

MK: a pluripotential embryonic stem-cell-derived neuroregulatory factor

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Summary

MK is a gene encoding a secreted heparin-binding polypeptide originally isolated by differential screening for genes induced by retinoic acid (RA) in HM-1 embryonal carcinoma cells. Here we report that MK is expressed at high levels in both embryonal carcinoma and pluripotential embryonic stem cells and their differentiated derivatives. MK expression in these cell types is unaffected by the presence or absence of RA. Recombinant MK protein (rMK) was produced by transient expression in COS cells and purified by heparin affinity chromatography. rMK is a weak mitogen for 10T1/2 fibroblast cells but inactive as a mitogen for Swiss 3T3 fibroblasts. rMK is a potent mitogen for neuroectodermal precursor cell types generated by treatment of 1009 EC cells with RA but has no mitogenic or neurotrophic effects on more mature 1009-derived neu-

ronal cell types. rMK is active as an in vitro neurotrophic factor for E12 chick sympathetic neurons and its activity is markedly potentiated by binding the factor to tissue-culture plastic in the presence of heparin. Stable 10T1/2 cells lines have been established which express MK. These cells do not exhibit any overt evidence of cell transformation but extracellular matrix preparations derived from these cells are a potent source of MK biological activity. It is concluded that MK is a multifunctional neuroregulatory molecule whose biological activity depends upon association with components of the extracellular matrix.

Key words: embryonic stem cells, growth factor, heparin, extracellular matrix, neurotrophic factor.

Introduction

Murine embryonal carcinoma (EC) and pluripotential embryonic stem (ES) cells have proved to be valuable experimental systems for the identification of growth and differentiation regulatory factors with actions in early development (reviewed by Heath et al., 1990). In particular, both EC and ES cells have been found to secrete at least two distinct species of heparin-binding growth factors (Heath et al., 1989). One of these factors has been firmly identified (Heath et al., 1989) as murine FGF-4 (K-FGF/Hst). FGF-4 is a secreted member of the fibroblast growth factor (FGF) family with analogous biological functions to the FGF-2 (bFGF) prototype FGF, including the ability to induce mesoderm formation in animal pole explants of *Xenopus laevis* (Paterno et al., 1989). The identity of the second, biochemically distinct, heparin-binding growth factor species purified from EC-cell-conditioned medium has, however, remained obscure.

It has recently emerged that a number of growth factors exhibit high affinity for immobilised heparin, but are unrelated in sequence to the FGF family of growth factors. Thus, HB-GAM is an $18 \times 10^3 M_r$ protein which was isolated by

heparin affinity chromatography from adult brain as a neurite outgrowth-promoting factor for foetal rat CNS neurons. It proved, upon sequence analysis, to be entirely distinct from bFGF, although similar in terms of physical properties (Rauvala, 1989; Merenmies and Rauvala, 1990). An identical agent was subsequently isolated from adult brain preparations in other laboratories under the names pleiotropin (PTN, Li et al., 1990; Courty et al., 1991) and heparin-binding neurotrophic factor (HBNF, Bohlen et al., 1990). Tezuka et al. (1990) also isolated cDNA sequences encoding HB-GAM/HBNF/PTN by differential cloning of genes expressed in osteogenic tissues.

HB-GAM/HBNF/PTN is 65% identical in amino acid sequence to the secreted heparin-binding protein MK, which was first described as a retinoic acid-induced gene product in HM-1 EC cells (Kadomatsu et al., 1988; Tomomura et al., 1990a). In situ hybridisation studies showed that the MK gene is transcribed in a number of embryonic cell types including the egg cylinder, nervous system, lung and kidney (Kadomatsu et al., 1990). The identification of these two novel heparin binding growth factors prompted us to examine HB-GAM and MK expression in murine ES and EC cells.

We report here that, in contrast to the original discovery of MK as a gene induced upon differentiation of HM-1 EC cells, its product proves to be abundant in both germ line competent ES cells and their differentiated derivatives; its expression in these cell types is not significantly modified by the presence or absence of retinoic acid. Furthermore, purified recombinant MK protein proves to have both mitogenic and neurotrophic effects on a variety of neuronal cell types *in vitro*. A striking feature of the biological action of MK is that its activity is strongly enhanced by association with components of the extracellular matrix in the presence of heparin.

Material and methods

Cell culture and reagents

Cell culture medium comprised DME:F12 (50:50 vol:vol; Gibco) supplemented with foetal calf serum (10% by volume, selected batches). All cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂ in air. ES cell culture medium was additionally supplemented (unless otherwise indicated) with 10 ng/ml recombinant leukemia inhibitory factor (Smith et al., 1988) and 10⁻⁴ M betamercaptoethanol (Sigma, tissue-culture grade). In some experiments, the FCS used for cell culture was charcoal stripped to remove endogenous RA by the method described by VanderBurg et al. (1988).

Swiss 3T3 (Flow laboratories) and 10T1/2 fibroblasts were propagated as mass cultures according to the '3T3' regime described by Todaro and Green (1963). Embryonal carcinoma cells (P19 and 1009) and embryonic stem cells (E14TG2a) were maintained as described (Rathjen et al., 1990).

