

C-Met signalling in an HGF/SF-insensitive variant MDCK cell line with constitutive motile/invasive behaviour

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SUMMARY

The Met protein is a receptor tyrosine kinase for hepatocyte growth factor/scatter factor (HGF/SF), a multifunctional growth factor with mitogenic, motogenic and morphogenic properties. A morphologically altered variant of the MDCK cell line, MDCK-1, spontaneously exhibits a number of features associated with a partial HGF/SF-Met induced phenotype (less adhesive colonies in culture, enhanced invasion and motility, nascent tubule formation), but paradoxically does not respond to HGF/SF treatment. Although the overall cell surface expression and distribution of Met were found to be similar in parental MDCK cells and the MDCK-1 cell line, p145^{met} autophosphorylation (\pm HGF/SF) was significantly reduced in MDCK-1 cells in vitro and in vivo when compared with parental MDCK cells. In contrast, EGF induced cell proliferation and EGF receptor autophosphorylation to similar levels in both cell lines. The basal levels of protein tyrosine phosphorylation were higher in MDCK-1 cells when compared with parental MDCK cells, including that of two prominent

proteins with molecular masses of ~185 kDa and 220 kDa. Moreover, both p185 and p220 are present and tyrosine phosphorylated in Met immunoprecipitates from MDCK-1 cells (\pm HGF/SF), but not parental MDCK cells. In addition, Met immunocomplexes from MDCK-1 cells exhibited an approximately 3-fold increased tyrosine kinase activity in vitro when compared with MDCK cells, correlating with the higher basal levels of total phosphotyrosine. Treatment of MDCK-1 cells with the tyrosine kinase inhibitor herbimycin A reverted the cell phenotype to a more MDCK-like morphology in culture, with a concomitant reduction in the tyrosine phosphorylation predominantly of p220. Taken together these data suggest that aberrations in Met activity and associated signalling render MDCK-1 cells insensitive to HGF/SF, and may also mediate alterations in MDCK-1 cell behaviour.

Key words: Met, HGF/SF, Scattering, MDCK-1, Tyrosine phosphorylation.

INTRODUCTION

The product of the *met* proto-oncogene (Cooper et al., 1984) is the transmembrane tyrosine kinase p190^{met}, which has been identified as the receptor for hepatocyte growth factor/scatter factor (HGF/SF) (Bottaro et al., 1991; Naldini et al., 1991a). HGF/SF is a polypeptide growth factor capable of exerting a wide range of effects on target cells expressing Met in vitro, including the induction of cell proliferation, differentiation, invasion and scattering (for reviews see Gherardi and Stoker, 1991; Rosen et al., 1994; Warn, 1995). The latter property involves the break up of epithelial cell colonies into single, highly motile cells (Stoker et al., 1987) and is associated with a marked reorganisation of the F-actin cytoskeleton (Dowrick et al., 1991). Because of its biological properties, a putative role for HGF/SF as a mediator of various physiological processes involving epithelial-mesenchymal conversions has been suggested (Tsarfaty et al., 1992; Sonnenberg et al., 1993), including embryogenesis, development, tissue repair and angiogenesis (reviewed by Rosen et al., 1994). In addition, there is now substantial evidence that HGF/SF and Met are

involved in the development of tumours and their progression to malignancy (reviewed by Rosen et al., 1994; Warn, 1994; Cortner et al., 1995).

Met is synthesised as a single polypeptide 170 kDa precursor which is co-translationally glycosylated (Giordano et al., 1989a). Terminal glycosylation and proteolytic cleavage generate the mature disulphide-linked heterodimer p190^{met}, comprising a 50 kDa α -chain and a 145 kDa β -chain (Giordano et al., 1989b). The β -chain spans the plasma membrane and contains a tyrosine kinase (TK) domain within the cytoplasmic region (Park et al., 1987; Gonzatti-Haces et al., 1988; Tempest et al., 1988). HGF/SF binds to the extracellular portion of Met and induces autophosphorylation on tyrosine residues within the β -chain (Bottaro et al., 1991; Naldini et al., 1991a,b). It has now been established that autophosphorylation of two tyrosine residues (Tyr¹²³⁴ and Tyr¹²³⁵) within the TK domain are essential to induce the full intrinsic TK activity of Met (Naldini et al., 1991c; Longati et al., 1994). The phosphorylation of other tyrosine residues outside of the TK domain provides docking sites for various src-homology 2 (SH2) containing proteins including phosphatidylinositol-3 kinase (PI-3K),

members of the c-src cytoplasmic tyrosine kinase family, phospholipase C- γ (PLC γ), a ras-GTPase activating protein (rasGAP), Grb2 and Shc (Bardelli et al., 1992; Faletto et al., 1993; Ponzetto et al., 1994; Pelicci et al., 1995). Met-mediated tyrosine phosphorylation of these proteins and/or their translocation to the inner face of the plasma membrane promotes a cascade of intracellular signalling which ultimately modulates cell behaviour (reviewed by van der Geer et al., 1994).

In view of its fundamental role in various biological phenomena, the mechanisms by which cells regulate and modify their response to HGF/SF is important to address. It has previously been shown that Met activity is negatively regulated by phosphorylation of a serine residue located in the juxta-membrane domain of the β -chain (Gandino et al., 1994), and this is mediated by both protein kinase C (PKC) and calcium-sensitive kinases (Gandino et al., 1990, 1991). A tyrosine phosphatase has also been shown to associate with and dephosphorylate Met following HGF/SF stimulation (Villa Moruzzi et al., 1993). In addition, truncated forms of Met have been described in which alternative post-translational processing gives rise to receptors retaining ligand binding capacity, but which are unable to transduce intracellular signals in response to HGF/SF due to lack of a functional TK domain (Prat et al., 1991; Crepaldi et al., 1994). It has been suggested that these forms of Met act to suppress the HGF/SF response in some cells. Conversely, constitutively activated forms of Met have been described, arising as a consequence of various events including gene rearrangements (Park et al., 1986), gene overexpression (Giordano et al., 1989b), defective post-translational processing (Mondino et al., 1991), alternative splicing (Lee and Yamada, 1994, 1995), point mutations/deletions (Zhen et al., 1994; Weidner et al., 1995) and/or autocrine stimulation (Iyer et al., 1990; Rong et al., 1992).

In this paper we describe a variant of the MDCK cell line, termed MDCK-1, which was isolated and cloned from parental MDCK cells in culture on the basis of its failure to scatter in response to HGF/SF stimulation. The results presented suggest that HGF/SF insensitivity may be linked to the spontaneous development of many features associated with partially activated Met in the MDCK-1 cell line. The possible mechanisms involved in this phenomenon are discussed.

MATERIALS AND METHODS

Cells, reagents and antibodies

All reagents, unless specified, were purchased from Sigma (Poole, UK). The Madin-Darby canine kidney (MDCK) cell line (Madin and Darby, 1958) was obtained from Michael Stoker (ICRF Cell Interactions Laboratory, Cambridge, UK). A spontaneous variant line, MDCK-1, was isolated from MDCK cells in culture and cloned by M. Perryman and M. Stoker (ICRF Cell Interactions Laboratory, Cambridge, UK) on the basis of its failure to scatter in response to HGF/SF, and was a generous gift from them. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin/100 μ g/ml streptomycin (Gibco) and 5% foetal calf serum (FCS, Gibco). Cells were maintained at 37°C, in a humidified atmosphere of 5% CO₂. Mouse recombinant HGF/SF was purified by heparin-Sepharose CL 6B chromatography from the supernatant cultures of the mouse myeloma line NSO (M. Sharpe, K. Lane and E. Gherardi, unpublished work).

