The activated human *met* gene encodes a protein tyrosine kinase

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We have raised antibodies against a synthetic dodecapeptide corresponding to the carboxyl terminus of the predicted *met* gene product. Phosphorylation of 60 kDa and 65 kDa proteins on tyrosine residues was observed when immunoprecipitates of cells containing the activated human *met* gene were incubated with [γ-^{32}P]ATP. Phosphoproteins with the same molecular masses could be immunoprecipitated from cells metabolically labelled with [^{32}P]orthophosphate. When considered together, these observations indicate that the activated human *met* gene encodes 60 kDa and 65 kDa proteins that can catalyse autophosphorylation on tyrosine residues.

Antipeptide antibody met *oncogene* *Tyrosine kinase*

1. INTRODUCTION

The activated *met* gene present in the MNNG-HOS chemically transformed human cell line was originally identified by its ability to transform NIH3T3 mouse fibroblasts in DNA transfection experiments [1,2]. Activation of *met* involves a chromosomal rearrangement that generates a chimeric gene containing 5′-sequences (designated *tpr*) derived from chromosome 1, and 3′-sequences (designated *met*) derived from chromosome 7 [3,4]. DNA sequence analysis has demonstrated that the 3′-region of activated *met* is related to the tyrosine kinase domains of the human insulin and epidermal growth factor (EGF) receptors, indicating that in common with these two receptors activated *met* may also encode a protein tyrosine kinase [5]. To directly examine whether *met* encodes a protein tyrosine kinase we have raised antibodies against a synthetic peptide corresponding to the 12 amino acids at the carboxyl-terminus of the predicted *met* gene product and used these antibodies to precipitate *met* related products from cells containing the activated human *met* gene.

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2. MATERIALS AND METHODS

2.1. *Cell lines*

All cell lines including human HOS and MNNG-HOS [6] and untransformed and transformed NIH3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum.

2.2. *Antipeptide antibodies*

A peptide of sequence VDTRPASFWETS was synthesized by Cambridge Research Biochemicals, Cambridge, England. The peptide was coupled to Keyhole limpet haemocyanin and the conjugate used to immunize New Zealand White rabbits as described [7]. Rabbit serum was examined for its ability to bind the peptide, which was immobilized in microtitre dishes, by an enzyme-linked immunosorbent assay (ELISA) that utilized

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horseradish peroxidase [8]. Peroxidase activity, which is proportional to the amount of binding of antibody to peptide, was assayed with *o*-phenylenediamine after which the absorbance at 492 nm was measured [8]. Antipeptide antibodies used in some experiments were purified by chromatography on affinity columns of immobilized synthetic peptide [7].

2.3. *Immunoprecipitations and kinase assays*

Lysates of unlabelled cells and of cells that were incubated for 5–6 h in phosphate-free DMEM (without added foetal calf serum), containing 0.35–0.7 $mCi \cdot ml^{-1}$ carrier-free [^{32}P]orthophosphate (Amersham International), were prepared as described [7]. Antibody-protein A-Sepharose complexes for immunoprecipitations were prepared by incubating 20 μl of protein A-Sepharose [50% (v/v) in phosphate-buffered saline] with an equal volume of either phosphate-buffered saline containing 1 μg of affinity-purified antibody or unpurified serum for 20 min at 22°C. The washed antibody-protein A-Sepharose complexes were then incubated with cell lysates either in the absence or presence of immunizing peptide at 1 mM for 3–16 h at 4°C. To assay for kinase activity, immunoprecipitates from unlabelled cells were washed 5 times with HNTG [20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol] and were resuspended in 40 μl of HNTG containing 5 mM $MnCl_2$ and 3 μCi [γ-^{32}P]ATP (5000 $Ci \cdot mmol^{-1}$; Amersham International). After incubation at 22°C for 15 min, sample buffer containing 2-mercaptoethanol was added, and precipitated proteins were solubilized by boiling for 3 min before being subjected to electrophoresis through a 7.5% polyacrylamide gel containing SDS [9]. Immunoprecipitates from [^{32}P]orthophosphate-labelled cells were washed as described [7] and proteins subjected to SDS-PAGE. To detect phosphorylated proteins, the gels were fixed, dried and subjected to autoradiography at −70°C for 1–16 h using Fuji RX film.

2.4. *Phosphoamino acid analysis*

Phosphorylated proteins were located in polyacrylamide gels by autoradiography, eluted from the gels after trypsin digestion [7] and hydrolysed with constant-boiling HCl at 110°C for 75 min. The lyophilized hydrolysate was dissolved in a solution containing phosphoserine, phosphothreonine and phosphotyrosine and subjected to anion-exchange high-performance liquid chromatography (HPLC) [10]. The positions of elution of authentic phosphoamino acids were determined by fluorescence spectroscopy after post-column derivatization with *o*-phthalaldehyde [11]. Radioactivity in the eluent fractions (0.75 ml) was detected by Cerenkov counting.

