Kinase Cascades and Gene Expression Control in Insulin Action

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Insulin-activated protein kinases in fat and other cells
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Introduction
Many aspects of the signalling system that links the insulin receptor to the wide range of intracellular effects of insulin remain rather poorly understood. Originally, it appeared that a common feature of the intracellular effects might be the dephosphorylation of regulatory enzymes in metabolism, such as glycogen synthase, pyruvate dehydrogenase and triglyceride lipase, brought about by the activation of either appropriate protein phosphatase activities or cyclic-AMP phosphodiesterase activity [1, 2]. It has been proposed that small-molecular-weight mediators such as phosphoinositol glycans may be involved [3, 4] but doubts remain concerning the extent to which such compounds play a central second-messenger role in insulin action [2, 5]. Moreover, it has become increasingly evident that insulin also causes the increased phosphorylation of many proteins in cells including the ribosomal protein S6, acetyl-CoA carboxylase and ATP-citrate lyase [1, 2]. Several laboratories have also demonstrated that exposure of cells to insulin results in the activation of many distinct intracellular protein kinases. Thus, it has been proposed that protein serine kinases, perhaps arranged in a cascade initiated by the activated tyrosine kinase activity of the insulin receptor itself, may play a central role in the insulin signalling system [1, 2, 6, 7] (Fig. 1). Indeed, it is possible that all the decreases in protein phosphorylation are in fact secondary to a primary activation of protein kinases causing increases in the activity of cyclic-AMP phosphodiesterase [8, 9] or protein phosphatases [10], or the inhibition of specific protein kinases. Most likely, a combination of all three mechanisms is involved.

In this article, we focus on two areas of our research into insulin action which have a bearing on this topic. The first is the effects of insulin on the phosphorylation of the insulin receptor in different cell types. It is generally agreed that the first event in insulin signalling is the activation of the intrinsic tyrosine kinase activity of the β-subunits, which results in the autophosphorylation of multiple tyrosines in these subunits, as well as increased phosphorylation of the insulin receptor [11]. However, it is not clear how increased tyrosine kinase activity of the insulin receptor may bring about the activation of the intracellular protein kinases which are largely specific for serine/threonine residues. In this context, it is of considerable interest that substantial increases in serine and threonine phosphorylation of the insulin receptor can be observed following addition of insulin to certain cells since the protein kinases involved may also be important early components of protein kinase cascades. The second area that we will focus on is the characterization of and interrelations between the various insulin-activated serine/threonine kinases in the cytoplasm of cells,
Fig. 1

Means whereby a cascade of serine/threonine protein kinases in the insulin signalling system may bring about both increases and decreases in the phosphorylation of specific proteins within cells

![Diagram of kinase cascades]

INCREASES IN
SPECIFIC PROTEIN
PHOSPHORYLATION

DECREASES IN
SPECIFIC PROTEIN
PHOSPHORYLATION

with some emphasis on the kinases that might be involved in the increased phosphorylation of acetyl-CoA carboxylase, ATP-citrate lyase and an acid-soluble 22 kDa protein in fat cells.

Studies on the phosphorylation of the insulin receptor in various cell types

Two-dimensional thin-layer mapping of radiolabelled phosphopeptides has proved to be a powerful technique for studying the phosphorylation of the insulin receptor both in vitro and within intact cell preparations [12, 13]. The examples given in Fig. 2 allow comparisons to be made between the pattern of phosphorylation of the receptor in transfected cultured COS cells (which express about $2 \times 10^8$ human insulin receptors per cell) with that found in freshly isolated rat liver cells (which contain about 25,000 receptors per cell). One important similarity is that, in both cases, insulin stimulates a marked increase in the phosphorylation of the three tyrosines in the kinase domain (corresponding to Tyr-1158, -1162 and -1163 of the human receptor and giving rise to phosphopeptides A1, A2, B2, B3 and C1) although it is noticeable that a larger proportion of the rat liver receptors become phosphorylated on all three tyrosines in this domain (peptides A1 and A2). However, there are other more marked differences such as the complete lack of phosphorylation of the two tyrosines (1328 and 1334) near the C-terminus of the liver receptor. Most relevant in the context of this article are the substantial differences in the patterns of phosphoserine- and phosphothreonine-containing peptides. In the transfected COS cells, there is very little evidence of such peptides from receptors extracted from cells incubated in the absence of insulin. By contrast, in the presence of insulin there are increases in the phosphorylation of at least five phosphoserine-containing peptides (peptides S1–S5) and one phosphothreonine-containing peptide (peptide T) (Fig. 2). Studies from a number of different laboratories indicate that the latter peptide contains Thr-1348 [14–16]. Only one of the phosphoserine-containing peptides has been identified so far (peptide S5 which contains Ser-1305/1306) [16, 17] and this peptide is by no means the most prominent. Transfected Chinese hamster ovary (CHO) cells which also express about $1 \times 10^8$ human insulin receptors (CHO-T cells) give a very similar pattern of phosphopeptides. The situation in fresh liver cells is quite different [18, 19]. In the absence of insulin there is a considerable amount of phosphorylation of the receptor on serines giving rise to peptides Sa–Sc. Addition of insulin causes only quite modest changes including an increase in peptide Sa and probably a decrease in peptide Sb. Unlike the transfected COS and CHO-T cells, insulin caused no increase in the phosphorylation of the peptides corresponding to S5 and T of the human receptor. Further studies have shown that addition of glucagon to liver cells increases the phosphorylation of peptides Sa and Sc but these changes are not accompanied by any detectable alteration in the tyrosine kinase activity of the receptor [19]. In contrast, we have found recently that exposure of liver cells to phorbol esters results in a decrease in insulin receptor tyrosine kinase activity (D. F. Lynch and J. M. Tavare, unpublished work) as
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Fig. 2

