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Characterization of insulin-stimulated protein serine/threonine kinases in CHO cells expressing human insulin receptors with point and deletion mutations

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The activation of insulin-stimulated protein-serine/threonine kinases has been investigated in CHO cell lines transfected with cDNAs encoding either wild-type or mutant human insulin receptors. (1) Insulin treatment of CHO cells over-expressing wild-type insulin receptors resulted in the rapid and substantial (5–10-fold) activation of cytosolic protein kinases which phosphorylated myelin basic protein, Kemptide and two peptide substrates based on sites phosphorylated on ribosomal protein S6 *in vivo*. (2) Further fractionation of cytosolic extracts by MonoQ chromatography revealed two peaks of insulin-stimulated myelin basic protein kinase activity which were highly related to the previously described mitogen-activated protein (MAP) kinases ERK1 and ERK2. In addition, at least two major peaks of S6 kinase activity were resolved, which exhibited properties similar to the 70 kDa and 90 kDa S6 kinases described by others; the predominant effect of insulin was on the activity of the 90 kDa enzyme and was in excess of 10-fold. (3) MonoQ fractionation of extracts from parental CHO cells, or cells expressing kinase-deficient receptors, showed all insulin-stimulated peaks of activity to be almost completely absent. (4) Further studies demonstrated that substitution of tyrosine residues 1162 and 1163 (or 1162 alone) with phenylalanine led to a substantial reduction in the ability of insulin to stimulate these protein kinase activities when assayed in cytosolic extracts. In contrast, deletion of 69 amino acids from the *C*-terminus of the insulin receptor β-subunit caused a leftward shift in the insulin dose–response curve of the MAP kinase activity, but apparently not in that of the 90 kDa S6 kinase activity.

INTRODUCTION

Insulin-stimulated insulin receptor autophosphorylation and protein-tyrosine kinase activity are important requirements for cellular signalling by this hormone (Ellis *et al.*, 1986; Chou *et al.*, 1987; Ebina *et al.*, 1987; McClain *et al.*, 1987; Debant *et al.*, 1988). The interaction of insulin with its receptor also results in the rapid phosphorylation of a number of intracellular proteins on serine and threonine residues (e.g. acetyl-CoA carboxylase and ribosomal protein S6; Denton, 1986). Recently, considerable advances have been made in the purification, cloning and characterization of some of the insulin-stimulated protein-serine/threonine kinases that may be responsible for the changes in phosphorylation. These include ribosomal protein S6 kinases and mitogen-activated protein (MAP) kinases (Ray & Sturgill, 1987; Boulton *et al.*, 1990*a*, 1991*a*). Thus insulin, and indeed other growth factors whose receptors possess intrinsic protein-tyrosine kinase activity, may bring about many of their effects on intracellular processes by regulation of protein phosphorylation cascades, perhaps initiated on tyrosine residues (Hoshi *et al.*, 1988; Ahn *et al.*, 1990; Gomez *et al.*, 1990; Miyasaka *et al.*, 1990; Tsao *et al.*, 1990; Boulton *et al.*, 1991*b*).

The MAP kinases represent a group of closely related proteins with apparent molecular masses of approx. 42–44 kDa that are able to phosphorylate myelin basic protein *in vitro*. Activation of these enzymes often appears to require concomitant phosphorylation of threonine and tyrosine residues (Ray & Sturgill, 1988; Anderson *et al.*, 1990; Boulton *et al.*, 1991*b*). Recently, two closely related MAP kinases have been identified and cloned (termed ERK1 and ERK2; Extracellular Regulated Kinase; Boulton *et al.*, 1990*a*,*b*; 1991*a*).

Several protein kinases have been identified that phosphorylate ribosomal protein S6 in response to insulin and other growth factors (Erikson *et al.*, 1991; Lane & Thomas, 1991; Sturgill & Wu, 1991). Cloning of the cDNA for some of these enzymes has revealed two distinct families of ribosomal protein S6 kinases; those with an apparent molecular mass of around 70 kDa (Kozma *et al.*, 1990; Banerjee *et al.*, 1990) and those of approx. 90 kDa (Jones *et al.*, 1988; Alcorta *et al.*, 1989), both of which require phosphorylation on serine/threonine residues for full activity (Ballou *et al.*, 1988; Price *et al.*, 1990).

Members of the 90 kDa family of ribosomal protein S6 kinases which have been inactivated by treatment with protein phosphatase 2A *in vitro* can be subsequently reactivated upon phosphorylation by MAP kinases (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn & Krebs, 1990). This suggests that these kinases may form part of an insulin-stimulated protein kinase cascade. However, the precise molecular link between the insulin receptor protein-tyrosine kinase and these serine/threonine protein kinases remains unsolved (Gomez *et al.*, 1990; Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991).

Site-directed mutagenesis of the insulin receptor cDNA and expression of receptor mutants in a variety of cell lines (most commonly CHO, NIH 3T3 or Rat1 fibroblasts) has proven a useful means by which to dissect functions of the receptor that are important in signalling (reviewed in Ellis *et al.*, 1991). However, although the sensitivity of cells to insulin increases upon over-expression of wild-type insulin receptors, the

Abbreviations used: MAP kinase, mitogen-activated protein kinase; ERK, extracellular regulated kinase.

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magnitudes of the responses are generally rather small (e.g. insulin often has only approx. 2-fold effects on glucose transport and glycogen synthesis). In the current study we demonstrate that, in CHO cells expressing the human insulin receptor cDNA, insulin has substantial effects (often > 10-fold) on the activity of members of the MAP and ribosomal protein S6 kinase families, while having little effect on these activities in parental CHO cells. We have therefore examined the ability of insulin to activate those protein-serine/threonine kinases in CHO cells expressing mutant human insulin receptors in order to begin to dissect the molecular mechanism by which the insulin receptor activates protein-serine/threonine kinases.

MATERIALS AND METHODS

Materials

All reagents were as previously described (Tavaré & Dickens, 1991), except for myelin basic protein and cyclic AMP-dependent protein kinase inhibitor peptide which were from Sigma (Poole, Dorset, U.K.), and microcystin-LR from Calbiochem–Novabiochem. Kemptide (LRRASLG), and the S6 peptides RRLSSLRA (8-mer) and KEAKEKRQEQIAKKRRLSSLRASTSKSESSQK (32-mer) were synthesized by Dr. Graham Bloomberg (Department of Biochemistry, University of Bristol). Casein kinase II substrate peptide (RRREEETEEE) was synthesized by Ms. L. DeOgny (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). Anti-peptide antiserum 837, specific for ERK1 and ERK2, was a gift from Dr. M. H. Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas). Purified *Xenopus* S6 kinase II and MAP kinase, and antiserum 2168.1 raised against recombinant *rsk* (also reactive with *Xenopus* S6 kinase II), were generously provided by Dr. J. L. Maller (Howard Hughes Medical Institute, Denver, CO, U.S.A.).