Antibodies against the extracellular domain of human Met (mouse monoclonal antibody, clone DO-24) and against the phosphotyrosine epitope (mouse monoclonal antibody, clone 4G10) were purchased from Upstate Biotechnology (New York, US). An antibody against the C terminus of mouse Met (rabbit polyclonal antibody, clone SP 260) was raised as previously described (Iyer et al., 1990). Rabbit anti-mouse peroxidase-conjugated antibodies, goat anti-rabbit FITC-conjugated antibodies, and rabbit anti-mouse-FITC and -rhodamine-conjugated antibodies were purchased from Dako (High Wycombe, UK).

Cell migration and collagen gel assays

For cell migration assays, cells were grown to confluence in 24-well culture plates (Corning, Stone, UK), and a series of artificial wounds made through cell monolayers using a plastic pipette tip. Cells were then incubated either in the absence or presence of 10 ng/ml HGF/SF for 24 hours. Cells were finally fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) and stained with Coomassie Blue solution. Cells were observed with a Nikon inverted phase microscope and photographs taken using T-max 400 film (Kodak).

The collagen gel invasion assays were performed essentially as described by Behrens et al. (1989). In brief, collagen gels were prepared by mixing eight parts of sterile collagen solution (collagen rat tail, type I, Collaborative Biomedical, Milton Keynes, UK) with one part sterile 10 \times concentrated DMEM and one part sterile 0.2 M Hepes solution. The pH of the final solution was adjusted to 7.4 and, following gelation at 37°C, cells were seeded at a density of 5 \times 10⁴ cells per 35 mm dish in either the absence or presence of 10 ng/ml HGF/SF. The medium was changed every day over the course of the experiment (4 days). The number of invading cells were counted daily over 10 random fields of view (0.33 mm²) using a Nikon inverted phase microscope.

For investigating tubule formation within collagen type I gels, collagen gels were prepared as described above, and cells resuspended at 5 \times 10⁴ cells/ml of collagen gel prior to gelation in a 24-well plate. DMEM medium containing 10% FCS was applied to each well and was changed every 2 days. When the cells had formed well sized spherical cysts (after approximately 1 week in culture), HGF/SF (ranging from 0.1-100 ng/ml) was added and the cultures maintained for up to 5 weeks. Cells were visualised and photographed as described above.

Immunofluorescence and flow cytometry

Cells for immunofluorescence staining were seeded on 1 cm diameter glass coverslips. Three PBS washes were performed between each subsequent step and all incubations were carried out at 37°C. Cells were fixed in 4% paraformaldehyde/PBS for 20 minutes and non-specific sites blocked for 1 hour with 1:10 diluted rabbit serum (Dako). Cells were then incubated for 1 hour with an anti-Met antibody (DO-24, diluted 1:100), followed by a 1 hour incubation with a 1:40 dilution of anti-mouse rhodamine-conjugated antibody. Finally, coverslips were mounted on glass slides in citifluor (Citifluor Ltd, London, UK) and fluorescence observed using a Zeiss Standard R epifluorescence microscope. Photographs were taken using T-max 400 film.

Flow cytometry analysis of Met was performed using a fluorescence activated cell scanner (FACScan). MDCK and MDCK-1 cells were removed from tissue culture flasks by brief incubation with trypsin/EDTA. Cells were centrifuged at 270 g for 3 minutes at 4°C and washed three times with PBS between subsequent steps. All incubations were carried out for 30 minutes at 4°C. For cell surface staining of Met, cells were incubated firstly with 5 μ g/ml of DO-24 antibody followed by a 1:40 diluted rabbit anti-mouse FITC-conjugated antibody. Following all incubations and washes, cells were resuspended in 1% paraformaldehyde/PBS and transferred to FACScan tubes containing FACScan buffer (Becton Dickinson,

Cowley, UK). The degree of green (FITC) fluorescence was measured using the FL1 channel of a FACScan (Becton Dickinson) and data was analysed using the LYSIS, version 1.1 software package (Becton Dickinson).

Immunoprecipitation and western immunoblotting

Cells were grown to approximately 70% confluence on 10 cm diameter Petri dishes. Following treatments as indicated, cells were solubilised by washing twice with ice-cold PBS, and resuspending in 1 ml of ice-cold lysis buffer (20 mM Pipes, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate). Cell lysates were clarified by centrifugation at 15,000 *g* for 15 minutes at 4°C, and protein determination was performed on the soluble protein supernatants using a standard Pierce assay (Pierce, Rockford, US).

For western analysis, 10 µg of protein samples were resuspended 3:1 into 4× Laemmli buffer (0.4 M Tris-HCl, pH 6.8, 0.4 M DTT, 8% SDS, 39% glycerol, 0.04% bromophenol blue), boiled for 5 minutes, and resolved by SDS-PAGE. The proteins were then electrotransferred onto nitrocellulose membrane (ECL-hybond, Amersham, Little Chalfont, UK). Following transfer, the membranes were first blocked for 1 hour with a 5% solution of BSA in TBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and then incubated for 1 hour with a 1:2,000 dilution of anti-phosphotyrosine antibody (clone 4G10), followed by a 1:1,000 dilution of horseradish peroxidase-conjugated second stage antibody. Tyrosine phosphorylated proteins were detected using the enhanced chemiluminescence reagents (ECL, Amersham) and exposure to Kodak X-OMAT AR film. The developed films were scanned and the integrated optical density of individual bands was determined by laser densitometry (Pharmacia, GelScan XL 2.1).

For immunoprecipitation of Met, 0.5 mg of protein sample was adjusted to 1 ml with lysis buffer, and incubated for 2 hours at 4°C with 50 µl of a 20% suspension of Protein-A-agarose to pre-clear the samples. The samples were then centrifuged at 15,000 *g* for 1 minute in an Eppendorf desktop centrifuge to pellet the agarose beads, and the supernatants incubated at 4°C overnight with 1 µl of anti-Met antibody (clone DO-24). Samples were then incubated for 3 hours at 4°C with 50 µl of a 20% suspension of Protein-A-agarose to allow coupling to the immune complex. The agarose pellets were centrifuged as described above, and washed and re-centrifuged a further four times with lysis buffer. Samples for SDS-PAGE were resuspended into 25 µl of 2× Laemmli buffer. Samples for both the Met immunocomplex *in vitro* kinase assay and the non-radioactive tyrosine kinase assay were used in the respective procedures described below.

In vitro kinase assay

Met proteins were immunoprecipitated as described above, and the final Met-immunocomplex washed an additional two times in buffer A (25 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM sodium vanadate, 10 µM cold ATP, 0.1% BSA and 0.1% Triton X-100). The kinase assay was performed in 20 µl of buffer A containing 10 µCi of [γ -³²P]ATP (specific activity 3,000 Ci/mmol⁻¹; Amersham). The reaction was carried out at 30°C for 15 minutes and stopped by adding 8 µl of boiling 4× Laemmli buffer. The eluted proteins were separated by 6% SDS-PAGE, and the gels subjected to alkali treatment for 1 hour (1 N NaOH at 55°C). The gels were finally dried and subjected to autoradiography at -70°C with intensifying screens.