Two-dimensional maps of the phosphopeptides of insulin receptors from intact transfected COS cells expressing human insulin receptors and rat liver cells in the presence and absence of insulin.

Full details of procedures are given in [16, 18]. The key relates to the phosphopeptides derived from insulin receptors of COS cells. Phosphorylation of Tyr-1158, -1162 and -1163 in the kinase domain gives rise to a family of five phosphopeptides which are mono-(C2), bis- (B2 and B3) or tris- (A1 and A2) phosphorylated. The same peptides are evident in the maps of the rat liver receptor. Phosphopeptide B1 contains Tyr-1328 and Tyr-1334 and is not present in the rat liver map. Phosphoserine- and phosphothreonine-containing peptides are indicated in the figure and are discussed further in the text.

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<th>Human</th>
<th>Rat</th>
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<td>Control</td>
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<td>Insulin</td>
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observed in other cells [20, 21]; however, the accompanying changes in phosphorylation of the rat liver receptor remain to be established.

The relationship between tyrosine and serine/threonine phosphorylation of the insulin receptor can also be explored by studying the changes in patterns of receptor phosphorylation in CHO cells expressing mutated insulin receptors. Recent studies by Tavare and Dickens in collaboration with Dr L. Ellis (Texas A & M University) and Dr R. Roth (Stanford University) have demonstrated the following. First, in intact Y1162F and Y1162/3F cells there is a complete loss of insulin-stimulated phosphorylation of Thr-1348, whereas insulin-stimulated serine phosphorylation is still apparent albeit reduced in magnitude [22]. This observation suggests that distinct signalling pathways are involved in bringing about the increased phosphorylation of these sites in cells. Second, in hIRACT69 cells which express human insulin receptors with a 69-amino-acid deletion from the C-terminus of the β-subunit there is little or no difference in insulin-stimulated phosphorylation of not only Tyr-1158, -1162 and -1163 but also of the major phosphoserine-containing peptides (S1 and S2), which are retained in this mutant. As expected, phosphopeptides corresponding to Tyr-1328 and -1334, Ser-1305/1306 and Thr-1348 were not present as these sites are in the deleted portion of the receptor (J. M. Tavare et al., unpublished work).

Overall, it is evident from the above studies that the insulin receptor can probably be phosphorylated within cells by a number of different serine/threonine protein kinases, some of which may play an important role in intracellular signalling. Almost certainly, complex inter-relationships exist between the changes in tyrosine and serine/threonine phosphorylation of the receptor. The marked differences in patterns of phosphorylation observed in the receptors of transfected COS or CHO cells and fresh rat liver cells are presumably a reflection of the different amounts or activities of the receptor, protein kinases and protein phosphatases present in the two cell types. The amino acid sequences of the rat and human receptors are nearly identical.
Studie on insulin-activated serine/threonine kinases in fat and CHO cells

The number of insulin-activated cytoplasmic serine/threonine kinases that have been reported is increasing rapidly (Table 1). In particular, we and others have demonstrated the presence of more than eight in rat epididymal fat cells. In the case of our own studies, we have made extensive use of Mono-Q chromatography in order to separate the kinases including mitogen-activated protein (MAP)/myelin basic protein kinases, S6-peptide kinases, ATP-citrate lyase kinases, acetyl-CoA carboxylase kinase and casein kinase 2 [23, 24] (C. Schmitz-Peiffer, A. C. Borthwick & R. M. Denton, unpublished work). Activation varies from about 1.5-fold to more than four-fold. We have focused our attention mainly on acetyl-CoA carboxylase kinase, casein kinase 2 and ATP-citrate lyase kinases and these are considered briefly in turn.

