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Determination of Insulin Signaling Pathways in Hepatocytes

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ABSTRACT. Diabetes is a major cause of morbidity and mortality, and associated with a high risk of atherosclerosis, and liver, kidney, nerve and tissue damage. Defective insulin secretion in pancreas and/or insulin resistance in peripheral tissues is a central component of diabetes. It is well established that, regardless of the degree of muscle insulin resistance, glucose levels in diabetic and non-diabetic individuals are determined by the rate of hepatic glucose production. Moreover recently studies using liver-specific insulin receptor knockout mice show the paramount role of the liver in insulin resistance and diabetes. Insulin exerts a multifaceted and highly integrated series of actions via its intracellular signaling systems. The first major section of this review defines the major insulin-mediated signaling pathways including phosphatidylinositol 3-kinase and mitogen activated protein kinases. The second major section of the review presents a summary and evaluation of methods for determination of the role and function of signaling pathways, including methods for determination of kinase phosphorylation, the use of pharmacological inhibitors of kinase and dominant-negative kinase constructs, and the application of new RNA interference methods.

Keywords: Insulin signaling, Diabetes, Insulin resistance, Protein kinase, RNA interference, Dominant negative construct.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia caused by defective insulin secretion and/or defective tissue response to insulin. Diabetes is associated with a high risk of atherosclerosis and kidney, nerve, and tissue damage. Also a higher incidence of hepatic disease including hepatic cancer and non-alcoholic hepatitis has been reported to be associated with diabetes. Liver plays an important role in regulation of metabolic homeostasis and is one of major organ response to insulin and glucagon. Hepatic insulin level and cellular responsiveness are altered in diabetes. It is well established that, regardless of the degree of muscle insulin resistance, glucose levels in diabetic and non-diabetic individuals are determined by the rate of hepatic glucose production, which in turn is regulated by insulin (Cherrington, 1999). Disruption of insulin action in liver by tissue-specific knockout of the insulin receptor leads to severe glucose intolerance and resistance to the blood glucose-lowing effect of insulin. This provides evidence of a direct role of liver in post-prandial glucose homeostasis, and suggests that a considerable portion of the decrease in blood glucose following insulin administration is due to a suppression of hepatic glucose production rather than an increase in muscle glucose uptake (Michael *et al.*, 2000).

The numerous and varied cellular effects of insulin, including increased glucose transport, antilipolysis, glycogenesis, promotion of DNA and protein synthesis, cell division, regulation of gene expression, apoptosis and osmolarity are mediated by the cell surface insulin receptor (Kim *et al.*, 2003b, 2004b). Ligand activation of this receptor tyrosine kinase (RTK) results in autophosphorylation of the receptor as well as recruitment and phosphorylation of a number of intracellular proteins that then serve to transduce the signal for insulin actions. The best characterized of these are the insulin receptor substrate (IRS). Following tyrosine phosphorylation, each of these intracellular substrates associates with one or more molecules through specific recognition sites, terms SH2 domains, to generate a downstream signal. Insulin receptor signaling results in activation of phosphati-

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dylinositol 3-kinase (PI3K) and a variety of downstream effectors, including Akt (PKB; protein kinase B), ribosomal p70 S6 kinase and atypical protein kinase C (PKC). Activation of the insulin receptor also leads to activation of mitogenic-activated protein kinase (MAPK) signaling pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (Kim *et al.*, 2004b).

In this review, an overview of the hepatic signaling pathways in response to insulin will be described along with the methods for identifying which signaling pathways and components are involved in mediating insulin action.

INSULIN-MEDIATED SIGNALING PATHWAYS

Insulin receptor

The insulin receptor is a transmembrane heterotetramer consisting of two alpha (extracellular; 135 kDa) and two beta (transmembrane; 95 kDa) subunits linked by disulfide bonds. During transport to the cell surface, a single high molecular weight proreceptor is proteolytically cleaved at a tetrabasic amino acid sequence (arginine-lysine-arginine-arginine) located at the junction of the alpha and beta subunits, and oligosaccharide chains are added at specific sites of glycosylation. Interactions between the two alpha subunits, and between the alpha and beta subunit, are stabilized by disulfide bridges (Cheatham and Kahn, 1992).

Unoccupied alpha-subunits on the cell membrane surface inhibit the intrinsic tyrosine kinase activity of the beta subunit and may be viewed as a regulatory subunit of the catalytic intracellular subunit. The beta subunit is composed of a short extracellular domain, a transmembrane domain, and a cytoplasmic domain which possesses intrinsic tyrosine kinase activity. The cytoplasmic domain contains the ATP binding site and autophosphorylation sites. Binding of insulin to the alpha subunits of the receptor induces conformational changes leading to activation of the RTK activity resulting in transphosphorylation of the beta subunits and endocytic internalization of the receptor via clathrin-coated vesicles (Cheatham and Kahn, 1992). Some of the tyrosine phosphorylated residues of the beta subunits of the receptor present binding sites for the subsequent recruitment of signaling molecules. The insulin receptor utilizes a family of soluble adaptors or scaffolding molecules, such as insulin receptor substrates (IRSs 1-4) and Shc molecules to initiate its signaling cascade through other effectors.