Cell lines

All of the cell lines used in this study were derived from parental CHO.K1 cells, which express approx. 2000–3000 rodent insulin receptors per cell. CHO.T cells are transfected with the human insulin receptor cDNA (Ellis *et al.*, 1986). CHO.YF1 and CHO.YF3 cells express mutant human insulin receptors in which tyrosine residues 1162 (YF1) or 1162 and 1163 (YF3) have been replaced with phenylalanines (Ellis *et al.*, 1986). CHO.ΔCT69 cells express a human insulin receptor in which the *C*-terminal 69 amino acids (residues 1287–1355) have been deleted from the β-subunit (Chin *et al.*, 1991). CHO.K1030R cells express a human insulin receptor in which lysine-1030 has been replaced with arginine at the putative ATP-binding site, resulting in a receptor which is devoid of protein-tyrosine kinase activity (Ebina *et al.*, 1987). All cell lines express between 5×10^5 and 1×10^6 insulin receptors per cell, except the CHO.K1030R cell line (50000 receptors per cell).

All transfected cell lines were maintained in Ham's F-12 medium (Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 10% (v/v) foetal calf serum (Gibco Ltd., Paisley, Scotland, U.K.), 50 units of benzylpenicillin/ml, 50 μg of streptomycin/ml and 400 μg of G418 (Gibco)/ml at 37 °C under an atmosphere of CO_2/air (1:19). Parental CHO.K1 cells were maintained in an identical manner but in medium lacking G418. Cells were plated at an initial density of 2×10^5 or 1×10^6 cells per 30 mm or 100 mm dish respectively, and grown to near-confluence (2–3 days) before being incubated for 16 h in Hams F-12 medium containing 0.1% foetal calf serum. Cells were then incubated for a further 2 h in serum-free medium prior to insulin treatment.

Preparation of cell extracts

Insulin-stimulated protein-serine/threonine kinase activities were measured either in crude cell lysates or after partial purification by MonoQ f.p.l.c. For measurement of kinase activities in crude cell extracts, 30 mm dishes of serum-starved cells were incubated at 37 °C in 1 ml of serum-free medium supplemented with the concentrations of insulin and for the times indicated in the Figure legends. The medium was aspirated and the cells washed with 2 ml of ice-cold phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-NaH_2PO_4, pH 7.4) and rapidly extracted by scraping into 0.25 ml of ice-cold buffer A [50 mM-β-glycerophosphate (pH 7.4)/1.5 mM-EGTA/1 mM-benzamidine/1 mM-dithiothreitol/0.5 mM-Na_3VO_4/0.1 mM-phenylmethanesulphonyl fluoride/1 μM-microcystin-LR/1 μg of each of pepstatin, antipain and leupeptin/ml]. The resulting cell extracts were clarified by centrifugation at 10000 *g* for 10 min at 4 °C. Extracts were then assayed immediately for kinase activity. Such an extraction procedure resulted in complete cell lysis, as judged by release of cytosolic lactate dehydrogenase activity into the supernatant.

Cell extracts for fractionation by MonoQ chromatography were prepared from 100 mm dishes of cells (10 dishes of cells per treatment) which were incubated in 5 ml of serum-free medium with or without 10 nM-insulin for 10 min at 37 °C. The medium was then removed and cell monolayers were washed with 2 ml of ice-cold phosphate-buffered saline and immediately extracted by scraping into 0.5 ml of ice-cold buffer A. The extract was gently homogenized in a hand-held glass homogenizer (5 strokes), clarified by centrifugation at 10000 *g* for 10 min at 4 °C and snap-frozen in 1 ml aliquots in liquid nitrogen for storage at −70 °C until required.

Fractionation of insulin-stimulated kinases by MonoQ chromatography

Cell extracts (between 1 and 3 mg of protein) from CHO.K1, CHO.T and CHO.K1030R cells were rapidly thawed to 4 °C and any remaining particulate matter removed by filtration through a low-protein-binding 0.2 μm-pore-size filter (Sartorius; SM16534). All further operations were carried out at 4 °C. The cell extracts were loaded on to a MonoQ HR 5/5 column (Pharmacia) at a flow rate of 0.5 ml/min, pre-equilibrated with buffer B [50 mM-β-glycerophosphate (pH 7.4)/1 mM-EGTA/1 mM-dithiothreitol]. The column flow-through was collected and the column was washed with buffer B until the absorbance at 280 nm returned to baseline. The column was developed, at a flow rate of 0.75 ml/min, with a linear gradient to 0.5 M-NaCl in buffer B. Fractions (1 ml) were collected into tubes containing 50 μl of 50 mM-β-glycerophosphate (pH 7.4)/2 mM-Na_3VO_4 and assayed the same day.

Protein kinase assays

Crude cell extracts or fractions from MonoQ chromatography were assayed for kinase activity by incubation in 96-well microtitre plates in a final volume of 50 μl containing 20 mM-Mops (pH 7.4)/10 mM-$MgCl_2$/1.5 mM-EGTA/1 mM-dithiothreitol/0.1 mM-Na_3VO_4/2 mM-microcystin-LR/1 μM-cyclic AMP-dependent protein kinase inhibitor peptide/0.1 mM-[γ-^{32}P]-ATP (200–500 c.p.m./pmol) and substrate peptides (0.1 mg of myelin basic protein/ml, 0.5 mM-Kemptide, 0.5 mM-8-mer S6 peptide, 0.5 mM-32-mer S6 peptide or 0.5 mM-casein kinase II substrate peptide). Reactions were initiated by the addition of sample (20 μl) and terminated, after 15 min at 30 °C, with 20 μl

of 2 M-HCl. Samples (30 μl) were removed from the reaction mixtures, spotted on to P81 phosphocellulose paper squares (Whatman) and immersed in ice-cold 150 mM-H_3PO_4. The papers were rinsed three times with fresh acid, once in ethanol, dried and then counted for radioactivity in 10 ml of water by the Čerenkov method. Non-specific ^{32}P incorporation was determined in identical assays lacking substrate. In kinase assays using crude cytosolic extracts, less than 30% of the total ATP was hydrolysed during the course of the reaction and ATPase activity present in MonoQ fractions was negligible.