Non-radioactive tyrosine kinase assay

Met proteins were immunoprecipitated as described above, and the final Met-immunocomplex washed an additional two times in buffer B (25 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM sodium vanadate, 5 mM ATP, 0.1% BSA and 0.1% Triton

X-100). The method for the detection of tyrosine kinase activity in Met immunoprecipitates was adapted from a tyrosine phosphatase kit purchased from Boehringer Mannheim (Lewes, UK). In brief, streptavidin coated wells were incubated with a solution containing a tyrosine phosphorylated artificial peptide fragment conjugated to biotin (at a final concentration of 0.6 nM) for 30 minutes at 37°C. The wells were then washed three times with PBS, and incubated for 1 hour at 37°C with 1 U/ml of alkaline phosphatase (Boehringer Mannheim), allowing for the dephosphorylation of the tyrosine residues on the immobilised peptide substrate. Following a further three PBS washes, Met immunoprecipitates in buffer B were added to the wells and incubated for 1 hour at 37°C with shaking. The wells were then washed four times with PBS, before adding a mouse anti-phosphotyrosine HRP-conjugated antibody for 1 hour at 25°C (Boehringer Mannheim, clone 3-365-10, 0.5 U/ml). The wells were finally washed five times with PBS prior to adding the provided peroxidase substrate (ABTS). The absorbance at 405 nm with a reference wavelength of 490 nm was measured using an ELISA plate reader (model MR5000, Dynatech, Billingshurst, UK). The tyrosine kinase activity in each sample was calculated from the increase in absorbance above alkaline phosphatase controls, and values expressed relative to unstimulated MDCK cells.

RESULTS

MDCK-1 cells exhibit a distinctive 'semi-scattered' morphology which is not affected by HGF/SF

In the absence of HGF/SF, MDCK cells formed tight, discrete colonies under subconfluent culture conditions (Fig. 1A). Following the addition of HGF/SF for 24 hours, parental MDCK cell colonies dispersed into individual and highly motile cells (Fig. 1B). In contrast, MDCK-1 cell colonies displayed a 'semi-scattered' morphology in culture, that is, colonies formed with irregular edges and isolated cells were present along the edges and between colonies (Fig. 1C). Confluent monolayers of MDCK-1 cells resembled those of parental MDCK cells and there was no overgrowth or foci formation (data not shown). The saturation density of MDCK-1 cells was 1.29×10^5 cells/cm², a somewhat lower density than MDCK cells at confluence (4.52×10^5 cells/cm²) and was due to their larger mean size associated with a more flattened morphology (compare Fig. 1A and C). Interestingly, MDCK-1 cells displayed no further changes in colony morphology or cell shape following HGF/SF treatment (Fig. 1D). In addition, HGF/SF had no effect on the proliferation of MDCK or MDCK-1 cells, either in the absence or presence of serum (data not shown).

Time lapse video microscopy demonstrated that whereas MDCK cells were largely non-motile in the absence of HGF/SF, isolated MDCK-1 cells were highly motile, exhibiting extensive membrane ruffling at leading edges, and displaying prominent pseudopodial extensions (Fig. 2). Such cells showed contact inhibition of movement when encountering adjacent MDCK-1 cell colonies, whereby transient and unstable cell-cell contacts were formed. This continued cell movement associated with weak cell-cell adhesion, was also a feature of the smaller MDCK-1 colonies. Within larger colonies however, cell contacts appeared more stable, although movements within the colonies continued. This altered phenotype was quite distinct from HGF/SF-treated ('fully scattered') MDCK cells, which were observed as individual and highly motile cells with no cell-cell contacts (Fig. 1B).

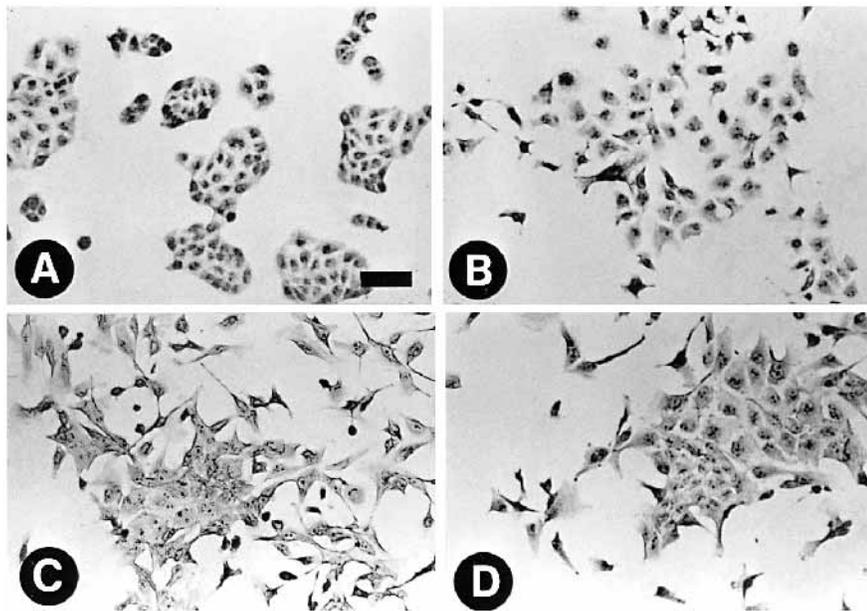


Fig. 1. Colony morphologies of MDCK and MDCK-1 cells. MDCK cells (A and B) and MDCK-1 cells (C and D) were grown to ~25% confluence, and cultured for a further 24 hours either in the absence (A and C) or presence (B and D) of 10 ng/ml HGF/SF. Bar, 100 μ m.

Motility data, as calculated from the distances travelled by cells across the time-lapse video screen, demonstrated that individual MDCK-1 cells (mean velocity = 8.4 cm/hour) were as motile as HGF/SF-treated MDCK cells (mean velocity = 8.6 cm/hour). MDCK-1 cells within a colony were considerably less motile (mean velocity = 2.5 cm/hour), but nonetheless more so than parental MDCK cells in the absence of HGF/SF (mean velocity = 0.1 cm/hour). HGF/SF treatment had no effect on the motility of isolated MDCK-1 cells (mean velocity = 8.8 cm/hour), or the proportion of cells displaying the highly motile phenotype.

The effect of HGF/SF on MDCK and MDCK-1 cell migration was further investigated using a wounding assay. In the absence of HGF/SF, MDCK cells failed to migrate into wounds etched through a confluent cell monolayer (Fig. 3A). However, following treatment for 24 hours with HGF/SF, a

large number of MDCK cells migrated into and effectively closed the wound gap (Fig. 3B). In contrast, a small proportion of MDCK-1 cells spontaneously migrated into wounds in the absence of HGF/SF, and wound edges appeared irregular, demonstrating the 'semi-scattered' phenotype (Fig. 3C). The extent of MDCK-1 migration was not affected by treatment with HGF/SF (Fig. 3D), even when doses of up to 200 ng/ml HGF/SF were used (data not shown).