3. RESULTS AND DISCUSSION

The amino acid sequence of portions of the protein encoded by the *met* gene may be deduced from partial DNA sequence analysis [5]. To facilitate the analysis of proteins encoded by *met* we have raised antibodies to a synthetic peptide that corresponds to the carboxyl-terminus of the predicted amino acid sequence. Sera from rabbits immunized with peptide conjugated to Keyhole limpet haemocyanin were analysed for their abilities to bind the synthetic peptide using an ELISA. The results obtained using immune serum from one rabbit are shown in fig.1. Binding of the antiserum to the synthetic peptide was detected at dilution of the serum up to 1 in 40960 indicating that high titres of the antipeptide antibody had been obtained. The specificity of the antiserum was confirmed in control experiments where we failed to detect

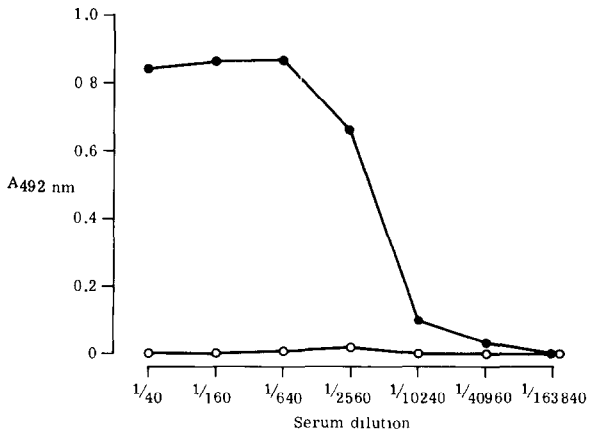

Fig.1. Titres of antipeptide antibodies present in preimmune serum (○) and in immune serum (●) from the same rabbit determined by ELISA. The absorbance at 492 nm is proportional to the amount of antibody bound to peptide.

binding of the preimmune serum to peptide (fig.1).

Evidence that the *met* gene encodes a protein kinase was obtained in experiments in which proteins immunoprecipitated with antipeptide antiserum were incubated in the presence of [γ-^{32}P]ATP. Immunoprecipitated proteins were analysed by SDS-polyacrylamide gel electrophoresis and in vitro phosphorylation of proteins was detected by autoradiography. The results of these analyses are shown in fig.2. Phosphorylation of a 60 kDa protein and less extensive phosphorylation of a 65 kDa protein were observed in immunoprecipitates of MNNG-HOS cells and of a line of NIH3T3 mouse fibroblasts (22-1a) transformed by the activated human *met* gene. Precipitation of the cellular component responsible for this phosphorylation was blocked when immunoprecipitations were carried out either in the presence of an excess of immunizing peptide or using preimmune, instead of immune serum. No phosphorylation of the 60 and 65 kDa proteins was observed in experiments with cells that do not express the activated *met* gene. The cell lines examined included untransformed NIH3T3 cells, a line of spontaneously transformed NIH3T3 cells designated ST10 [12] and HOS cells, the parent cell line from which the MNNG-HOS cells were originally derived by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [6].

Evidence that the major site of protein phosphorylation is on tyrosine was obtained by subjecting an acid hydrolysate of the ^{32}P-labelled 60 kDa phosphoprotein, which had been extracted from a polyacrylamide gel, to HPLC on an anion-exchange column in admixture with unlabelled phosphoserine, phosphothreonine and phosphotyrosine. The results in fig.3 show that a single major peak of radioactive material coeluted with phosphotyrosine. Little or no radioactive material eluted at positions corresponding to phosphoserine and phosphothreonine. Parallel experiments with acid hydrolysates of the ^{32}P-labelled 65 kDa phosphoprotein demonstrated that the major site of phosphorylation of this protein also occurred on tyrosine residues (not shown).

Many protein tyrosine kinases, including the EGF and insulin receptors, can catalyse their own phosphorylation [13,14], and by analogy it seemed probable that phosphorylation of the 60 and 65 kDa proteins may result from autophosphory-

