power field was 11.7±1.6 and 3.9±0.6 after addition of 9G6, respectively at 1 and 10 µg/ml, as compared to 52.5±3.9 in the absence of 9G6.

CCM and SpF also showed a hormetic effect on the growth of SK-BR-3 cells, i.e. stimulation at lower concentrations and inhibition at higher concentrations. With ZR-75-1 cells only a slight stimulation of growth was observed (Fig. 7D).

DISCUSSION

A factor present in conditioned medium of COLO-16 cell cultures, causing spreading and growth arrest of human SK-BR-3 mammary cancer cells was purified using fast cell spreading as a biological assay. The major activity is associated with a 50 kDa protein, which could be purified in an overall yield of 0.5 to 1 µg from 16 liters of conditioned medium. This preparation is at least 1,000-fold more active compared with the starting medium with respect to spreading activity. This means that the spreading factor is still active at

a concentration of 1 ng/ml, similar to that of several other growth factors (Peles et al., 1992).

In the course of the purification we also detected two other fractions with lower activities. They were found in variable and batch-dependent amounts. The most likely explanation for these minor fractions is that they could have originated from partial proteolytic degradation in the course of purification. The possibility cannot be excluded that these additional activities represent modified forms of the 50 kDa protein. Due to their variable appearance and their low yield we have so far been unable to further characterize these proteins.

Activation of the target cells by the 50 kDa factor is associated with tyrosine phosphorylation in a 185 kDa protein that is immunoprecipitated by a monoclonal antibody 3B5 directed against the intracellular domain of the p185^{erbB2} receptor. This suggests a direct or indirect correlation between ligand binding and Neu/HER-2 receptor phosphorylation.

This conclusion is further corroborated by inhibition of spreading activity by two antibodies (mAb 9G6 and IRC 12) that are specific for the extracellular domain of the Neu/HER-2 receptor. Other antibodies that also react with this domain of the Neu/HER-2 receptor have no effect. But this could be the result of a lower affinity or of an interaction with Neu/HER-2 that does not inhibit binding of SpF with its receptor. The antibody mAb 3B5 raised against an intracellular part of the receptor, and that also recognizes the denatured form of the antigen (see above), is also ineffective in blocking the spreading effect of the 50 kDa factor.

Taken together, the tyrosine phosphorylation of the p185^{erbB2} receptor and inhibition of cell spreading by Neu/HER-2-specific antibodies support the hypothesis that the 50 kDa spreading factor is a putative ligand of Neu/HER-2. Several such ligands have been reported recently. The novel aspect of our findings is that phosphorylation of p185^{erbB2} by a 50 kDa ligand seems to be associated in SK-BR-3 cells with inhibition of growth and stimulation of a spectrum of motility-related phenomena such as cell surface ruffling, chemokinesis and chemotaxis. This classifies SpF as a motogenic rather than as a mitogenic factor at least for SK-BR-cells. ZR-75-1 cells respond in a similar way, though at higher ligand concentrations, in line with the lower expression levels of the p185 receptor. Immunofluorescence analysis reveals that stimulation of the target cells is associated with a fast clustering of the p185 receptor into microvillar structures. Both, p185^{erbB2} tyrosine phosphorylation and receptor redistribution take place immediately after ligand addition. We therefore do not know if receptor phosphorylation precedes and/or is necessary for redistribution or if this aggregation is the trigger for subsequent phosphorylation. At a later stage of the stimulation process, p185 is preferentially found in the filopodia. This suggests that receptor aggregation is not a step leading to internalization.

Recently, Neu/HER-2 ligands have collectively been referred to as the neuregulins, comprising a large number of proteins with molecular masses ranging from 25 kDa to 75 kDa. 25 kDa ligands are produced by macrophages (Tarakhovsky et al., 1991) and kidney cells (Huang and Huang, 1992). A 30 kDa and a 75 kDa factor from mammary carcinoma cells have been isolated by Lupu et al. (1990, 1992). The Neu differentiation factor (NDF), or heregulin, is a 44/45 kDa glycoprotein that was isolated from human breast tumour cell-conditioned medium as a specific stimulator of tyrosine phosphorylation in p185^{erbB2} (Peles et al., 1992, 1993; Wen et al., 1992; Holmes et al., 1992). Marchionni et al. (1993) described a number of glial growth factors, derived by alternatively splicing, and yielding 31 kDa, 34 kDa, 45 kDa and 59 kDa products acting as mitogens for Schwann cells.

Finally, ARIA, a family of proteins ranging in molecular mass from 33 kDa to 42 kDa and probably derived from the same ARIA gene, have also been recognized as putative Neu ligands (Falls et al., 1993).

None of the previously described factors shows any biochemical or immunological resemblance to the 50 kDa spreading factor. Neither did we find similarity with cytokines known to be secreted by COLO-16 cells. The spreading factor described in this paper is thus a new factor that belongs to the increasing family of Neu/HER-2 ligands. It is not clear yet if it is also a product of the neuregulin gene family (Holmes et al., 1992); this needs to be verified by future protein sequencing.

A direct effect on p185^{erbB2} appears to be the most simple, although not exclusive, mechanism. p185 could for instance serve as part of the signal transduction cascade in which another receptor binds the spreading factor. For instance, p185 was shown to be a substrate for the EGF receptor-tyrosine-kinase (Kokai et al., 1988; Connely and Stern, 1990) and a similar stepwise effect could take place in the action of the 50 kDa motility factor. An alternative theory takes into consideration the possibility that the factor could function as a cell-substratum adhesion or haptotactic molecule. This hypothesis is very unlikely, since fast plasma membrane motility is stimulated in both round and spread cells.

Although this is the first report pointing to the association of a p185^{erbB2} ligand with cell motility, our results did not come as a complete surprise, since recent studies by Carothers Carraway et al. (1993) showed a physical association between the p185 receptor and the microfilament system via a transmembrane glycoprotein complex. This association was found in the microvillar fraction of cells of a rat mammary adenocarcinoma and could also be active in SK-BR-3 cells, connecting the ligand with microfilament organizing systems, possibly through an Src homology-2 domain recognizing the phosphorylated tyrosine(s) in p185^{erbB2}. In line with this is the inability to block the SpF effect by *Pertussis* toxin, suggesting that the signal transducing mechanism does not pass via a G-protein-mediated pathway (data not shown).

Overexpression of p185^{erbB2} has been associated with a variety of, sometimes opposite, biological phenomena; namely, growth inhibition (Dati et al., 1990) and growth stimulation (Holmes et al., 1992), oncogenic transformation (Bernstein et al., 1993; Noguchi et al., 1993) and differentiation (Maguire et al., 1989). Similarly, ligand binding has resulted in the expression of different phenotypes (Lupu et al., 1992; Peles et al., 1992; Marchionni et al., 1993). These different reactions may be due to different ligands or to differences in the response of different cells, in line with the well-documented multifunctional character of peptide growth factors (Sporn and Roberts, 1988; Stoker and Gherardi, 1991; Wen et al., 1992). The c-*erb*B2 gene is frequently amplified in human breast cancer (King et al., 1985; Slamon et al., 1987) and this phenomenon seems to be cancer-specific (De Potter et al., 1989b). The production of the ligands in human cancers has not been analysed, so far. Further characterization of the

50 kDa factor and other factors may lead to the development of probes for the analysis of the role of the p185^{erbB2} ligands in breast cancer development and progression.

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