Although PKC and tyrosine phosphatase-mediated down-regulation of Met have been previously described (Gandino et al., 1990; Villa Moruzzi et al., 1993), we observed that depletion or inhibition of PKC using either PMA or staurosporine, and inhibition of tyrosine phosphatases using vanadate failed to restore the scattering response of MDCK-1 cells to HGF/SF treatment (data not shown). In addition, the spontaneous development of MDCK-1 cell phenotypes was

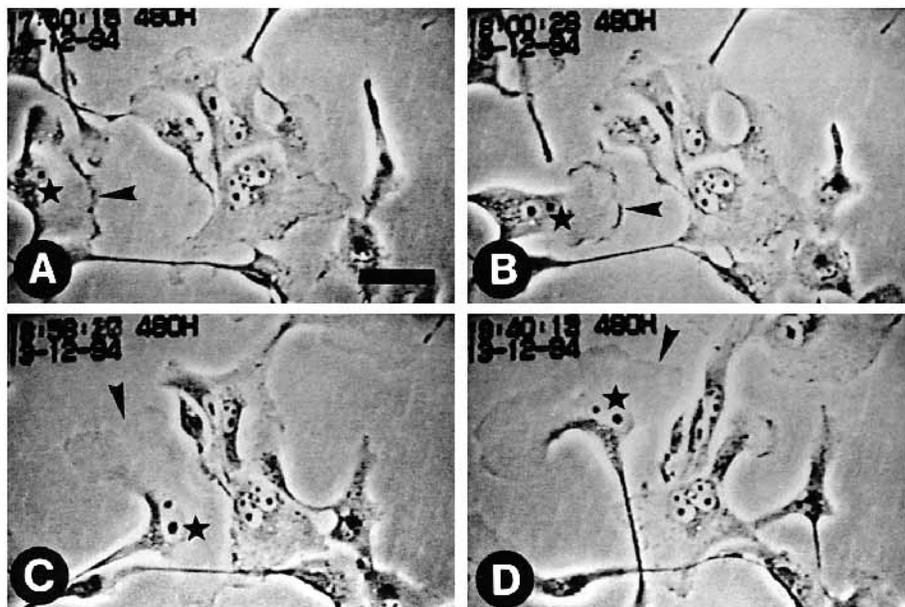


Fig. 2. Successive time-lapse video images of MDCK-1 cells in culture. The times (hours: minutes: seconds) are indicated in the top left of each photograph. The starred cell represents the same cell throughout and arrowheads indicate areas of membrane ruffling at the leading edge of this cell. Bar, 50 μ m.

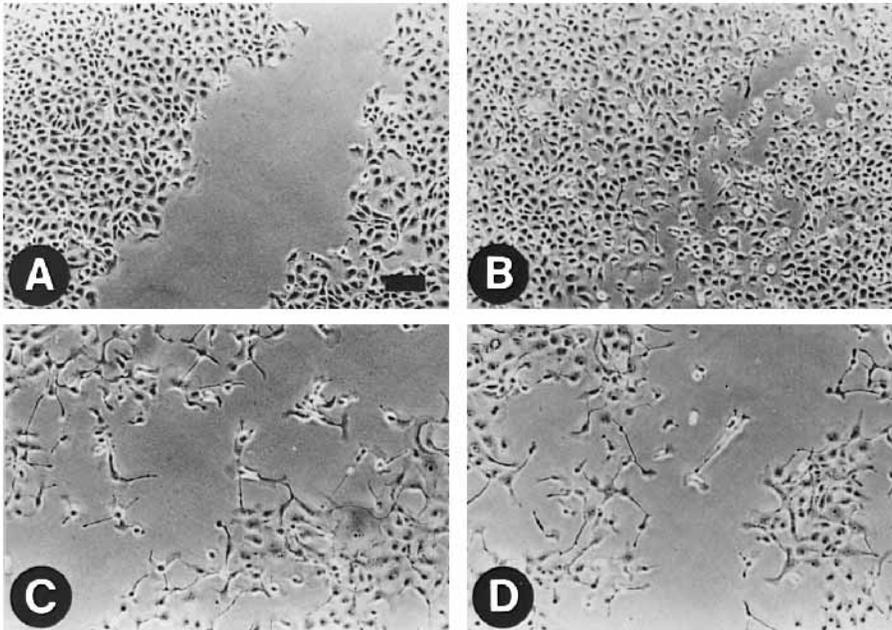


Fig. 3. The migratory properties of MDCK cells (A and B) and MDCK-1 cells (C and D). Cells were grown to confluence, and an artificial wound etched through the monolayer prior to 24 hour incubation with either control medium (A and C) or medium supplemented with 10 ng/ml HGF/SF (B and D). Bar, 100 μ m.

shown unlikely to be due to autocrine stimulation by secreted factors, since the tissue culture supernatant from MDCK-1 cells had no effect on MDCK cell morphology (data not shown).

MDCK-1 cells exhibit spontaneous invasive behaviour and display partial tubule formation in collagen gels

The ability of MDCK and MDCK-1 cells to invade collagen type I gels was investigated and found to be markedly different (Fig. 4). In the absence of HGF/SF, MDCK cells failed to invade the collagen gels. However, their invasive potential was greatly enhanced following the addition of HGF/SF, with increasing invasion observed over a 4-day period. In contrast, MDCK-1 cells were able to invade collagen gels to a smaller but nonetheless significant extent in the absence of HGF/SF, although clearly not to the level of HGF/SF-treated parental

MDCK cells. Furthermore, MDCK-1 cells appeared to reach an invasive plateau after 2 days in culture, with no further increase occurring after this time. Following the addition of

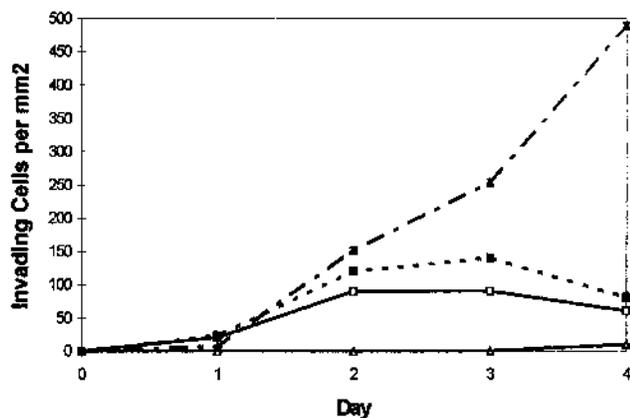


Fig. 4. The invasion of MDCK (Δ , \blacktriangle) and MDCK-1 (\square , \blacksquare) cells into collagen type I gels. Cells were seeded in either the absence (Δ , \square) or presence (\blacktriangle , \blacksquare) of 10 ng/ml HGF/SF, and the number of cells invading the gel were quantified over a 4 day period.

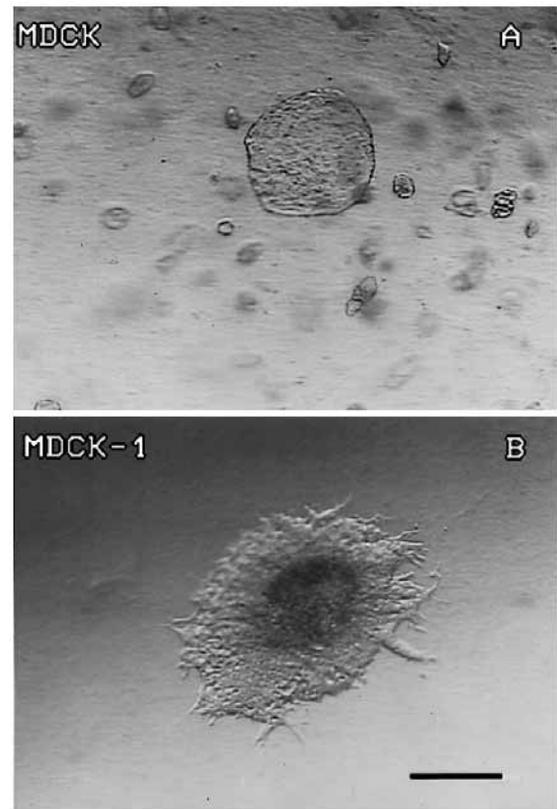


Fig. 5. The partial tubule formation displayed by MDCK-1 cells when grown in collagen type I gels in the absence of HGF/SF. MDCK cells (A) and MDCK-1 cells (B) were seeded within collagen as described in Materials and Methods in the absence of HGF/SF. Photographs were taken at 2 weeks. Bar, 50 μ m.