Immunoblotting

Proteins in samples (0.5 ml) from MonoQ fractions, derived from extracts of insulin-treated CHO.T cells, were precipitated by incubation with 10% (w/v) trichloroacetic acid for 60 min at 0 °C. The protein pellets were collected by centrifugation at 10000 *g* for 20 min, neutralized and separated by SDS/PAGE (8% or 10% polyacrylamide). Proteins were transferred to Immobilon (Millipore) and Western-blotted with antisera specific to either ERK1/ERK2 or recombinant *rsk* (1:500 dilution), followed by chemiluminescent detection as described (Tavaré *et al.*, 1991).

RESULTS

Rapid activation of protein kinases by insulin in CHO.T cells

CHO.T cells were treated with 10 nM-insulin and the incubation terminated at time points up to 30 min. Soluble cytosolic extracts were rapidly prepared and assayed for protein kinase activity towards myelin basic protein (hereinafter referred to as MAP kinase; see later discussion), Kemptide and S6 peptide (8-mer). As shown in Fig. 1, all three kinase activities were rapidly stimulated by insulin, with the maximal response occurring at ~ 5 min. Kinase activity able to phosphorylate Kemptide and S6 peptide (8-mer) remained elevated for a further 5 min before declining to values of approx. 50% and 70% respectively of the maximal activity by 30 min. In contrast, MAP kinase activity was rather more transiently stimulated, and declined to approx. 50% of its maximal activity by 15 min.

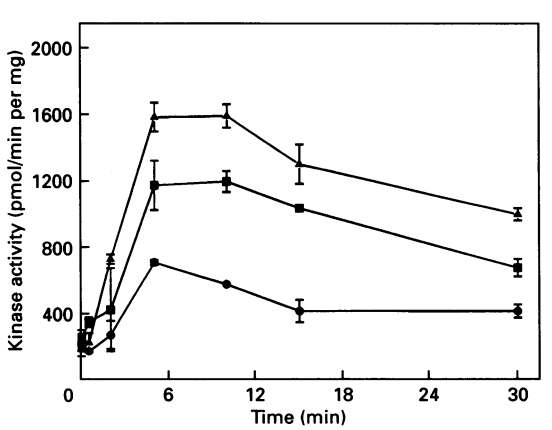

Fig. 1. Time course of activation of insulin-stimulated protein kinases in CHO.T cells

CHO.T cells were treated for the indicated times with 10 nM-insulin and cytosolic extracts prepared. Kinase activity in the extracts was assayed using myelin basic protein (●), S6 peptide (8-mer) (■) or Kemptide (▲) as substrates, as described in the Materials and methods section. The data points shown represent the means ± S.E.M. for three sets of dishes, each assayed in duplicate.

Insulin-stimulated kinase activities in cytosolic extracts of CHO cells expressing human insulin receptor mutants

CHO.K1, CHO.T, CHO.YF1, CHO.YF3, CHO.ΔCT69 and CHO.K1030R cell lines were treated with or without 10 nM-insulin for 5 min and protein kinase activities were assayed in cytosolic extracts. The data are shown in Fig. 2, where kinase activities for each substrate are expressed as a fraction of the insulin-stimulated kinase activity measured in the CHO.T extract for each individual substrate.

MAP kinase activity is shown in Fig. 2(*a*). In all cell lines the basal MAP kinase activity was essentially the same (approx. 312 ± 31 pmol/min per mg of protein). The parental CHO.K1 cell line showed a small 1.4-fold insulin effect, and this was increased to 3.8-fold in cells expressing wild-type human insulin receptors (CHO.T cells). Cells expressing receptors with Y1162F (CHO.YF1 cells) and Y1162/1163F (CHO.YF3 cells) substitutions exhibited 2.1- and 1.8-fold insulin effects respectively. MAP kinase activity in extracts from CHO.ΔCT69 cells was stimulated approx. 4-fold. The incorporation apparent at 10 nM-insulin was 23 ± 8% greater (mean ± S.E.M. of three experiments; $P < 0.05$) in extracts from CHO.ΔCT69 cells than from CHO.T cells. Extracts of CHO.K1030R cells treated with or without insulin showed levels of MAP kinase activity similar to those observed in the parental CHO.K1 cells.

Fig. 2(*b*) shows the S6 peptide (8-mer) kinase activity in the same cell extracts. Introduction of the various mutant insulin receptors had little effect on basal S6 peptide kinase activity (approx. 247 ± 93 pmol/min per mg of protein). The insulin effect on S6 peptide kinase activity in parental CHO.K1 cells was low (1.6-fold) compared with that observed in CHO.T cells which express wild-type receptors (4.8-fold). Cells expressing the Y1162F and Y1162/1163F mutant insulin receptors exhibited 2.7- and 2.2-fold insulin effects respectively. However, in contrast with the results with MAP kinase activity (Fig. 2*a*), the insulin effect on S6 peptide kinase activity in CHO.ΔCT69 cells (5.5-fold) was not significantly different from that observed in CHO.T cells expressing the wild-type receptor. Abolition of the kinase activity of the receptor by substitution of lysine for arginine at the ATP-binding site (CHO.K1030R cells) resulted in a return to a level of insulin stimulation (1.5-fold) similar to that observed in parental CHO.K1 cells.

Kemptide kinase activity in the different cell types is shown in Fig. 2(*c*) with, again, little significant difference in basal activity between the various cell lines (approx. 185 ± 33 pmol/min per mg of protein). The insulin effect on Kemptide kinase activity in parental CHO.K1 cells was negligible (1.8-fold) when compared with that observed in extracts from CHO.T cells (10.3-fold). Cells expressing the Y1162F and Y1162/1163F mutant insulin receptors again exhibited greatly diminished insulin-responsiveness (3.4- and 3.7-fold effects respectively). Removal of 69 amino acids from the *C*-terminus of the β-subunit (CHO.ΔCT69 cells) had little effect on the insulin stimulation of Kemptide kinase activity (9.8-fold) when compared with cells expressing the wild-type receptor. Cells expressing the kinase-inactive insulin receptor (CHO.K1030R cells) showed an insulin-sensitivity (1.5-fold) not significantly different from that observed in the parental CHO.K1 cells.

Dose response of insulin-stimulated protein kinases in CHO.K1, CHO.T and CHO.ΔCT69 cells

We investigated further the apparent increase in the insulin sensitivity of MAP kinase observed in CHO.ΔCT69 cells. The response of CHO.K1, CHO.T and CHO.ΔCT69 cells to various concentrations of insulin was examined for MAP kinase (Fig.