Insulin increases the activity of acetyl-CoA carboxylase in fat cells by about twofold apparently because a greater proportion of the enzyme is in a citrate-insensitive polymerized form [25]. This is associated with an increased phosphorylation of the enzyme on at least two serine residues [25-27]. One site is Ser-21 which in vitro can be phosphorylated by casein kinase 2, but phosphorylation of this site does not appear to result in any change in the activity of the enzyme [26, 27]. The other

<table>
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<td>Summary of serine/threonine protein kinases that are activated in cells incubated with insulin</td>
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<table>
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<tr>
<th>Fat cells*</th>
<th>Activated by phosphorylation</th>
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<tr>
<td>Acetyl-CoA carboxylase kinase</td>
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<tr>
<td>ATP-citrate lyase kinase</td>
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</tr>
<tr>
<td>Casein kinase 2</td>
<td>*</td>
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<tr>
<td>Insulin receptor kinase(s)</td>
<td>*</td>
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<tr>
<td>p44 MAP kinase (ERK 1)</td>
<td>*</td>
</tr>
<tr>
<td>p42 MAP kinase (ERK 2)</td>
<td>*</td>
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<tr>
<td>Protein kinase C</td>
<td>*</td>
</tr>
<tr>
<td>Raf-1 kinase</td>
<td>*</td>
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<tr>
<td>p70 Ribosomal protein S6 kinase</td>
<td>*</td>
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<tr>
<td>p90 Ribosomal protein S6 kinase</td>
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serine or serines are within another peptide which is as yet unidentified and which has been designated the 'l-peptide'; the active polymerized form of the enzyme from insulin-treated fat cells is phosphorylated within this peptide [23, 25, 26]. We have recently separated an insulin-activated acetyl-CoA carboxylase kinase from fat pad extracts. This kinase is eluted from Mono-Q with 0.17 M NaCl pH 7.4 and appears to phosphorylate acetyl-CoA carboxylase within a peptide which, on the basis of two-dimensional thin-layer analysis, h.p.l.c. and Superose-6 chromatography, is the same or similar to the 'l-peptide'. There is an increase in acetyl-CoA carboxylase activity which parallels the phosphorylation of the enzyme by this kinase [23]. Co-eluting from Mono-Q with the kinase is a potent inhibitor of acetyl-CoA carboxylase activity which we have recently shown to be CoA [28]. Indeed, acetyl-CoA carboxylase is a better substrate for the activating kinase in the presence of CoA [23, 28]. A further interesting development has been our recent finding that Mono-Q fractions containing acetyl-CoA carboxylase kinase activity also contain S6-peptide kinase activity similar to that of the 90K-S6 kinase family, and attempts to separate the activities by subsequent procedures have not proved successful to date. However, purified 90K-S6 kinase preparations from frog oocytes and rabbit skeletal muscle kindly given to us by the laboratories of Professor J. Maller (University of Colorado, USA) and Professor P. Cohen (University of Dundee) did not phosphorylate acetyl-CoA carboxylase (K. J. Heesom, S. K. Moule, N. J. Edgell & R. M. Denton, unpublished work).

Casein kinase 2 is eluted from Mono-Q at 0.40 M NaCl and its activity is increased about 1.6-fold following exposure of rat epididymal fat pads or cells to insulin [29]. One substrate for casein kinase 2 in fat cells is acetyl-CoA carboxylase as mentioned above. Another is probably an acid soluble 22 kDa protein which we first showed in 1980 to exhibit marked increases in serine and threonine phosphorylation following exposure of cells to insulin [29]. We have developed a means of purifying this protein from adipose tissue and investigated its properties in some detail [24, 30]. In particular we have found that this protein is a poor substrate for many different protein kinases except casein kinase 2 for which it is an excellent substrate. Moreover, casein kinase 2 phosphorylates the protein on both serine and threonine residues which, on the basis of two-dimensional thin-layer analysis, appear to correspond at least in part to the sites exhibiting increased phosphorylation in insulin-
treated fat cells [30, 31]. The function of the 22 kDa protein remains to be established. The only amino acid sequence information we have obtained to date is from the N-terminus of a phosphorylated peptide and this shows homology with the sequence adjacent to one of the phosphorylation sites of 'G-substrate' [30]. This suggests that the protein may have a role in the regulation of protein phosphatase activities.