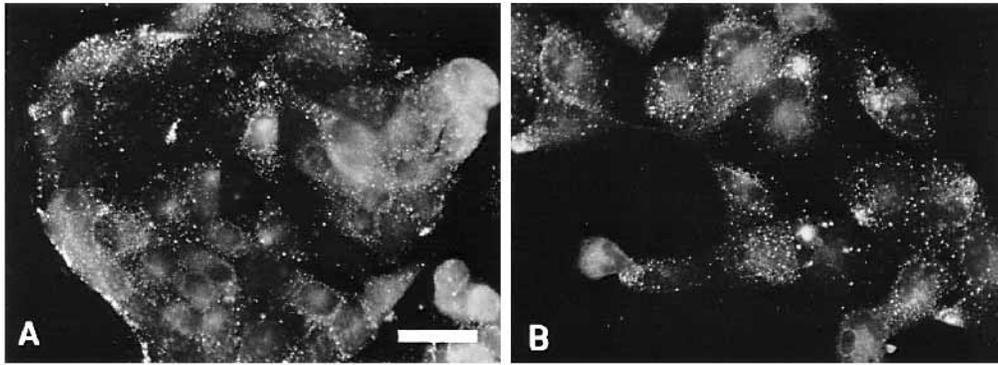


Fig. 6. Immunofluorescence staining of Met in MDCK cells (A) and MDCK-1 cells (B). Cells were stained using an antibody raised against the extracellular domain of Met (clone DO-24). The photographs show mainly basal cell surfaces. Bar, 100 μ m.

HGF/SF, the invasive potential of MDCK-1 cells was not significantly increased above basal levels over the 4 day period.

When grown within collagen type I gels for 2 weeks, MDCK cells grew into large spherical cysts consisting of many cells in the absence of HGF/SF (Fig. 5A). Following the addition of HGF/SF, extensive branching tubule formation occurred over the course of several days as reported previously (data not shown; Montesano et al., 1991). In contrast, within a few days of growth in collagen, MDCK-1 cells formed small cysts with highly irregular edges, and displayed apparent invasion into the surrounding matrix. Moreover, after 2 weeks in culture, structures resembling nascent tubules were observed (Fig. 5B). These structures never reached the complexity of the branching tubules observed in HGF/SF-treated parental MDCK cells over the time period investigated (5 weeks). Furthermore, HGF/SF treatment had no effect on further tubule development in MDCK-1 cells (data not shown).

MDCK and MDCK-1 cells display comparable Met expression and distribution on the cell surface

The level of Met expression and its distribution on the surface of MDCK and MDCK-1 cells were investigated. Immunofluorescence microscopy revealed that Met was expressed mainly as large dot-like aggregates of varying sizes on the surfaces of both cell lines (Fig. 6A,B). These aggregates appeared to be expressed over the whole cell surface of both MDCK and MDCK-1 cells to similar levels. However, whereas Met was often distributed more intensely along the borders of parental MDCK cell colonies in areas where a free edge was apparent, this pattern of Met distribution was not observed in MDCK-1 cells (Fig. 6A,B). Flow cytometric analysis provided further evidence that the overall cell surface expression was similar in both cell lines. When probed with an antibody against the extracellular domain of Met, the increase in fluorescence was identical in MDCK and MDCK-1 cells (Fig. 7).

Aberrant protein tyrosine phosphorylation in the MDCK-1 cell line

The basal level of protein tyrosine phosphorylation in MDCK-1 cells was compared to that of parental MDCK cells by immunoblotting with anti-phosphotyrosine antibodies (clone 4G10). Following overnight serum starvation, a number of proteins from the cell lysate of MDCK-1 cells were phosphorylated on tyrosine to a greater extent when compared with MDCK cells in the absence of HGF/SF stimulation (Fig. 8, lanes 1, 3). Proteins with molecular masses of approximately 60 kDa, 70 kDa, and 185 kDa were all phosphorylated on

tyrosine in MDCK-1 cells significantly above levels in parental MDCK cells, together with several proteins forming a broad band between 115 and 130 kDa. Moreover, we detected a tyrosine phosphorylated 220 kDa protein (p220), which was unique to MDCK-1 cells. Following stimulation with 100 ng/ml HGF/SF, MDCK cells showed a significant increase in the tyrosine phosphorylation of proteins with molecular masses of approximately 42 kDa, 44 kDa, 54 kDa, 60 kDa, 100 kDa and 145 kDa (p145^{met}), together with a cluster of proteins in the range of 105-130 kDa (Fig. 8, lane 2). In contrast, MDCK-1 cells displayed only a small increase in the tyrosine phosphorylation of a 145 kDa protein (p145^{met}) above basal levels following HGF/SF stimulation (Fig. 8, lane 4). There was no increase in the tyrosine phosphorylation of other proteins above basal levels, even when doses of up to 800 ng/ml HGF/SF were used (data not shown).

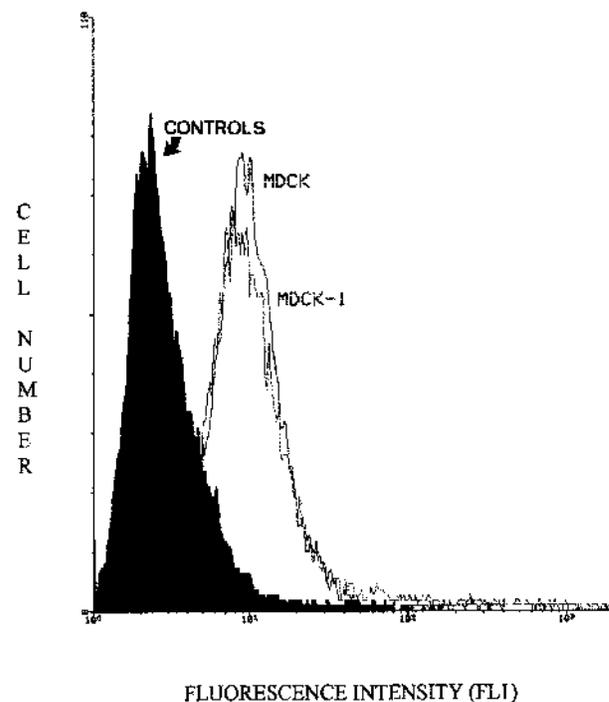


Fig. 7. Flow cytometric analysis of cell surface Met expression in MDCK and MDCK-1 cells. Non-fixed cells were probed with an antibody against the extracellular domain of Met (clone DO-24). Experimental conditions are indicated on the chart. Controls represent second stage antibody staining only.