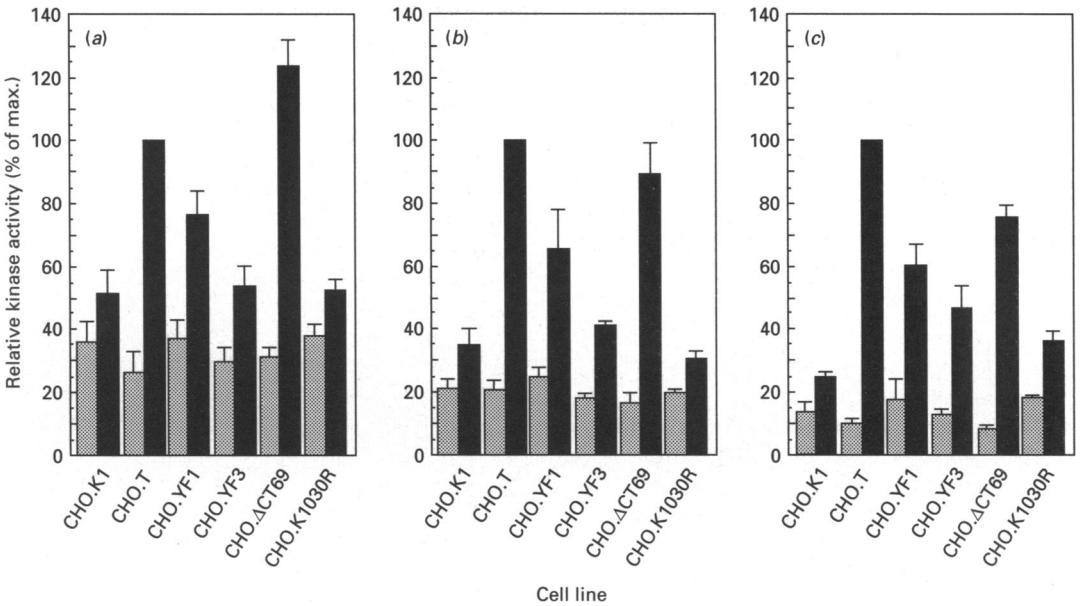

Fig. 2. Insulin-stimulated kinase activities in cytosolic extracts of CHO cells expressing human insulin receptor mutants

CHO.K1, CHO.T, CHO.YF1, CHO.YF3, CHO.DCT69 and CHO.K1030R cells were treated for 5 min with (■) or without (▨) 10 nM-insulin, and cytosolic extracts were prepared. Kinase activities in the extracts were assayed using myelin basic protein (*a*), S6 peptide (8-mer) (*b*) and Kemptide (*c*) as substrates. The data shown represents the means ± S.E.M. for three experiments performed in duplicate with separate cell preparations.

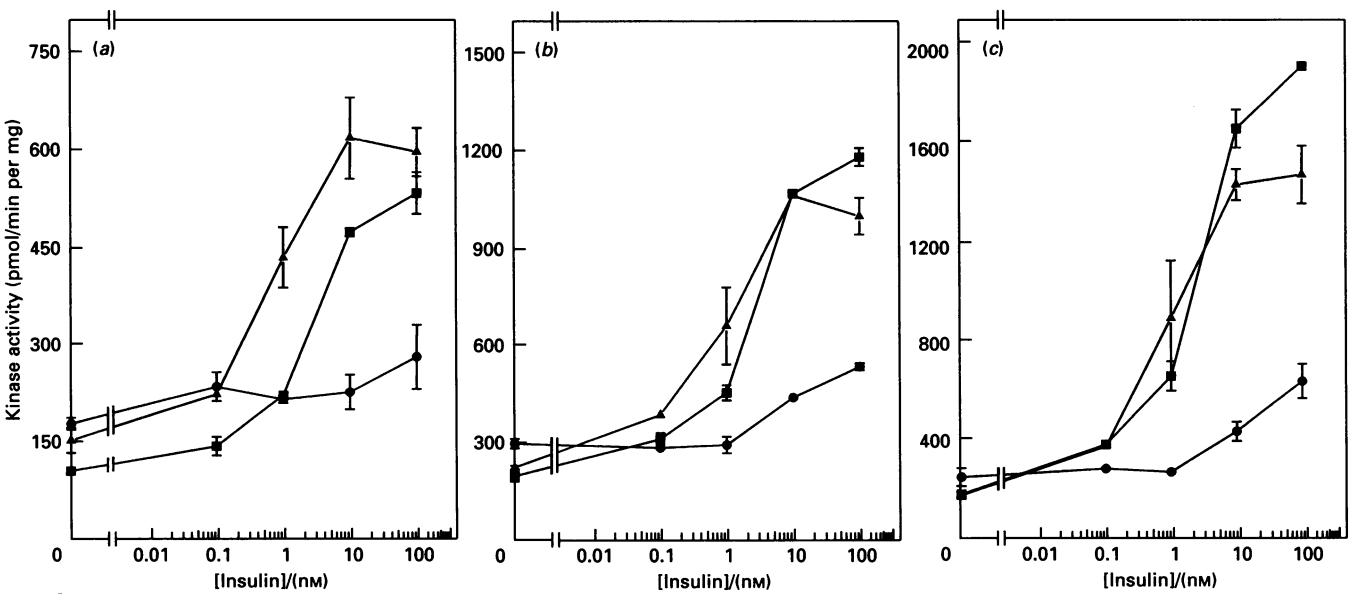

Fig. 3. Dose–response curves of insulin-stimulated kinases in CHO.K1, CHO.T and CHO.ΔCT69 cells

CHO.K1 (●), CHO.T (■) and CHO.ΔCT69 (▲) cells were treated for 5 min with insulin at the concentrations indicated and cytosolic extracts prepared. Kinase activities in the extracts were assayed using (*a*) myelin basic protein, (*b*) S6 peptide (8-mer) and (*c*) Kemptide as substrates, as indicated. The data shown are the means ± S.E.M. of two sets of dishes, each assayed in duplicate.

3*a*), S6 peptide (8-mer) kinase (Fig. 3*b*) and Kemptide kinase (Fig. 3*c*) activities in cytosolic cell extracts.