P19 EC cell differentiation was induced by exposure to RA as described by Rudnicki and McBurney (1987). E14TG2a ES cell differentiation was achieved either by exposure to 10⁻⁷ M RA (all *trans*; Sigma, added from a 10⁻² M stock dissolved in tissue-culture trade DMSO) or by culture of cells at low density in the absence of leukemia inhibitory factor (Smith et al., 1988). 1009 EC cell differentiation was achieved by plating 1009 cells at 10⁴ cells/well into 5 mm 6-well cluster dishes in 4 ml of medium (DME:F12 10%FCS). RA was added from a 10⁻² M stock (dissolved in DMSO) after 24 hours to a final concentration of 5×10⁻⁷ M.

Cell multiplication

Induction of DNA synthesis in experimental cell populations was determined by [³H]thymidine incorporation as described by Heath (1987).

Stable cell lines

6×10⁶ 10T1/2 fibroblasts were co-transfected with pXMT2-MK by the calcium phosphate method of Chen and Okyama (1987) using 10 µg of pXMT2-MK and 10 µg of PGK-Neo. The day after transfection, the cells were plated into 10×10 cm tissue-culture dishes and cultured in the presence of 300 µg/ml G418 (Sigma) for 14 days. A random sample of G418 resistant colonies were picked under a dissecting microscope and expanded in mass culture for further analysis. A matrix preparation was obtained from selected clones by release of cells with EDTA as described by Rathjen et al. (1990).

Purification of rMK

Conditioned media

rMK was purified from media conditioned by COS cells transfected with the MK expression plasmid pXMT2MK. 5×10⁷ COS cells were transfected with 50 µg of the PXMT2MK by electro-

poration (Biorad gene pulser, 330 V, 500 µF) and plated into 175 cm² tissue-culture flasks in 75 ml of culture medium. The following day the medium was changed to 100 ml DME:F12 supplemented with 10 µg/ml transferrin (Sigma) and 10 µg/ml Heparin (BDH). The cells were cultured for a further 48 hours and the cell-culture medium collected and passed through a 0.22 µm filter prior to processing for purification.

COS-cell-conditioned medium (200 ml) was pumped onto a 1 ml Hitrap heparin affinity column (Pharmacia) at a flow rate of 200 µl/minute at 4°C. The column was washed with 10 ml of 50 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and the rMK eluted by washing the column with 5 ml of phosphate buffer (pH 7.4) containing 2 M NaCl. The 5 ml heparin affinity eluate was then desalted (pharmacia PD-10 desalting column) and used for biological assays.

Final purification was obtained by reverse phase chromatography on a Brownlee rp-300 microbore column. The heparin affinity eluate was loaded directly onto the column at a flow rate of 1 ml/minute and then washed with solvent A (0.1% trifluoroacetic acid in water) for 5 minutes at a flow rate of 1 ml/minute. The flow rate was adjusted to 0.1 ml/minute over 1 minute and protein eluted by a gradient (0-60% at 1%/minute) of solvent B (0.1% trifluoroacetic acid in acetonitrile (Far UV grade BDH)). rMK, purified by reverse-phase electrophoresis, was analysed by SDS-PAGE (Phastgel system Pharmacia 20% linear gels). An initial standard sample of rMK purified by rpHPLC was subjected to amino acid analysis for protein quantification. In subsequent experiments, rMK was quantified by comparison of peak height (280 nm) with data obtained from the standard sample. The yield of rMK produced by these methods varied from 18 µg to 23 µg (3 experiments from 200 ml of initial conditioned media) and agreed well with the protein concentration of the heparin affinity eluate determined by dye binding assay (Biorad, bovine serum albumin standard). Recoveries were not determined.

Tissue-culture substrata and media

Tissue-culture substrata were coated where indicated with a solution containing 0.1 mg/ml poly-L-lysine in distilled water for 5 minutes. Substrata were rinsed three times in water and allowed to dry. In some experiments, the substrata were further coated with either EHS laminin (Sigma 10 µg/ml in PBS, 2 hours at room temperature) and rinsed with DMEM immediately before use, or heparin (10 µg/ml (BDH) in PBS, 2 hours at room temperature), or a mixture of both. In some experiments, MK was coated by incubation for 2 hours at room temperature, over prepared substrata at designated concentrations to a maximum of 100 ng/ml. Growth factors were added to the culture medium at the nominated concentrations.

Assay for neurotrophic activity

The sympathetic ganglia from embryonic day-12 (E12) chicks were dissected out and dissociated into single cells using standard techniques (Wakade et al., 1982). Briefly, the ganglia were trypsinised for 30 minutes in Ca²⁺- and Mg²⁺-free phosphate-buffered saline, triturated and the resultant single-cell suspension preplated over tissue-culture plastic in DMEM:10% FCS for a period of 45 minutes to enrich for neurons. They were plated at a concentration of 2.0×10³ cells per 16 mm well of a 24-well cluster under the appropriate experimental conditions. After incubation at 37°C for 2 days in the presence or absence of MK, the resulting cell survival and the proportion of neuronal cell bodies with neurites longer than 2 cell diameters were determined by phase-contrast microscopy.

Isolation of RNA

Cell pellets (10⁷-10⁸ cells) were washed in phosphate-buffered

saline (PBS) and lysed in 5 ml of 30 mM Tris pH 7.5, 150 mM NaCl, 15 mM MgCl₂, 0.4% Nonidet P40. Nuclei were removed by centrifugation for 15 minutes at 3000 g. An equal volume of Tunes (10 mM Tris, pH 7.5, 7 M urea, 0.35 M NaCl, 1 mM EDTA, 2% SDS) was added to the supernatant. The cytoplasmic RNA was extracted twice with methanol/chloroform and once with chloroform before ethanol precipitation and recovery by centrifugation. The pellet was washed with 70% ethanol before being dissolved in DEPC-treated water.