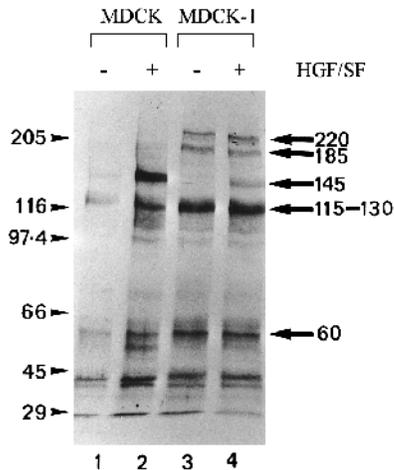


Fig. 8. Phosphotyrosine staining of MDCK and MDCK-1 cell lysates. Cells were serum starved overnight prior to stimulation with 100 ng/ml HGF/SF for 10 minutes where indicated. 8% SDS-PAGE/anti-phosphotyrosine immunoblotting was performed on the Triton X-100 soluble extracts from MDCK cells (lanes 1 and 2) and MDCK-1 cells (lanes 3 and 4). 10 μ g of protein extract was loaded per lane. Positions of molecular mass markers and different tyrosine phosphorylated proteins mentioned in the text (in kDa) are indicated.

MDCK and MDCK-1 cells exhibit similar responses to EGF

To investigate whether the insensitivity of MDCK-1 cells to HGF/SF is more general to other growth factors, the cellular and biochemical responses of MDCK and MDCK-1 cells to EGF was compared. EGF (ranging from 1 to 300 ng/ml) enhanced the proliferation of both cell lines to similar extents, but had no effect on cell scattering (data not shown). In addition, EGF induced the tyrosine phosphorylation (autophosphorylation) of the EGF receptor (EGFR, 170 kDa) in both MDCK cells (Fig. 9A) and MDCK-1 cells (Fig. 9B) *in vivo*, together with a number of other proteins with molecular masses of approximately 42 kDa, 44 kDa, 52 kDa, 70 kDa, 85 kDa, 100 kDa, 120 kDa and 185 kDa, with only slight variations in the levels of tyrosine phosphorylation displayed between the two cell lines.

MDCK-1 cells display variations in Met-associated signalling

Immunoprecipitation of Met from MDCK and MDCK-1 cells yielded the following results (Fig. 10A,B). Parental MDCK cells exhibited a basal level of p145^{met} tyrosine phosphorylation *in vivo* which increased approximately 2.5-fold following HGF/SF stimulation (Fig. 10A, lanes 1 and 2, B). The *in vitro* autokinase activity of p145^{met} also increased in MDCK cells following HGF/SF stimulation, although this was less obvious due to the high basal activity observed in the absence of HGF/SF (Fig. 11, lanes 1 and 2). In addition, a 170 kDa protein (p170) was also tyrosine phosphorylated *in vitro* in the absence of HGF/SF stimulation, and displayed a slight increase in tyrosine phosphorylation following HGF/SF stimulation (Fig. 11, lanes 1 and 2). In contrast, MDCK-1 cells in the absence of HGF/SF displayed a much lower level of basal p145^{met} tyrosine phosphorylation *in vivo* (Fig. 10A, lane 3, B) and autokinase activity *in vitro* (Fig. 11, lane 3). Following

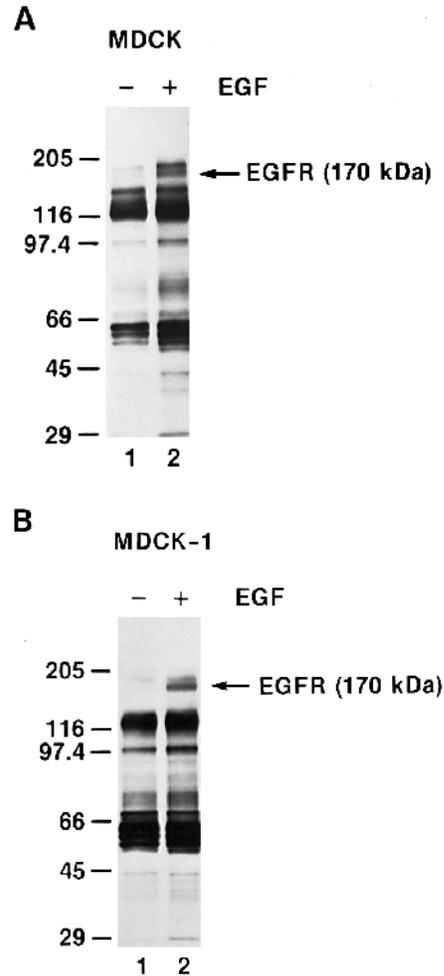


Fig. 9. Effect of EGF on protein tyrosine phosphorylation in MDCK cells (A) and MDCK-1 cells (B). Cells were serum starved overnight prior to stimulation with 100 ng/ml EGF for 10 minutes where indicated. 8% SDS-PAGE/anti-phosphotyrosine immunoblotting was performed on the Triton X-100 soluble extracts. 10 μ g of protein extract was loaded per lane. Positions of molecular mass markers (in kDa) and the EGFR are indicated.

HGF/SF stimulation, both p145^{met} phosphorylation (Fig. 10A, lane 4, B) and autokinase activity (Fig. 11, lane 4) increased in MDCK-1 cells above basal levels, but neither reached the respective levels observed in parental MDCK cells \pm HGF/SF. In addition, the *in vitro* tyrosine phosphorylation of p170 was not observed either in the absence or presence of HGF/SF stimulation (Fig. 11, lanes 3 and 4).

Another outstanding feature associated with Met immunoprecipitates from MDCK-1 cells was the presence of two proteins designated p185 and p220, both of which were constitutively phosphorylated on tyrosine *in vivo* (Fig. 10A, lanes 3 and 4). Neither p185 or p220 were observed in Met immunoprecipitates from parental MDCK cells \pm HGF/SF (Fig. 10, lanes 1 and 2). The degree of tyrosine phosphorylation of p185 and p220 was not increased by HGF/SF stimulation *in vivo* (Fig. 10A, compare lanes 3 and 4). Moreover, both p185 and p220 were tyrosine phosphorylated *in vitro* in the Met immunocomplex kinase assay \pm HGF/SF, with a slight increase in phosphorylation observed following HGF/SF stimulation (Fig. 11, lanes 3 and 4). The presence of these proteins in Met immunocomplexes was not due to non-specific interactions, since immunoprecipitations with control mouse serum or Protein A-agarose alone under identical conditions did not yield either p185 or p220. Furthermore, immunoprecipitation of Met using a different antibody against the C terminus (clone

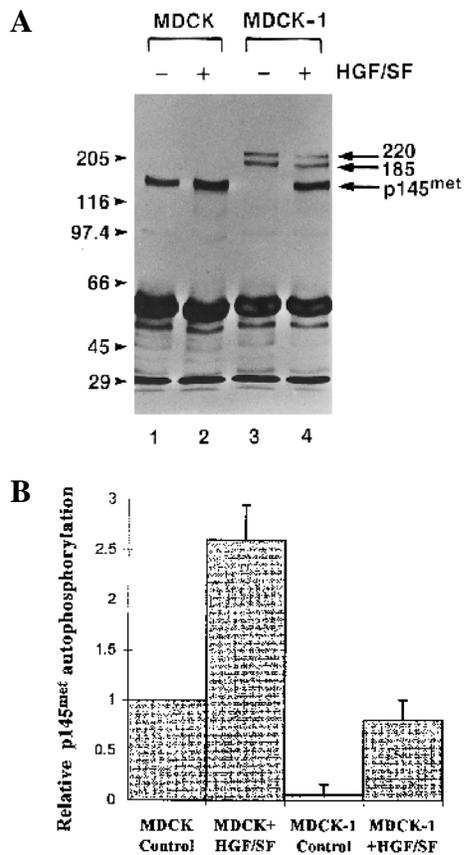


Fig. 10. (A) Phosphotyrosine staining of Met immunoprecipitates from MDCK cells (lanes 1 and 2) and MDCK-1 cells (lane 3 and 4). Cells were serum starved overnight prior to stimulation with 100 ng/ml HGF/SF for 10 minutes where indicated. Cells were then lysed and 8% SDS-PAGE/anti-phosphotyrosine immunoblotting performed on Met immunoprecipitates. The positions of molecular mass markers and different tyrosine phosphorylated proteins mentioned in the text are indicated (in kDa). (B) Densitometry measurements of p145^{met} tyrosine phosphorylation from A. Values are expressed relative to Met phosphorylation in control parental MDCK cells. Error bars represent the s.e.m. from three individual experiments. Experimental conditions are indicated under each column.