Insulin had little effect on any of these protein kinase activities in parental CHO.K1 cells unless used at supraphysiological concentrations (> 10 nM). As would be expected, based on previous studies of other bioeffects of insulin (e.g. glucose transport and thymidine uptake), CHO.T cells show a leftward shift in the dose response to insulin compared with CHO.K1 cells (ED_{50} 2.5 ± 0.9 nM) for activation of all three protein kinase activities (Fig. 3). CHO.ΔCT69 cells displayed a further leftward shift in insulin-sensitivity for the activation of MAP kinase (Fig. 3*a*; ED_{50} 0.6 ± 0.2 nM). At 1 nM-insulin, the activity seen in the CHO.ΔCT69 cell extracts (510 ± 96 pmol/min per mg) was significantly greater than that in the CHO.T extracts (254 ± 23 pmol/min per mg; means ± S.E.M., $n = 4$, $P < 0.005$). A slight leftward shift in the insulin dose response was also apparent for the activation of S6 peptide (8-mer; Fig. 3*b*) and Kemptide (Fig. 3*c*) kinase activities. However, this was not significant and was not observed in a separate experiment, in which the dose responses for S6 peptide (8-mer) and Kemptide kinases in

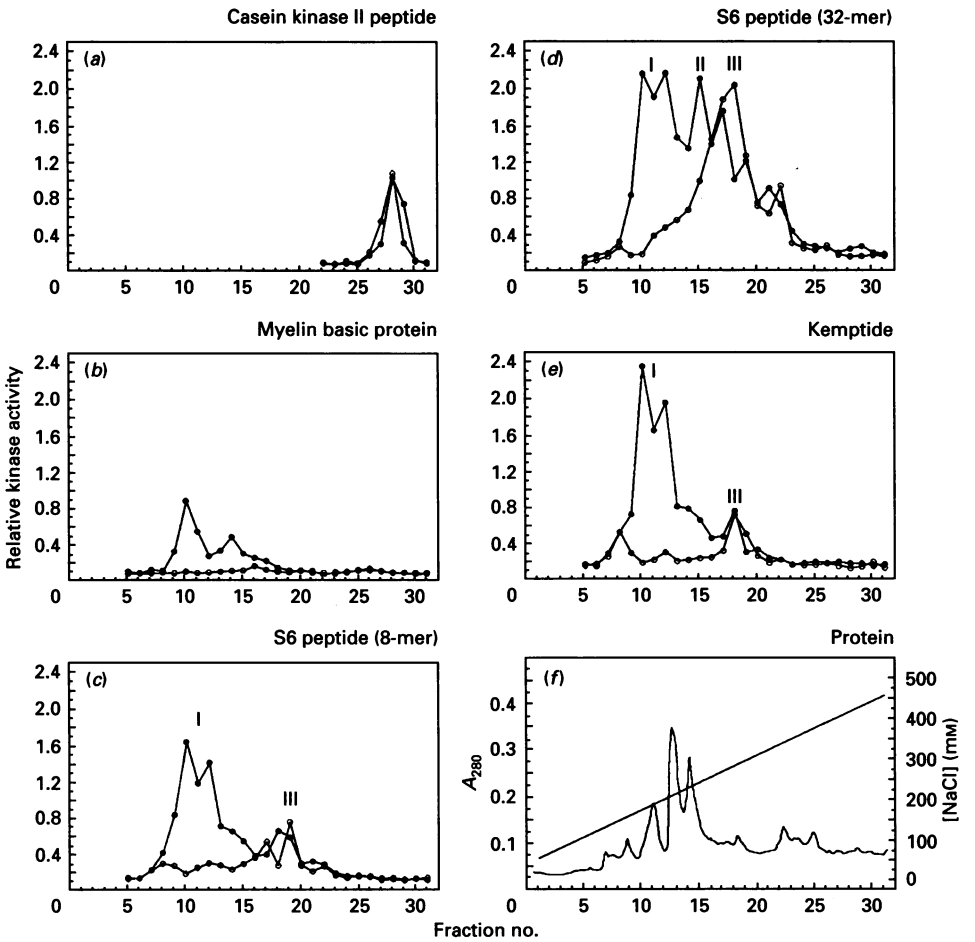

Fig. 4. MonoQ fractionation of insulin-stimulated kinase activities in cytosolic extracts of CHO.T cells

CHO.T cells were treated for 5 min with (●) or without (○) 10 nM-insulin. Cytosolic extracts were prepared and fractionated by anion-exchange chromatography on MonoQ as described in the Materials and methods section. Samples of each fraction were assayed for protein kinase activity towards casein kinase II substrate (*a*), myelin basic protein (*b*) S6 peptide (8-mer; *c*), S6 peptide (32-mer; *d*) or Kemptide (*e*). Kinase activities are expressed relative to the peak casein kinase II activity, which was found to be essentially the same for each cell line with no apparent effect of insulin. Also shown in the Figure is a characteristic absorbance profile (A_{280}; *f*). Similar results were obtained in six independent experiments using myelin basic protein, S6 peptide (32-mer) and casein kinase II as substrates, and in three experiments using S6 peptide (8-mer) and Kemptide as substrates.

CHO.ΔCT69 cells were superimposable with those observed in CHO.T cells. Thus it would appear that the insulin stimulation of MAP kinase activity in CHO.ΔCT69 cells is enhanced with respect to that in CHO.T cells, whereas the responses of S6 peptide (8-mer) kinase and Kemptide kinase are apparently normal.

MonoQ f.p.l.c. fractionation of insulin-stimulated protein kinase activities from cytosolic extracts of CHO.T cells

To resolve the individual components that make up the combined protein kinase activities measured in crude cytosolic extracts, we fractionated these extracts by anion-exchange chromatography on MonoQ. Fig. 4 shows the profile of kinase activity towards casein kinase II substrate peptide, myelin basic protein, S6 peptides (8-mer and 32-mer) and Kemptide in fractions eluting from MonoQ.

Casein kinase II eluted late in the gradient, at approx. 400 mM-NaCl, with insulin having no significant effect on its activity (22.9 ± 3.5 and 25.7 ± 3.3 pmol/min per mg of protein for the peak fractions from control and insulin-stimulated cells respectively; Fig. 4*a*). This was found to be the case regardless of the cell line used (results not shown). Indeed, the absolute casein kinase II activity in both cytosolic extracts and peak MonoQ fractions remained relatively constant for each CHO cell line.

Cytosolic MAP kinase activity from insulin-treated cells was fractionated into two distinct peaks eluting from MonoQ at approx. 150 mM- and 210 mM-NaCl, while MAP kinase activity in the basal state was essentially undetectable (Fig. 4*b*).

Insulin-stimulated S6 kinase activity, as measured using the short S6 peptide (8-mer; Fig. 4*c*), eluted as a doublet early in the gradient with maxima at 135 mM- and 160 mM-NaCl (peak I). The insulin effect observed on activity in these fractions was approx. 8–10-fold. A second, much smaller, peak of S6 kinase activity eluted later in the gradient at approx. 270–290 mM-NaCl (peak III). However, the size of this peak was somewhat variable between experiments and was unaffected by insulin.