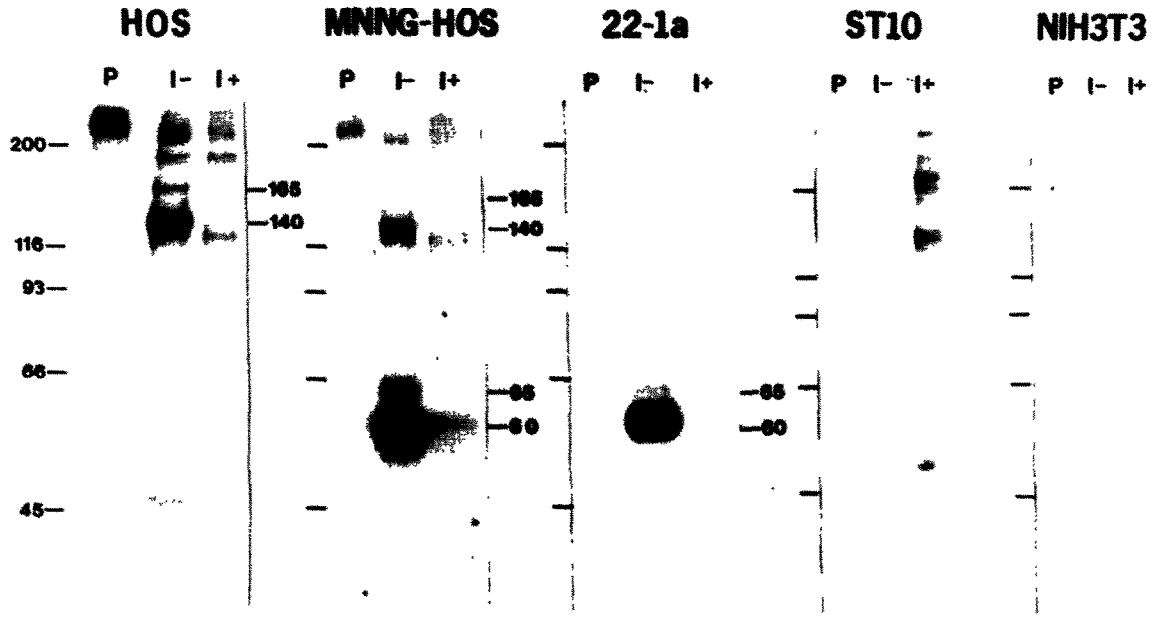

Fig.2. Autoradiograph of immunoprecipitated proteins that had been incubated in vitro with [γ-^{32}P]ATP and analysed by SDS-PAGE. Immunoprecipitations were carried out with preimmune serum (P) and with immune serum either in the absence (I−) or presence (I+) of immunizing peptide. Molecular mass standards are indicated in kDa.

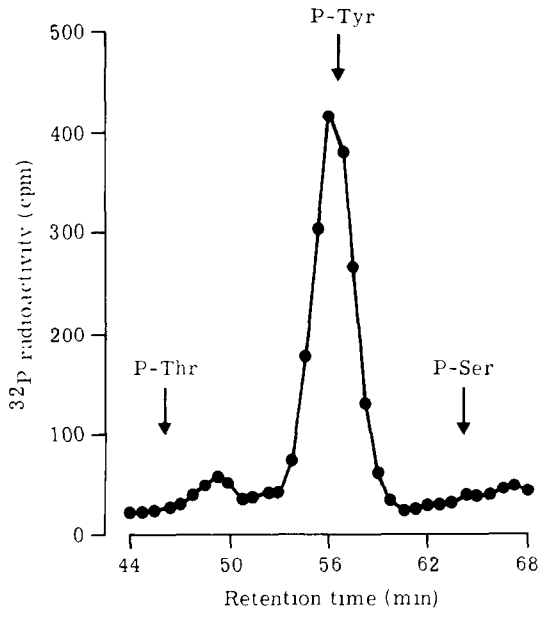

Fig.3. Anion-exchange HPLC of an acid hydrolysate of the 60 kDa protein that was radiolabelled when an immunoprecipitate from 22-1a cells was incubated with [γ-^{32}P]ATP. The elution positions of the unlabelled phosphoamino acid markers, indicated by arrows, were determined by fluorescence spectroscopy.

lation of products encoded by the activated *met* gene. To obtain evidence that the activated *met* gene encodes these 60 and 65 kDa proteins, anti-peptide antibodies were used to precipitate products of the *met* gene from cells that had been metabolically labelled with [^{32}P]orthophosphate. Affinity purified antipeptide antibodies were used for these studies because non-specific precipitation of phosphoproteins was observed in preliminary experiments using unpurified antiserum. As expected, 60 and 65 kDa phosphoproteins were precipitated from MNNG-HOS and from 22-1a cells (fig.4). These phosphoproteins were not