First strand DNA synthesis

2.5 µg of cytoplasmic RNA in 40 µl DEPC-treated water was heated at 65°C for 3 minutes, quenched on ice and added to 5 µl 10 × RTC buffer (BRL), 2.5 µl 10 mM dNTP mix, 1 µl (40 units) of RNasin (Promega), 0.5 µl (0.5 µg) oligo(dT) 12-18 (Pharmacia) and 1 µl (200 units) cloned MuLV-1 reverse transcriptase (BRL). The reaction was heated at 37°C for 1 hour and terminated by heating at 95°C for 5 minutes.

Oligonucleotides

Oligonucleotides were synthesised on an applied biosystems 380A DNA synthesiser. 5 MK had the sequence 5-GCAATTCAT-GAGCACCGAGGTTCTT-3'. The ATG underlined represents the initiation codon of murine MK (Matsubara et al., 1990; Tomomura et al., 1990b). The preceding nucleotides constitute an *EcoRI* site used to facilitate cloning of the amplified material. 3 MK had the sequence 5'-AAGTCGACGGCCTCCTGACTTAGTCCTT-3'. The first 8 residues constitute a *SalI* site used to facilitate cloning the amplified material. The rest of the oligonucleotide represents residues 415-434 of murine MK (Kadomatsu et al., 1988).

Polymerase chain reaction and cloning

1 µl of cDNA was amplified in a 50 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 µM each dNTP, 0.5 µM 5 MK and 3 MK oligonucleotide primers, 2 units Taq polymerase (Cetus). PCR was performed according to the following protocol: an initial denaturation for 5 minutes at 95°C was followed by 30 cycles of 1.5 minutes denaturation, annealing at 64°C for 1.5 minutes and extension for 1.5 minutes at 72°C. Following amplification the reaction was increased to 100 µl by addition of reaction buffer to which was added 5 µl 10% SDS and 1 µl of proteinase K (1 mg/ml). The reaction was heated at 55°C for 30 minutes before extraction with phenol/chloroform and ethanol precipitation. The amplified material was digested with *EcoRI* and *SalI* and subjected to electrophoresis in a 1.5% agarose gel. For cloning, the amplified band was excised from the gel and purified by means of a gene clean kit. The purified insert was cloned into *EcoRI/SalI* cut pBS KS+ (Stratagene).

MK was cloned into the expression plasmid pXMT2 in both orientations. For the sense orientation the pBSMK plasmid was cut with *SalI*, the ends were blunted by Klenow fill in and the insert released by digestion with *PstI*. The pXMT2 vector was prepared by cutting with *EcoRI* followed by blunting with Klenow and digestion with *PstI*. For the antisense orientation the insert was released from pBS/MK as an *EcoRI/SalI* fragment and cloned into *EcoRI/XhoI* cut pXMT2.

Northern blotting

RNA was analysed by electrophoresis in a formaldehyde/Mops-buffered agarose gel. Typically 15 µg of cytoplasmic RNA was run on a 1.25% agarose gel containing 2.22% formaldehyde. RNA samples were denatured by heating at 60°C for 10 minutes in loading buffer containing 50% formamide, 6.66% formaldehyde, 10% glycerol, 0.025% bromophenol blue and 10 µg/ml ethidium bromide. Samples were transferred to HYBOND N+(Amersham) by

capillary blotting for 5 hours in the presence of 40 mM NaOH. Probes for hybridisation were prepared by separating cloned inserts from plasmid vectors by agarose gel electrophoresis followed by purification using a Gene Clean Kit. DNA was labelled with ³²P by random priming (Boehringer). Prehybridisation was for 1 hour at 64°C in 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA. Radiolabelled probe (10⁸-10⁹ cts/minutes per µg) was added to the prehybridisation solution and hybridisation was allowed to proceed for 18 hours at 65°C. Filters were washed to a final stringency of 0.2× SSC/0.1% SDS at 64°C and exposed to X-ray film at -70°C with intensifying screens.

Results

Expression of MK transcripts in ES and EC cells

The presence of MK and HB-GAM transcripts in ES cells was examined by polymerase chain reaction (PCR) amplification of cDNA derived from E14TG2a ES cell poly(A)⁺ mRNA using primers corresponding to the nucleotide sequence of the predicted amino terminus and carboxy terminus of both proteins. A strong MK amplification product was obtained after 30 cycles of amplification. Significant amplification of an HB-GAM product was also observed although at lower levels. The PCR-amplified cDNAs were cloned into plasmid vectors for further analysis of MK gene expression.