When the longer S6 peptide (32-mer; Fig. 4*d*) was used to assay S6 protein kinase activities in MonoQ fractions, the double peak of activity seen with the 8-mer peptide (peak I) was again observed, with an insulin effect in excess of 10-fold. A complex additional peak of insulin-stimulated activity was also resolved, eluting at 240–290 mM-NaCl. This was made up of a peak at 240 mM-NaCl (peak II) which was also stimulated by insulin, and a further peak eluting at 270–290 mM-NaCl (co-migrating with

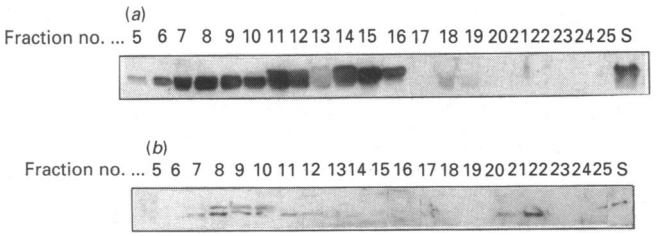

Fig. 5. Western blot analysis of MonoQ fractions from insulin-stimulated CHO.T cells

Cytosolic extracts of CHO.T cells incubated with 10 nM-insulin were fractionated by MonoQ chromatography. Fractions 5–25 inclusive were separated by SDS/PAGE and proteins reactive towards anti-ERK1/ERK2 (*a*) or anti-*rsk* (S6 kinase II; *b*) antisera were detected by Western blotting as described in the Materials and methods section. Standard samples of purified MAP kinase (*a*) or *Xenopus* S6 kinase II (*b*) were treated in a similar fashion (lanes S).

peak III observed using the 8-mer S6 peptide). Peak III was thus much more evident when the 32-mer S6 peptide was used, but it still exhibited little significant change in response to insulin (if anything a small decrease was evident; see Figs. 4*d* and 6*e*).

Basal and insulin-stimulated Kemptide kinase activity appeared to co-migrate almost exactly with kinase activity towards the short 8-mer S6 peptide (peak I), although a larger insulin effect was apparent for Kemptide kinase (10–12-fold; Fig. 4*e*).

It appears, therefore, that there are at least three major peaks of S6 peptide kinase activity that can be separated by MonoQ fractionation of CHO.T cell extracts, with peak I probably constituting most, if not all, of the insulin-stimulated S6 kinase activity detectable in crude cytosolic extracts when using the 8-mer peptide as a substrate (as seen in Figs. 1, 2 and 3).

Western blot analysis of MonoQ fractions from insulin-treated CHO.T cells with anti-peptide antibodies that recognize both ERK1 and ERK2 revealed a pattern of reactivity strikingly similar to that found in previous studies using Rat1 fibroblasts (Fig. 5*a*; see also Boulton & Cobb, 1991; Boulton *et al.*, 1991*b*). Elution of a lower-molecular-mass form (apparent mass 44 kDa) predominantly occurred between fractions 7 and 12 (Fig. 5*a*) and generally coincided with elution of the first peak of MAP kinase activity (Fig. 4*b*). A slightly higher-molecular-mass form (apparent mass 51 kDa) was also observed, eluting with two peaks at fractions 11 and 14/15 (Fig. 5*a*), which coincided with the first and second peaks respectively of MAP kinase activity (Fig. 4*b*). The 51 kDa form co-migrated with purified MAP kinase (Fig. 5*a*).

Western blot analysis using an antiserum raised to recombinant *rsk* and reactive towards *Xenopus* S6 kinase II (a member of the 90 kDa family of S6 kinases) demonstrated that a protein (apparent molecular mass 92 kDa) which was reactive towards

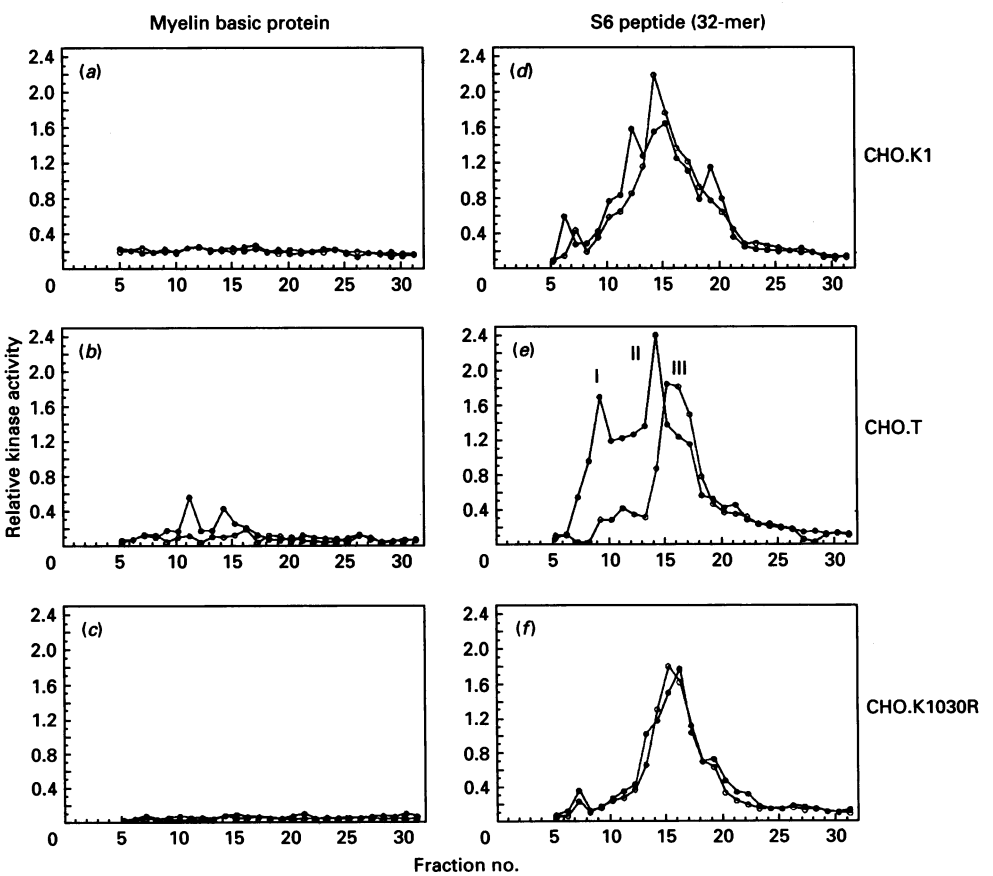

Fig. 6. MonoQ fractionation of MAP and S6 kinase activities in cytosolic extracts of CHO.K1, CHO.T and CHO.K1030R cells