SP 260) produced identical results (data not shown). Therefore, both p185 and p220 are immunoprecipitated specifically with anti-Met antibodies.

Met immunocomplexes from MDCK-1 cells demonstrate a higher tyrosine kinase activity in vitro

A non-radioactive in vitro TK assay was performed on Met immunoprecipitates from both MDCK and MDCK-1 cells \pm HGF/SF as described in Materials and Methods (Fig. 12). A low basal level of TK activity associated with Met in control MDCK cells, and this increased by approximately 40% following HGF/SF stimulation. More interestingly, Met immunocomplexes from MDCK-1 cells displayed a 3.5-fold increased TK activity in the absence of HGF/SF relative to control parental MDCK cells that did not increase following HGF/SF stimulation. The enhanced TK activity associated with Met immunocomplexes was shown to be specific since

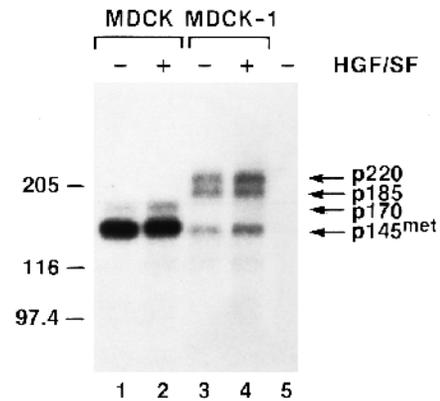


Fig. 11. In vitro kinase activity of Met and associated proteins from MDCK cells (lanes 1 and 2) and MDCK-1 cells (lanes 3 and 4). Cells were treated where indicated with 100 ng/ml HGF/SF for 10 minutes prior to solubilisation. Met immunocomplexes were then used in an in vitro kinase assay as described in Materials and Methods. Lane 5 represents control immunoprecipitation in the absence of cell lysate. The positions of molecular mass markers and the different proteins phosphorylated in vitro (in kDa) are indicated.

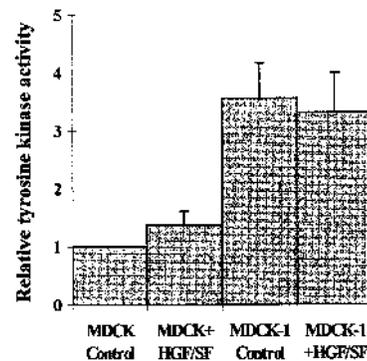


Fig. 12. In vitro tyrosine kinase (TK) activity associated with Met immunocomplexes from MDCK and MDCK-1 cells. Met immunoprecipitates were prepared from cells following stimulation with 100 ng/ml HGF/SF for 10 minutes, and assayed for in vitro TK activity as described in Materials and Methods. Experimental conditions are described under each column. Results are expressed relative to the TK activity associated with Met from control MDCK cells. Error bars represent the s.e.m. from triplicate samples.

control immunoprecipitations, using an isotype matched antibody (monoclonal IgG2a anti-rat thymocyte), did not precipitate any TK activity in either cell line (data not shown).

Herbimycin A reverts MDCK-1 cell morphology, with concomitant reduction in p220 tyrosine phosphorylation

Treatment of MDCK-1 cells with the tyrosine kinase inhibitor, herbimycin A, induced major changes in MDCK-1 cell morphology (Fig. 13). Following 24 hours treatment, the 'semi-scattered' appearance of MDCK-1 cell colonies reverted to a more parental MDCK-like morphology, whereby cell-cell contacts appeared to become stabilised, resulting in the formation of more adhesive cell colonies. Time-lapse video microscopy showed that this was associated with a considerable reduction in MDCK-1 cell motility (mean velocity = 1.2

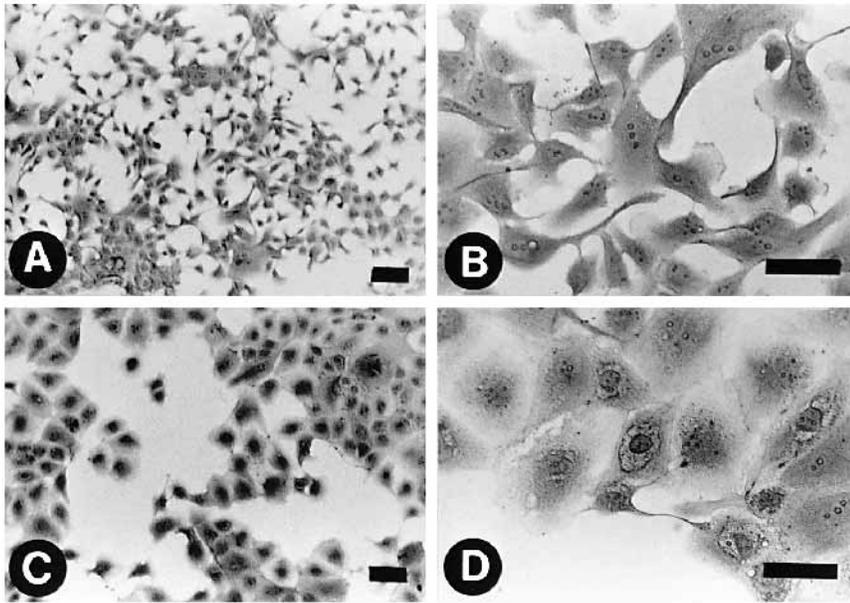


Fig. 13. The effect of herbimycin A on MDCK-1 cell morphology. MDCK-1 cells were grown to approximately 25% confluence and incubated for 24 hours in either control medium (DMSO, A and B) or medium supplemented with 1 μ M herbimycin A (C and D). Bars, 100 μ m.

cm/hour). HGF/SF did not induce the scattering of herbimycin A treated MDCK-1 cells, and herbimycin A also inhibited the scattering of parental MDCK cells following HGF/SF treatment (data not shown). To investigate a possible correlation between altered MDCK-1 cell morphology and aberrations in Met-associated signalling, protein tyrosine phosphorylation was examined both in the absence and presence of herbimycin A. Treatment (24 hours) with herbimycin A reduced the tyrosine phosphorylation of predominantly p220 in MDCK-1 cell lysates (Fig. 14A). p185, which is only observed as a very weak band in Fig. 14A, showed no reduced phosphorylation following herbimycin A treatment even after prolonged ECL exposure (data not shown). In addition, Met immunocomplexes from MDCK-1 cells treated with herbimycin A similarly displayed a reduction in the tyrosine phosphorylation of p220, with little if any reduction in that of p185 (Fig. 14B). These data indicate a correlation between the tyrosine phosphorylation of p220 and alterations in MDCK-1 cell morphology.