Northern blot analysis of total RNA from P19EC or E14TG2a ES cells with the MK cDNA revealed a strong signal from a molecule of an apparent size of 900 bp (Fig. 1) which was in accord with the previous reported size of the MK transcript (Tomomura et al., 1990b). No detectable signal was observed upon northern hybridisation of the same RNAs with the cloned HB-GAM cDNA. It thus appears that MK (but not HB-GAM) is an abundant transcript in both EC and ES cells. These results were unex-

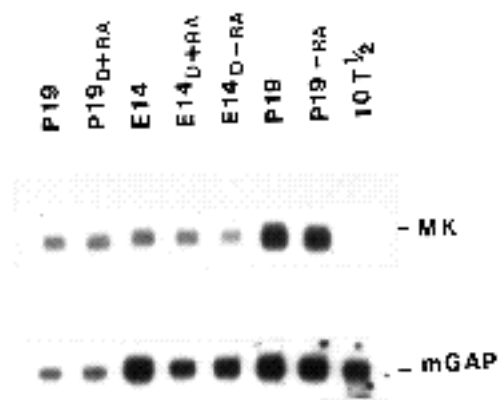


Fig. 1. Northern blot analysis of MK expression in murine EC and ES cells. Total cytoplasmic RNA was probed with ³²P-labelled MK cDNA (MK) and the blot reprobbed with a murine glyceraldehyde phosphate dehydrogenase cDNA (mGAP) as a loading control. Samples are: P19, P19 EC cell RNA; P19 D+RA, differentiated P19 EC cells derived by RA treatment; E14, E14 ES cells; E14 D+RA, E14 ES cells differentiated by exposure to RA; E14 D-RA, E14 ES cells differentiated by withdrawal of Leukemia inhibitory factor; P19 -RA, P19 EC cells propagated in retinoid depleted FCS; 10T1/2, 10T1/2 fibroblast RNA.

pected since MK transcripts were reported to be present at very low levels in HM-1 EC cells (Kadomatsu et al., 1988; Huang et al., 1990) and increased significantly upon exposure of the cells to retinoic acid. Northern hybridisation of P19 and E14TG2a cells differentiated by exposure to RA revealed that MK mRNA expression was not significantly altered by either exposure to 10^{-7} M retinoic acid or differentiation (by withdrawal of LIF) of ES cells in the absence of retinoic acid. One possible explanation for these results is that MK expression is induced in these cells by trace levels of retinoids present in FCS. However, the expression of MK mRNA in P19 cells grown in the presence of FCS depleted of retinoids by charcoal stripping was equivalent to that observed in untreated FCS (Fig. 1).

We conclude that MK is expressed in both undifferentiated stem cells and their differentiated derivatives and that its expression, in these cell types, is not detectably affected by exposure to RA. Twelve full-length MK cDNAs were also isolated by screening approximately 100,000 clones of a P19 EC cell cDNA library with the PCR-derived MK cDNA. This finding is in accord with the results of the northern hybridisation analysis and indicates that MK is a relatively abundant transcript in EC and ES cells.

Biological actions of MK protein

The biological function(s) of MK has not been clearly defined. Muramatsu and his colleagues have shown that MK protein derived from expression in eukaryotic cells has neurite-promoting actions on PC12 cells (Muramatsu and Muramatsu, 1991) although this activity was only observed at relatively high protein concentrations (100 ng/ml) where the possibility of contamination by other bioactive factors cannot be firmly excluded. Maruta and co-workers (Maruta et al., 1992) have more recently shown that bacterially expressed MK has neurite outgrowth promoting effects on spinal cord neurons but again the biological actions were manifest at high concentrations in which cross-reactivity with receptor systems for other neurotrophic factors cannot be firmly excluded.

Production of recombinant MK protein

The MK cDNA described above was cloned into the eukaryotic expression vector PXMT2, which was then transfected into COS cells. Serum-free, conditioned media were collected from the COS cells and the MK protein purified by heparin affinity chromatography. Characterisation of heparin affinity-purified rMK by reverse phase HPLC (rpHPLC) yielded a major biologically active protein product of approximate relative molecular mass 16,500 (Fig. 2). The identity of this species was confirmed by immunoblotting, using an antibody directed against MK expressed in bacteria (Maruta et al., 1992), and amino acid analysis.

A second protein species detected by SDS-PAGE of rpHPLC fractionated material had an apparent M_r of 65×10^3 and was devoid of both biological activity and anti-MK immunoreactivity. This species was also detected in conditioned media derived from COS cells transfected with the PXMT2 expression plasmid cloned in the antisense orientation. In addition, no biological activity could be detected in high-salt eluates of heparin affinity chromatography of

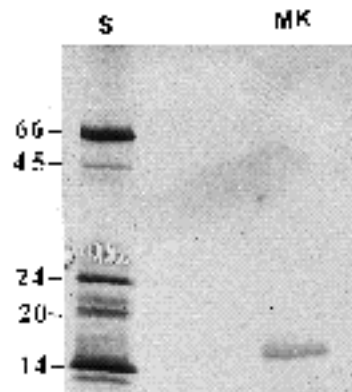


Fig. 2. SDS-PAGE of HPLC-purified rMK. S, protein standards; MK, 50 ng of rMK. The apparent molecular mass of protein standards is indicated ($\times 10^{-3}$).

media conditioned by cells expressing the antisense MK expression construct.

The use of rpHPLC as a purification step resulted in a substantial loss of MK bioactivity resulting, at least in part, from exposure to acid solvents. Similar findings have been reported for HBNF (Bohlen et al., 1990). It was also noted in the course of these experiments that biological activity present in either COS-cell-conditioned media or heparin affinity-purified samples was very unstable upon storage between -20°C and $+4^{\circ}\text{C}$. In order to circumvent this problem, MK preparations for biological assays described below were used within 5 days of purification and not subjected to the rpHPLC purification step (which was thereafter employed for quantitation of rMK protein concentration present in the heparin affinity eluates).