CHO.K1, CHO.T and CHO.K1030R cells were treated for 5 min with (●) or without (○) 10 nM-insulin, and cytosolic extracts were prepared and fractionated by anion-exchange chromatography on MonoQ as described in the legend to Fig. 4. Samples of each fraction were assayed for kinase activity towards myelin basic protein, casein kinase II substrate peptide, and S6 peptide (32-mer). Kinase activities are expressed relative to the peak casein kinase II activity. Profiles from CHO.K1 and CHO.K1030R extracts are representative of three independent experiments.

this serum eluted between fractions 7 and 10 (Fig. 5*b*). This protein appeared to co-migrate with purified *Xenopus* S6 kinase II (Fig. 5*b*). A lower, unidentified, band of reactivity (apparent molecular mass 89 kDa) was also observed, eluting between fractions 7 and 12 and also 21 and 22 (Fig. 5*b*).

MonoQ fractionation of MAP kinase and S6 peptide kinase activities in cytosolic extracts of CHO.K1, CHO.T and CHO.K1030R cells

Insulin-stimulated MAP kinase activity was undetectable in MonoQ profiles from parental CHO.K1 extracts (Fig. 6*a*). As expected, CHO.T cells exhibited two peaks of insulin-stimulated activity eluting at 150 and 210 mM-NaCl (Fig. 6*b*). The profile of MAP kinase activity from CHO.K1030R cells (Fig. 6*c*) was not significantly different from that observed with the parental CHO.K1 cells, confirming that the protein-tyrosine kinase activity of the insulin receptor is a pre-requisite for insulin stimulation of both peaks of MAP kinase activity.

Kinase activity towards the long S6 peptide (32-mer) was apparent in extracts from all three cell lines used. In the parental CHO.K1 cell line this activity was not stimulated appreciably by insulin and was present as a single broad peak centred around fraction 15 (Fig. 6*d*; probably equivalent to peaks II and III of Fig. 4*d*). Peak III was also present in MonoQ profiles from CHO.T (Fig. 6*e*) and CHO.K1030R cells (Fig. 6*f*), while insulin again stimulated S6 peptide kinase activity in peaks I and II in CHO.T cells (Fig. 6*e*). The profile of S6 peptide kinase activity from CHO.K1030R cells was, however, not significantly different from that observed in the parental CHO.K1 cell line (Fig. 6*f*).

DISCUSSION

Characterization of insulin-stimulated protein kinases in CHO.T cells

Insulin had a rapid and marked effect on protein kinase activities in CHO.T cells towards peptide substrates, even when assays were performed on relatively crude cytosolic extracts. Maximal activation was observed within 5 min of the addition of insulin (Fig. 1). Insulin effects were observed of approx. 3.8-fold for MAP kinase activity using myelin basic protein as substrate, approx. 5-fold for S6 peptide (8-mer) kinase and approx. 10-fold for Kemptide kinase (Figs. 1 and 2). Transfection of wild-type human insulin receptors into CHO.K1 cells resulted in a substantial increase in the responsiveness of the cells to insulin with respect to activation of all three protein kinase activities (as indicated by the leftward shift in dose–response curve for CHO.T versus CHO.K1 cells of Fig. 3).

Further fractionation of cytosolic extracts of CHO.T cells by MonoQ f.p.l.c. demonstrated that insulin had pronounced effects on MAP kinase activity, which was resolved into two distinct peaks (Fig. 4*b*). These peaks also contained activity that phosphorylated a 280 kDa protein in a crude rat brain microtubule preparation containing MAP2, thereby confirming their identity as MAP kinases (results not shown). The two peaks of insulin-stimulated MAP kinase activity in extracts from CHO.T cells (Fig. 4*b*) were eluted from MonoQ at very similar salt concentrations to MAP kinases (particularly ERK1 and ERK2) reported for other cell lines (Ahn *et al.*, 1990, 1991; Gomez *et al.*, 1990; Boulton *et al.*, 1991*a*,*b*). Indeed, Western blot analysis demonstrated that these peaks of MAP kinase activity co-eluted with proteins that were immunoreactive towards anti-ERK1/ERK2 antiserum (Fig. 5*a*). It is likely, therefore, that the first peak of MAP kinase activity to elute from MonoQ is ERK2 and the second peak is ERK1; these have reported apparent molecular masses of 42 and 44 kDa respectively on SDS/PAGE (Boulton & Cobb, 1991; Boulton *et al.*, 1991*b*).

The 90 kDa S6 kinase (similar to *Xenopus* S6 kinase II) has been reported to elute from MonoQ at 200–230 mM-NaCl, and utilizes both 32-mer and 8-mer S6 peptides and Kemptide as substrates. In contrast, the 70 kDa S6 kinase elutes rather later from MonoQ than the 90 kDa enzymes (240–390 mM-NaCl) and phosphorylates the 32-mer S6 peptide. The 8-mer S6 peptide, however, does not appear to be a good substrate for the 70 kDa enzyme (Erikson *et al.*, 1987; Dent *et al.*, 1990; Lavoinne *et al.*, 1991; Sturgill & Wu, 1991).

In our study, the 8-mer S6 peptide kinase activity from CHO.T cells was fractionated by MonoQ chromatography into two peaks: the major insulin-stimulated peak (peak I) which is eluted at 135–160 mM-NaCl, and a second peak (III) which is eluted at 270–290 mM-NaCl and is not significantly stimulated by insulin (Figs. 4*c* and 4*d*). The activity in peak I phosphorylates the 8-mer and 32-mer S6 peptides (Figs. 4*c* and 4*d*) and Kemptide (Fig. 4*e*), and thus has the characteristics of a 90 kDa S6 kinase. A protein reactive towards anti-*rsk* antiserum eluted in fractions from MonoQ (Fig. 5*b*) slightly earlier than the peak I of S6 kinase activity (Figs 4 and 6). The 90 kDa S6 kinase must be phosphorylated on serine and threonine to exhibit full activity (Ballou *et al.*, 1988) and it is likely that such phosphorylations may retard the elution of the kinase from MonoQ. Taken together, our evidence suggests that peak I contains a member of the 90 kDa S6 kinase family and that the Kemptide and 8-mer S6 peptide kinase activities present in the crude cytosolic extracts are predominantly a reflection of the 90 kDa S6 kinase activity.