Mono-Q chromatography of fat-cell extracts separates two insulin-activated ATP-citrate lyase kinases. The first, eluted at 0.25 M NaCl, is activated about 1.5-2-fold by insulin, also phosphorylates Kemptide and appears to correspond to the kinase first reported by Yu et al. [31]. The second is eluted at 0.08 M NaCl and its activity is increased more than fivefold following insulin treatment (C. Schmitz-Peiffer, A. C. Borthwick & R. M. Denton, unpublished work). The physiological importance of these kinases is a mystery as phosphorylation of ATP-citrate lyase has little effect on its catalytic activity and in the case of the latter kinase the serine phosphorylated is distinct from the major site of phosphorylation in insulin-treated cells.

We have also used Mono-Q chromatography to examine the effects of insulin on the activity of protein kinases in transfected CHO cells [32]. In C110-T cells insulin activates two myelin basic protein kinases and an S6-peptide kinase at least 10-fold. Western blotting results and other characteristics indicate that these kinases correspond to the MAP kinases, ERK1 and ERK2, and a 90 kDa S6 kinase, respectively. In cells expressing a kinase-negative mutant (K1030R) or Y1162F and Y1162F/Y1163F mutant forms of the insulin receptor, the insulin activation of all of these kinases is severely compromised and to a similar extent. In contrast, in cells expressing the hIRΔCT69 mutant receptor, the sensitivity of MAP kinase activity to insulin is increased (as demonstrated by a leftward shift in the dose response by about an order of magnitude). No comparable shift was evident in the insulin activation of the 90 kDa S6 kinase activity [32]. These findings suggest that the activation of these kinases is brought about in these cells through distinct intracellular signalling pathways. If this is so, it is not compatible with the hypothesis [33, 34] that the MAP kinases phosphorylate and activate the 90 kDa S6 kinase family in intact cells.

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Serine/threonine kinases and tyrosine phosphatases that act on the insulin receptor

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Introduction
Insulin initiates its effects by binding to cell-surface receptors, called insulin receptors. The insulin receptor is an insulin-activated tyrosine-specific protein kinase. The dominant form of the receptor has the structure $\alpha_2\beta_2$, with the subunits linked by disulphide bonds to give a $\beta-\alpha-\alpha-\beta$ structure. The $\alpha$-subunits ($M_r 135000$) appear to be located wholly extracellularly and function to bind insulin. In contrast, the $\beta$-subunits ($M_r 95000$) act as a transmembrane anchor and contain tyrosine kinase active sites within their cytosolic domains [1]. The tyrosine kinase catalyses very rapid autophosphorylation of its own $\beta$-subunit on multiple tyrosines. Insulin-stimulated autophosphorylation functions to activate the insulin receptor tyrosine kinase to phosphor-ylate other proteins and renders the tyrosine kinase constitutively active even when insulin is subsequently removed from the binding site [2].

Abolition of tyrosine kinase activity by mutation of Lys-1018 involved in ATP binding does not alter insulin binding, but abrogates cellular responses to insulin [3, 4]. Therefore the tyrosine kinase activity of the insulin receptor appears essential for signal transduction.

In intact cells insulin also induces phosphorylation of serine and threonine residues of the $\beta$-subunit (see, for example, [5]). This is believed to occur via the activation of protein serine/threonine kinases that are distinct from the receptor itself [6, 7]. We have been particularly interested in characterizing the insulin-stimulated insulin receptor serine kinase (IRSK). As this kinase can closely associate with the insulin receptor it has the potential to be an early cascade enzyme in insulin signalling [7]. Additionally, as phorbol ester- and cyclic AMP-stimulated insulin receptor serine phosphorylation apparently inhibit insulin receptor tyrosine kinase activity [8, 9], the IRSK has the potential also to control insulin receptor function, perhaps by limiting the lifetime of the activated receptor [10]. Studies into the identity, nature and mechanism of activation by insulin of IRSK, together with investigations into the location of sites of insulin-stimulated serine/threonine phosphorylation in the insulin receptor, are described in the first part of this article.

The second part of this article is concerned with the characterization of protein-tyrosine phosphatases (PTPases) active against the insulin receptor, and in particular the use of PTPases to probe the role of the various tyrosine autophosphorylation sites in the regulation of insulin receptor tyrosine kinase activity.

Insulin-stimulated insulin receptor serine kinase
At the time of the initiation of the IRSK project there was a dearth of in vitro systems that showed a high activity of the IRSK. Thus, although the insulin receptor underwent significant insulin-stimulated tyrosine and serine phosphorylation in intact cells, at that time most preparations of partially purified or purified insulin receptor described in the literature exhibited only insulin-stimulated tyrosine phosphorylation. This inhibited study of the IRSK. Thus the initial objective was to obtain a system in which a high activity of the IRSK could be demonstrated reproducibly. This was achieved by partially.