Mitogenic actions on fibroblast cell lines

Since FGF-4 does not account for the totality of heparin-binding mitogens present in EC-cell-conditioned medium (Heath et al., 1989), we examined the ability of rMK protein to induce DNA synthesis in quiescent 10T1/2 and Swiss 3T3 fibroblast cells. Unlike rFGF-4, MK was completely inactive as a mitogen for Swiss 3T3 cells (data not shown). This observation demonstrates that COS-cell-derived rMK is not detectably contaminated with FGF-like biological activities (such as bFGF) which might have been expected to co-purify with MK. rMK was, however, able to induce DNA synthesis in quiescent 10T1/2 cells (Fig. 3). The maximal induction of DNA synthesis observed was, however, approximately one fifth of that observed with rFGF-4. Half-maximal induction of DNA synthesis was manifest at approximately 1 nM rMK. We conclude that rMK is a significant but weak (in comparison to FGF-4) growth factor for 10T1/2 cells.

rMK is a mitogen for EC-cell-derived neuroectodermal cells

In view of the reported actions of MK on PC12 cells and the effects of the related factor HB-GAM on CNS neurons, we examined the action of rMK in two neuronal cell systems. 1009 EC cells differentiate into neurons with cholinergic properties (as well as an additional non-neuronal cell

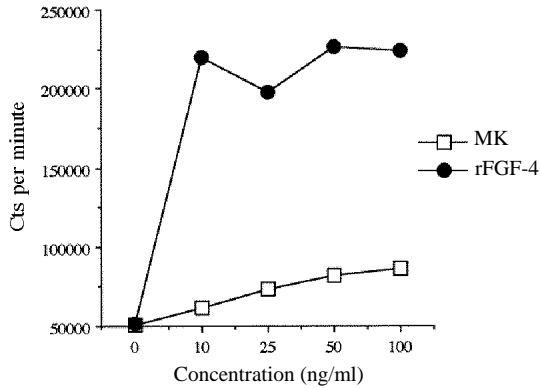


Fig. 3. Induction of DNA synthesis measured by [³H]thymidine incorporation in 10T1/2 fibroblasts by recombinant FGF-4 (gift of Dr D. Rogers, Genetics Institute) and rMK.

type) upon exposure to retinoic acid (Pfeiffer et al., 1981 and J. K. Heath unpublished data). RA-treated 1009 cells therefore represent a population of neuronal cells which, in view of their EC derivation, resemble the neurectodermal cell types of the early embryo.

rMK is a significant growth factor for neurectodermal cells derived by RA treatment of 1009 EC cells (Fig. 4) for 4 days with a half maximal response at approximately 1 nM. No mitogenic effect of rMK was observed on undifferentiated 1009 EC cells (data not shown).

RA-treated 1009 cells progressively form extensive networks of non-dividing neurons after culture for longer periods of time (7-14 days). No significant effect of exogenous rMK on DNA synthesis, cell numbers or neuron morphology could be observed after culture for 10 days in the presence of RA. This finding would suggest that the mitogenic effects of rMK on these cells are restricted to a cell type formed during the early phases of RA-induced differentiation, which gives rise to more mature cell types that are not overtly responsive to exogenous rMK.

rMK is a neurotrophic factor for sympathetic neurons

Since rMK was found to have significant biological actions on EC-cell-derived neuronal cell types in vitro, we examined the action of rMK on a number of other neuronal cell

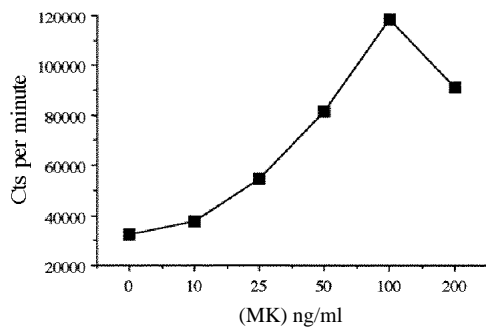


Fig. 4. Induction of DNA synthesis measured by [³H]thymidine incorporation in 1009 EC cells treated with RA for 4 days by rMK.

types from different sources. A striking finding from these investigations was that MK was able to enhance the survival of E12 chick sympathetic neurons in vitro, as well as promoting neurite outgrowth. The potency of rMK in this assay was, however, significantly affected by both the nature of the tissue-culture substratum employed and the mechanisms of rMK presentation to responding cells. Only minor effects of rMK on neuron survival and neurite outgrowth were observed when neurons were plated on polylysine/laminin and the rMK was added to the liquid phase of the cell cultures (Figs 5, 6D). However, marked increases in response were observed when the tissue-culture plates were first coated with heparin and the rMK presented to cells by prebinding it to the tissue-culture plate (Figs 5, 6B,C). The effect of heparin was minor at low (1 ng/ml) concentrations of rMK but became significant at rMK concentrations of 10 ng/ml (0.6 nM) and above. Under these conditions the response to rMK approached that obtained with nerve growth factor (NGF) added to cells in the soluble phase (Figs 5, 6E). These findings indicate that rMK can be a potent neurotrophic factor for sympathetic neurons if presented to cells in the insoluble phase in the presence of heparin.