DISCUSSION

The MDCK cell line has been used to investigate a variety of responses to HGF/SF *in vitro*, including cell scattering and motility (Stoker et al., 1987), matrix invasion (Weidner et al., 1990) and tubulogenesis (Montesano et al., 1991). In this report, we have characterised a variant of the MDCK cell line, MDCK-1, which spontaneously displays many features associated with partial Met-HGF/SF activation, yet fails to respond to HGF/SF stimulation. We have provided evidence to suggest that variations in Met-related, or Met-associated signalling may contribute to the spontaneous phenotypes of MDCK-1 cells in the absence of HGF/SF, in addition to the loss of HGF/SF sensitivity.

In the MDCK-1 cell line, we have shown that the loss of cellular responses to HGF/SF is not arising through a loss of Met expression from the cell surface. However, our results demonstrate that the extent of p145^{met} phosphorylation *in vivo*

is much reduced in MDCK-1 cells when compared with parental MDCK cells, both basally and in response to HGF/SF stimulation. This in turn relates to the reduced autophosphorylation of p145^{met} observed *in vitro* (autokinase activity), since it has previously been shown that autophosphorylation of p145^{met} on specific tyrosine residues is required to induce the full TK activity of Met (Naldini et al., 1991c; Longati et al.,

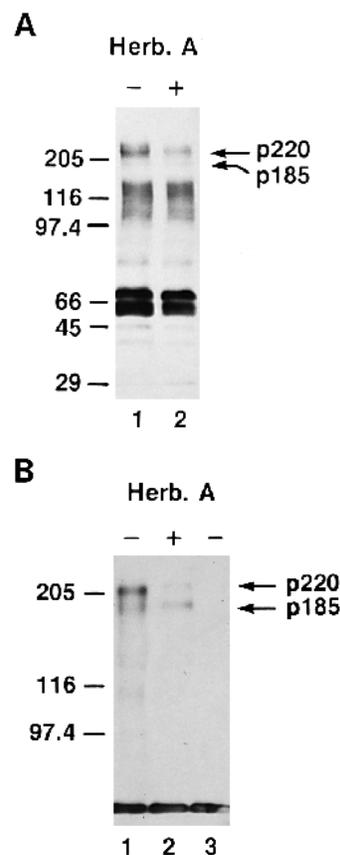


Fig. 14. Effect of herbimycin A on protein tyrosine phosphorylation in MDCK-1 cells. Cells were cultured for 24 hours either in the absence (DMSO, lanes 1) or presence (lanes 2) of 1 μ M herbimycin A. 8% (A) or 6% (B) SDS-PAGE/anti-phosphotyrosine immunoblotting was performed with the Triton X-100 soluble cell lysates (A) or Met immunoprecipitates (B) obtained as described in Materials and Methods. Lane 3 in B represents control Met immunoprecipitation in the absence of cell lysate. The positions of molecular mass markers (in kDa) and the tyrosine phosphorylated p185 and p220 proteins are indicated.

1994). It follows that reduced p145^{met} activity in MDCK-1 cells results in the lack of further autophosphorylation of tyrosine residues lying outside the TK domain, and subsequent failure to target down-stream signalling components possessing SH2 domains (Bardelli et al., 1992; Faletto et al., 1993; Ponzetto et al., 1994; Pelicci et al., 1995). In support of this hypothesis we have shown that one target of Met, the adapter protein p52 Shc (Pelicci et al., 1995), is tyrosine phosphorylated in MDCK-1 cells in response to only EGF, whereas parental MDCK cells display increased Shc phosphorylation in response to both EGF and HGF/SF (C. P. W., unpublished observations). In addition, some other proteins phosphorylated in MDCK cells in response to HGF/SF are not observed in MDCK-1 cells above basal levels, suggesting that additional downstream signalling molecules are not correctly targeted by p145^{met} in MDCK-1 cells. Thus, the MDCK-1 cell line may be of use in determining the absolute requirement of different signalling components in the variety of Met-mediated responses.

The reasons for reduced p145^{met} autophosphorylation in MDCK-1 cells remain unclear. It has previously been shown that activation of PKC down-regulates p145^{met} TK activity (Gandino et al., 1990). However, depletion/inhibition of PKC did not restore the HGF/SF response in MDCK-1 cells. Furthermore, PKC is also known to negatively regulate the EGF receptor (EGFR) via similar mechanisms (Cochet et al., 1984). Since tyrosine phosphorylation of the EGFR and other proteins in response to EGF stimulation in MDCK-1 cells is comparable to that of parental MDCK cells, PKC-mediated down-regulation of p145^{met} seems unlikely. Likewise, Ca²⁺-dependent kinases have been shown to down-regulate the autophosphorylation of both Met and the EGFR alike (Friedman et al., 1989; Gandino et al., 1991), suggesting that this too is an unlikely explanation for reduced p145^{met} activity in MDCK-1 cells. It is also unlikely that p145^{met} in MDCK-1 cells is maintained in a dephosphorylated state by an associated tyrosine phosphatase since vanadate treatment failed to restore the HGF/SF response.

The finding that there is no apparent difference in the EGF responses between MDCK and MDCK-1 cells suggests that the abnormality in MDCK-1 cells may be specific to Met. Our data suggest that the two constitutively tyrosine phosphorylated proteins p185 and p220 may play a role in this specificity, since both are specifically immunoprecipitated from MDCK-1 cells with Met antibodies. It is not certain at this stage whether these proteins are related to Met, or constitutively associated with Met in MDCK-1 cells. In addition, we have shown that despite reduced p145^{met} autokinase activity in vitro, Met-immunocomplexes from MDCK-1 cells display a marked increase in overall TK activity when compared with parental MDCK cells. It is likely therefore, that p185 and/or p220 contribute to the enhanced TK activity observed in vitro since both are phosphorylated, a common feature associated with receptor and non-receptor TKs (van der Geer et al., 1994).

The enhanced TK activity associated with Met in vitro also correlates with the higher basal levels of protein tyrosine phosphorylation observed in MDCK-1 cell lysates in vivo, which in turn may be involved in the development of the altered MDCK-1 cell phenotype. In support, the potent tyrosine kinase inhibitor herbimycin A reverts the morphology of the MDCK-1 cells towards a parental MDCK-like morphology, with a con-

comitant reduction in the tyrosine phosphorylation of p220. This suggests that the tyrosine phosphorylation of p220 may be partly responsible for alterations in MDCK-1 cell phenotypes. However, the cause-effect relationship between these events remains to be established. Herbimycin A has previously been shown to revert the morphologies of various cells transformed by a variety of oncogenic TKs including src, fyn, lck, bcr-abl and erbB2 (Uehara et al., 1988; Fukazawa et al., 1991). Thus, the MDCK-1 cell line would appear to represent a variant line in which constitutive activation of a Met-related or a Met-associated TK results in altered cell behaviour, mimicking some aspects of morphological transformation consistent with constitutive Met activation. It will be of interest to investigate the possibility that this may be directly associated with the loss of responses to HGF/SF through reduced p145^{met} activity. Attempts are currently underway to identify both p185 and p220, and to investigate their putative roles in MDCK-1 cell behaviour.

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