The use of the 32-mer S6 peptide as substrate revealed two additional peaks of S6 peptide kinase activity (peaks II and III; Fig. 4*d*). Peak II is observed only in the presence of insulin. The activity present in peak III, however, appeared, if anything, to decrease in the presence of insulin (Fig. 4). As there is little or no Kemptide or 8-mer S6 peptide kinase activity in this peak, and no protein exhibiting reactivity towards the anti-*rsk* (S6 kinase II) antiserum (Fig. 5*b*), it is likely that peaks II and/or III represent a member(s) of the 70 kDa S6 kinase family. If this is indeed the case, then it appears that the major insulin-stimulated S6 kinase activity in CHO.T cells is due to the 90 kDa enzyme.

Effects of point mutations in the human insulin receptor on insulin-stimulated protein kinases in transfected CHO cells

A K1030R point mutation at the ATP-binding site of the insulin receptor prevented insulin-stimulated MAP kinase and 90 kDa-S6 kinase activation when assayed both in crude cytosolic extracts (Fig. 2) or, more convincingly, after their separation by MonoQ f.p.l.c. (Fig. 6). The Y1162F and Y1162/1163F mutations attenuated the ability of insulin to stimulate the activity of both MAP kinase and 90 kDa S6 kinase activity when assayed in crude cytosolic extracts. The latter is in general agreement with Boulton *et al.* (1990*a*), who have reported previously that the Y1162F and Y1162/1163F mutations block insulin-stimulated S6 kinase activity using 40 S ribosomes as substrate. However, these results are not consistent with the observations of Debant *et al.* (1988), who found an apparently normal stimulation of S6 phosphorylation in CHO cells expressing the Y1162/1163F mutant insulin receptor. While we have not directly examined the state of S6 phosphorylation *in situ*, it is difficult to reconcile this difference of opinion unless the kinase(s) responsible for phosphorylating S6 ribosomal protein *in situ* are distinct from those that are found to phosphorylate S6 peptides and 40 S subunits in cell-free systems.

Recently, Cohen and his co-workers have purified an insulin-stimulated protein kinase from rabbit skeletal muscle, which they have designated ISPK1. This kinase phosphorylates the G-subunit of protein phosphatase-1, apparently promoting an increase in its phosphatase activity (Dent *et al.*, 1990; Lavoinne

et al., 1991). In the intact cell this could lead to both the dephosphorylation and activation of glycogen synthase and the dephosphorylation and inhibition of glycogen phosphorylase; the net result would be increased glycogen synthesis. ISPK1 appears to exhibit several properties characteristic of the 90 kDa S6 kinase (Lavoinne *et al.*, 1991). This hypothesis would, therefore, be entirely consistent with the parallel decrease in the ability of insulin to stimulate the 90 kDa S6 kinase (Figs. 2*b* and 2*c*) and glycogen synthesis (Debant *et al.*, 1988) in CHO.YF3 cells.

Effect of a *C*-terminal deletion mutation on insulin-stimulated protein kinases in transfected CHO cells

Deletion of 69 amino acids from the *C*-terminus of the insulin receptor β-subunit (CHO.ΔCT69 cells) appears to lead to an increase in the insulin stimulation of MAP kinase at both 1 and 10 nM-insulin (i.e. a leftward shift in dose–response curve; Fig. 3*a*) but apparently not of the 90 kDa S6 kinase (Figs. 3*c* and 3*d*), when compared with cells expressing the wild-type human receptor (CHO.T cells). The increased response observed at 10 nM-insulin in cytosolic extracts (Figs. 2 and 3) was confirmed after their separation by MonoQ f.p.l.c.; both peaks of MAP kinase activity showed an approx. 1.5-fold higher activity in CHO.ΔCT69 cells than in CHO.T cells (results not shown). Removal of the *C*-terminus of the β-subunit may, therefore, relieve a constraint upon the receptor, leading to enhanced MAP kinase activity at low concentrations of insulin. Interestingly, when immuno-isolated from transiently transfected COS cells, this receptor mutant has a significantly enhanced basal exogenous protein-tyrosine kinase activity (J. M. Tavaré, P. Ramos & L. Ellis, unpublished work). Deletion of 43 amino acids from the insulin receptor *C*-terminus has been reported to promote an augmented insulin-stimulated thymidine uptake into transfected Rat1 fibroblasts, while causing an abrogation of metabolic signalling (Thies *et al.*, 1989). Paradoxically, this mutation has no apparent effect on insulin receptor signalling when expressed in CHO cells (Myers *et al.*, 1991); thus we can draw no conclusion regarding the role of the MAP kinase identified in our study in mediating insulin's effects on thymidine uptake.

It has been demonstrated previously that MAP kinases can phosphorylate and activate 90 kDa S6 kinases *in vitro* (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn & Krebs, 1990). If this cascade occurs in intact cells (which has not been directly demonstrated), one might reasonably expect that any perturbation in MAP kinase activity would be accompanied by a parallel change in the activity of the 90 kDa S6 kinase, its putative substrate. As the sensitivity of the 90 kDa S6 kinase was unaffected by the 69-amino-acid deletion from the insulin receptor, it could be argued that this kinase cascade does not operate in CHO cells. The peak of 8-mer S6 kinase activity exhibits many properties of a 90 kDa enzyme. However, it elutes fractionally later from MonoQ than the protein reactive towards the anti-(90 kDa S6 kinase) antiserum, but this may well be a reflection of serine/threonine phosphorylation, which could retard elution of the active form of the enzyme. Perhaps the simplest explanation of our observations is that activation of the 90 kDa S6 kinase in CHO cells is dependent on other protein kinase(s) which may be acting in concert with the MAP kinase, and that these additional protein kinase(s) do not exhibit a shift in sensitivity in the CHO.ΔCT69 cells.

Note added in proof (received 7 July 1992)

Subsequently to the acceptance of this manuscript we obtained an antiserum reactive to the 70 kDa S6 kinase (a generous gift of Dr. G. Thomas, Friedrich Miescher Institute, Basel, Switzerland). Immunoblotting of MonoQ fractions with this antiserum revealed a broad peak of reactivity, at approx 70 kDa, eluting between fractions 13 and 23, with a maximum signal obtained in fractions 18 and 19. This corresponded almost exactly with peak III of S6 kinase activity (Fig. 4*d*) confirming that at least some of the S6 kinase activity observed in this peak may be due to an S6 kinase of the 70 kDa family.

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