These effects of cell substratum on MK function were further examined by testing the biological properties of a 'natural' substratum preparation that might be expected to more closely resemble the physiological presentation of MK in vivo. 10T1/2 fibroblasts were co-transfected with the MK expression plasmid pXMT2-MK and a PGK-Neo drug resistance plasmid. Stable cell lines were isolated by selection in G418 and a number of G418 resistant clones isolated by colony selection and expanded for analysis of expression of MK transcripts by PCR. No significant effects of MK expression on either cell morphology or growth rates were observed in these cell lines, in keeping with its weak

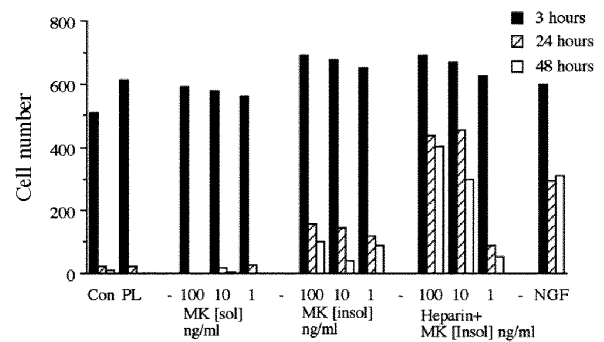


Fig. 5. Effects of rMK and substrate preparations on chick E12 sympathetic neuron survival in vitro. 2×10^3 neurons were plated onto tissue-culture dishes coated with poly-L-lysine and laminin. Cell numbers were determined after 3 hours, 24 hours and 48 hours culture. Additives are: Con, control COS-cell-conditioned media; PL, none; MK[sol], rMK added to the liquid phase of the tissue-culture media; MK[insol], rMK was incubated on prepared substrata prior to addition of cells; Heparin + MK[insol], substrata were incubated with heparin (10 μ g/ml) followed by rMK at the designated concentrations followed by addition of the test cells; NGF, nerve growth factor added at 50 ng/ml to the liquid phase of the culture.