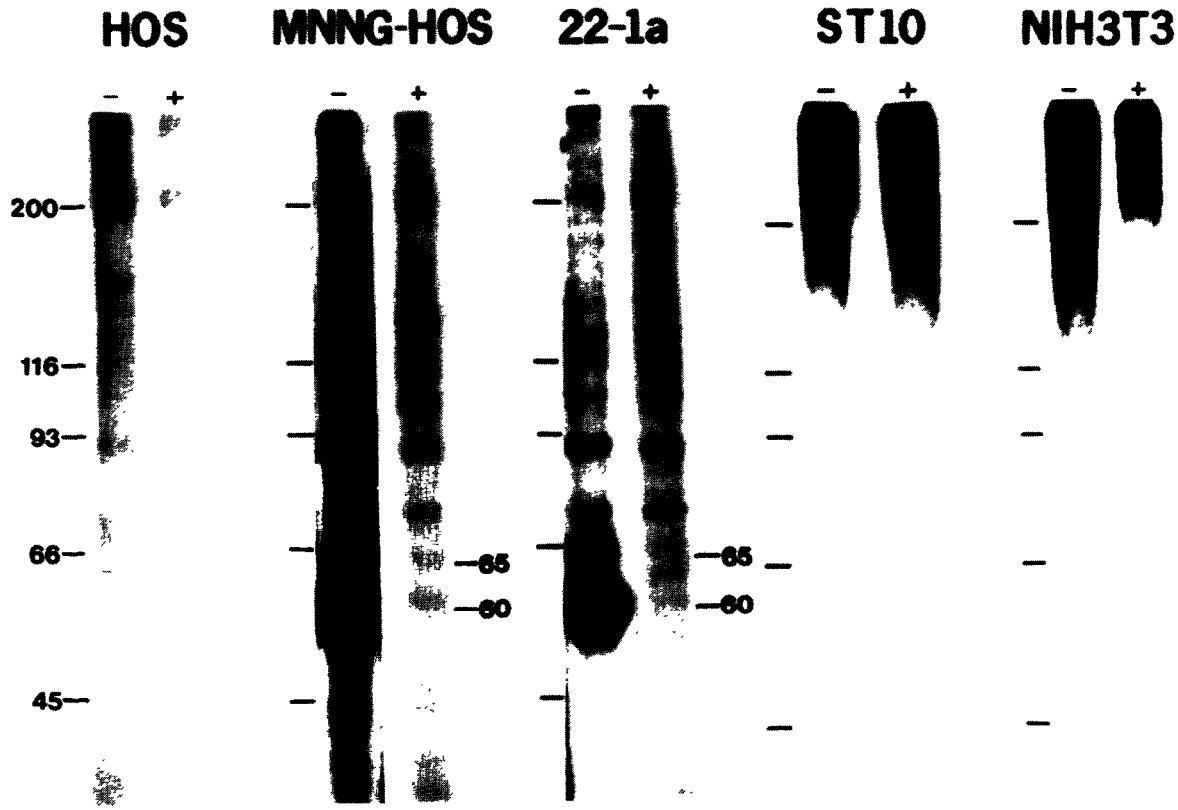

Fig.4. Autoradiograph of proteins immunoprecipitated from cells incubated with [^{32}P]orthophosphate and analysed by SDS-PAGE. Immunoprecipitations were carried out with affinity-purified antipeptide antibodies either in the absence (−) or presence (+) of immunizing peptide. Molecular mass standards are indicated in kDa.

detected when immunoprecipitations were carried out in the presence of an excess of immunizing peptide and were not detected in experiments with untransformed NIH3T3 cells, with spontaneously transformed NIH3T3 cells (ST10) or with the parent HOS cell line. When considered together, the data presented here provide evidence that the activated human *met* gene encodes 60 and 65 kDa proteins that can catalyse autophosphorylation on tyrosine. The relationship between these two proteins is not clear however. It is conceivable that the 60 kDa protein could be formed from the 65 kDa protein by degradation during cell lysis. Alternatively, one of these two proteins might be derived from the other by post-translational modification.

Although the proteins encoded by the unrearranged *met* gene have been studied less extensively, phosphorylation of a 140 kDa and a 165 kDa protein was detected when immunoprecipitates of HOS cells and MNNG-HOS cells, which both contain transcripts from the unrearranged *met* gene [3], were incubated with [γ-^{32}P]ATP (fig.2). Phosphorylation of these 140 and 165 kDa proteins was not observed with immunoprecipitates from cells, including K562 and HL60, that do not contain detectable levels of *met* transcripts [3] (not shown). Precipitation of the cellular component responsible for this phosphorylation was blocked when immunoprecipitations were carried out in the presence of an excess of immunizing peptide (fig.2). These results indicate that the normal *met* gene also encodes a protein tyrosine kinase and it is likely that ^{32}P-labelling of the 140 and 165 kDa proteins results from autophosphorylation of the products encoded by the normal *met* gene. Our failure to detect these 140 and 165 kDa phosphoproteins in cells labelled with [^{32}P]orthophosphate may reflect the low level of phosphorylation of these proteins under the conditions that we have used for metabolic labelling. Further experiments will be necessary to firmly establish the identity of the proteins encoded by the normal *met* gene and to determine the relationship between the 60 and 65 kDa proteins encoded by the activated *met* gene.

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