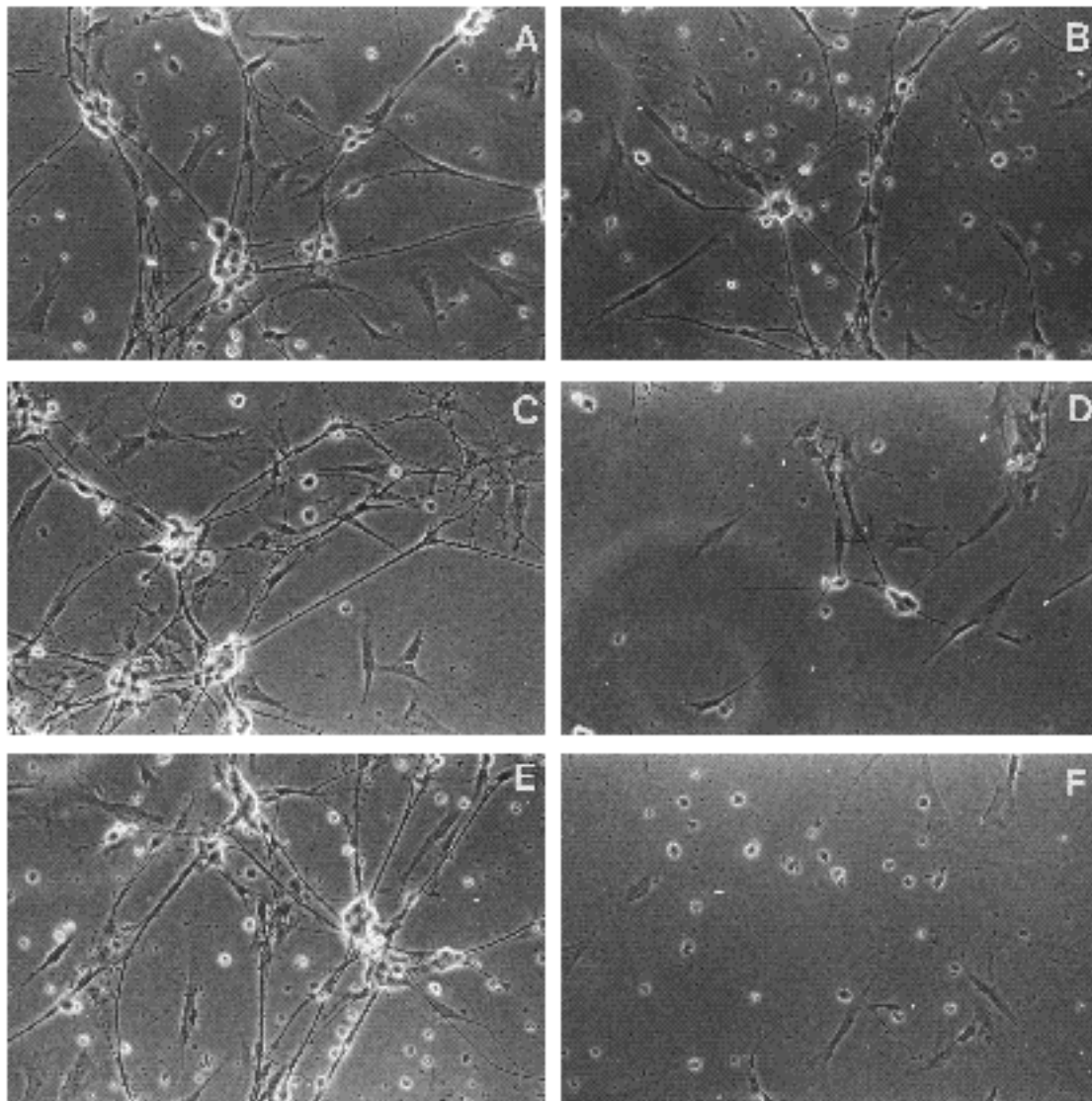


Fig. 6. Morphological appearance of E12 sympathetic neurons exposed to MK and experimental substratum preparations after 48 hours culture. Neurons were plated into tissue-culture dishes treated with poly-L-lysine and laminin. (A) Matrix preparation from 10T1/2 cells expressing MK from transfected plasmid (Clone 11). (B) 1 ng/ml rMK coated on substratum preparations in the presence of heparin. (C) 10 ng/ml rMK coated on substratum preparations in the presence of heparin. (D) 10 ng/ml rMK added to the liquid phase of the culture medium. (E) 50 ng/ml NGF. (F) Control COS-cell-conditioned medium.

effect on 10T1/2 cell proliferation when added exogenously. Four 10T1/2 clones which were found to express MK by PCR, a control clone (in which MK expression could not be detected by PCR) and non-transfected 10T1/2 cells were grown to confluence *in vitro* and a 'conditioned matrix' preparation obtained by removal of the cells from the tissue-culture dish by treatment with EDTA (Rathjen et al., 1990). Replating E12 sympathetic neurons onto the conditioned matrix produced a significant effect on both 24-hour neuronal survival and neurite outgrowth when sympathetic neurons were plated onto matrix derived from clones expressing MK (Figs 6A, 7), but not in the non-expressing control clone or non-transfected 10T1/2 cells (Fig. 7). Some interclonal variation was observed in the ability to support neuronal survival, which may reflect dif-

fering levels of functional MK protein expression between clones. These findings indicate that MK can be physically associated with matrix components in a biologically active form when expressed continuously in stably transformed fibroblast cell lines.

Discussion

Expression of MK

This paper demonstrates that MK is a major gene product of EC and ES cells and their immediate differentiated derivatives. This finding contrasts with the original results of Muramatsu and colleagues (Kadomatsu et al., 1988) who isolated MK from HM-1 EC cells by differential screening

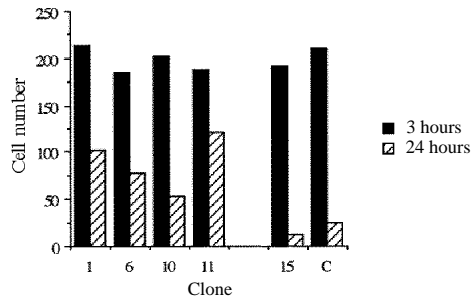


Fig. 7. E12 sympathetic neuron survival on ECM derived from MK-transfected 10T1/2 clones. 10^3 E12 neurons were plated onto ECM preparations from 10T1/2 cell clones which expressed MK from a transfected plasmid (clones 1, 6, 10 and 11) or from a clone recovered in the same experiment in which the MK expression plasmid had not integrated (clone 15). C, non-transfected 10T1/2 cells. Cell numbers were determined after 3 hours and 24 hours.

for genes induced by retinoic acid. Our data indicate that MK mRNA levels are not appreciably changed either by the presence or absence of retinoic acid. The basis of this difference is not completely clear; it seems that the effects of RA on MK gene expression in HM-1 cells are only transient. In particular, Huang et al. (1990) have shown that MK induction after RA treatment peaks after 48 hours of exposure and then subsides to non-induced levels after 96 hours, which are the equivalent of the time points examined here. The distinction between the two sets of data may therefore reflect the relatively low level of MK expression in HM-1 cells compared to EC or pluripotent ES cells. The fact that MK transcripts can be detected in embryonic ectoderm by *in situ* hybridisation techniques suggests that MK is normally expressed by pluripotential stem cells *in vivo*. The persistence of MK expression in differentiated cells also accords with the finding that MK is not confined to any single germ layer during early postimplantation development.

The physiological significance of the transient burst of MK expression in HM-1 cells in response to retinoic acid is not wholly clear. Since nuclear run-on experiments (Huang et al., 1990) indicate that MK gene transcription is only modestly induced by RA treatment of HM-1 cells (in contrast to steady-state mRNA levels) it may be concluded that the MK mRNA is for some reason especially unstable in HM-1 cells and the increase in steady-state MK mRNA levels has little general physiological significance. It is unlikely that MK protein acts as a mediator of some of the biological effects of RA (Maruta et al., 1992; Urios et al., 1991) since our data clearly show that it can be expressed in ES and EC cells in the absence of exogenous RA.

Biological function of rMK

The results reported here show that rMK produced by expression in COS cells and purified by heparin affinity chromatography has biological effects on three distinct cell types.

Recombinant MK is a significant but extremely weak mitogen for 10T1/2 fibroblasts, while at the same time being

inactive towards Swiss 3T3 cells. Furthermore, stable 10T1/2 cell lines were isolated that express MK but did not exhibit any overt evidence of morphological or proliferative changes associated with cell transformation. This contrasts with the effects of FGF-4 which is both a potent mitogen for these cells and powerful transforming gene when expressed under equivalent conditions (J. K. Heath and F. McDonald, unpublished data). Therefore, it appears that the effects of MK on fibroblast cell proliferation are influenced by the exact identity of the target cell line although the molecular basis of these differences is currently obscure. This may explain why some controversy exists regarding the mitogenic actions of rHB-GAM/HBNF/PTN on fibroblast cell lines (Li et al., 1990; Raulo et al., 1992; Bohlen et al., 1990); the activity observed may entirely depend on the fibroblast cell line employed. These findings also indicate that, at least in the expression system employed here, MK is not a transforming gene for mouse fibroblasts. This does not of course exclude the possibility that MK expression may be associated with oncogenic transformation of other cell types. Taken together, these observations show that the biological properties of MK are different from that of the heparin-binding growth factor activity isolated from P19 EC cells (Heath et al., 1989) which was very active in fibroblast mitogen assays. It is possible that the conditions of isolation of rMK expressed by COS cells results in a significant loss of activity, although this would seem unlikely in view of the potent effects of this factor in other systems and the absence of transforming activity *in vitro*. The most likely explanation for this discrepancy is that EC cells express an additional heparin-binding mitogen which is not a member of either the FGF or MK/HB-GAM family of growth factors. It is intriguing in this respect that the mitogenic activity of HB-GAM preparations derived from adult rat brain have been ascribed to a structurally distinct contaminating mitogenic factor designated p17 (Raulo et al., 1992).

The major biological effects of MK reported here involve actions on cells of direct or indirect neuroectodermal origins. Thus, rMK proves to be a potent mitogen for neuroectodermal cells derived from 1009 EC cells by prior treatment with RA for 4 days. This effect is transient, since no mitogenic or morphological effects could be observed at later stages when the cultures had formed extensive networks of non-dividing neurons. It is, however, possible that rMK has more subtle effects on this population *in vivo*, for example, controlling neurotransmitter expression by differentiated 1009-derived neurons. In light of the embryonic origins of 1009 cells and the expression of MK in both the primitive ectoderm and neuroectoderm of the normal embryo (Kadomatsu et al., 1990), this finding may indicate that MK is involved in the control of neuroectodermal proliferation in the early development of the *in vivo* nervous system. It will be of particular interest in this respect to examine the role of MK as a transforming gene in neuroectodermal tumours and cell lines.

The effects of rMK on neuronal cell behaviour are, however, clearly cell-type dependant, since the factor has potent neurotrophic and neurite outgrowth promoting effects on sympathetic neurons from later developmental stages. Together these findings indicate that MK is multifunctional

regulator of neuronal cell function with distinct actions on different cell types within the developing nervous system.

MK and the extracellular matrix

Previous studies of the biological actions of both MK and HB-GAM/HBNF/PTN have been difficult to interpret due to the low specific activity of the protein *in vivo*. It appears that, at least in the case of MK, this is due to at least two distinct phenomena. (1) It has been our experience of rMK protein produced in COS cells that the biological activity of the protein diminishes upon storage. The biochemical basis of this instability is at present unknown but may be due to the removal of co-factors required for protein stability during the purification procedure. In this respect the availability of stable cell lines expressing biologically active MK protein continuously may be a useful tool in further *in vitro* studies of MK function, as well as in defining the biological actions of MK in the adult nervous system by *in vivo* grafting techniques (Schinstine et al., 1991).

(2) Further determinants of MK bioactivity are the identity of the cellular substratum and the means by which rMK is presented to responding cells *in vitro*. This is most clearly demonstrated by the neurotrophic and neurite outgrowth promoting effects of rMK on sympathetic neurons. In this case, the effects of rMK were potentiated both by the addition of heparin to the tissue-culture substratum, and by allowing rMK to associate with the tissue-culture dish prior to plating the cells, rather than adding the soluble protein directly to the culture media. It is probable that these two effects are related since a 'conditioned matrix' tissue-culture substratum preparation derived from 10T1/2 cells expressing MK from a transfected expression plasmid also exhibited potent neurotrophic and outgrowth promoting actions. Since rMK exhibits a high affinity for immobilised heparin, it seems highly likely that MK protein requires to be physically associated with heparin in the extracellular matrix in order to exhibit maximal biological activity. This phenomenon is reminiscent of at least some members of the FGF family of heparin-binding growth factors in which, in at least one case, expression of heparin containing glycosaminoglycans has been found to be required for FGF function *in vitro* (Ornitz et al. 1992). The requirement for a physical association between MK and heparin (presumably in the form of HSPGs) for maximal activity could reflect either a stabilisation of the three dimensional structure of MK in a form available for receptor interaction, or a direct participation of HSPGs in the process of MK/receptor interaction.

Nevertheless, freshly prepared rMK presented to cells by substratum attachment in the presence of heparin is a very potent bioregulatory polypeptide with half maximal effects on sympathetic neuron survival and outgrowth at between 100-500 pM. This is consistent with action via association with a high affinity, signal-transducing receptor. It follows that these requirements for MK biological activity must be taken into account in attempting to characterise the identity of putative MK signal transducing receptor(s). The studies reported here also identify candidate target cell types that respond to physiologically plausible concentrations of MK in which such receptors may be characterised.

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