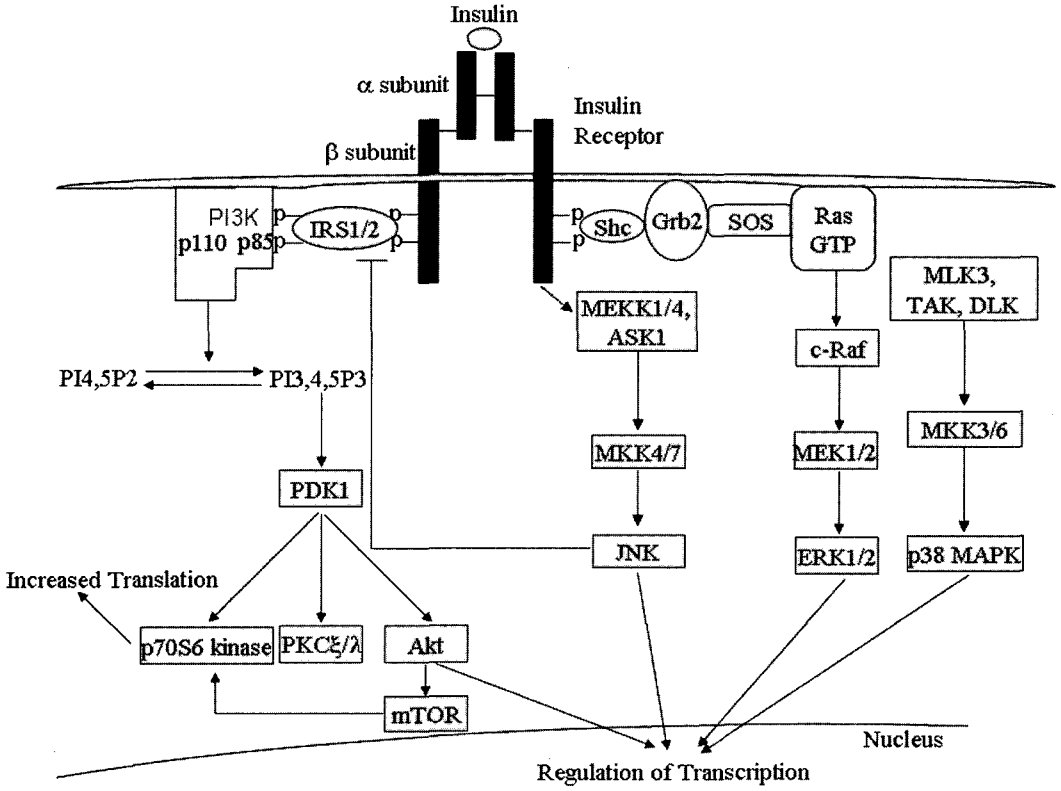

Fig. 1. Insulin-mediated signaling pathways.

Whereas the IRSs lack intrinsic catalytic activity, they have pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, and multiple phosphorylation motifs. The PH domains are globular protein domains of about 100~120 amino acids found in over 150 proteins to date. PH domains are primarily lipid-binding modules, although they are also involved in mediating protein-protein interactions. The PTB domain of IRS binds to phosphorylated NPXP motifs in the insulin receptor and is subsequently phosphorylated on multiple tyrosine residues by the activated insulin receptor kinase. Following phosphorylation, IRS attracts and binds additional effector molecules to the vicinity of the receptor, thereby serving to increase the diversity of the signaling pathways initiated by the insulin receptor. The primary effector that binds to IRSs in response to insulin receptor activation is the lipid kinase PI3K that produces PI3,4,5-triphosphate and subsequently activates Akt, PKC and p70 S6 kinase (Fig. 1).

The adaptor protein Shc exists in p46, p52 and p66 isoforms and possesses Src homology-2 (SH2) and PTB domains and three tyrosine-phosphorylation sites. In response to extracellular signals, Shc is phosphorylated on tyrosine residues and binds the growth factor receptor binding protein 2 (Grb2), which is constitutively associated with the guanine nucleotide exchange factor Son of Sevenless (SOS) (Kao *et al.*, 1997). Recruitment of the Grb2-SOS complex to the vicinity of Shc induces exchange of GDP to GTP on the membrane-bound GTPase Ras, thereby activating Ras. Activated Ras binds Raf and activates the serine/threonine Raf/MAPK kinase (MKK)/MAPK signaling pathway (Fig. 1) (Sasaoka and Kobayashi, 2000).

PI3K/Akt/p70 S6 kinase/atypical PKCs

There are four major classes of PI3Ks, designated Class I through IV; class I is also subdivided into Ia and Ib subsets. Class IV PI3Ks are not known to possess lipid kinase activity, but are serine/threonine kinases. The different classes of PI3Ks catalyze phosphorylation of the 3-OH position of phosphatidyl myo-inositol (PI) lipids, generating different 3-phosphorylated lipid products that act as second messengers. Class Ia PI3Ks are primarily responsible for production of 3-OH phosphoinositides in response to insulin. Class Ia enzymes are dimers composed of a 110-kDa catalytic subunit that is associated noncovalently to an 85- or 55-kDa regulatory subunit (Fig. 1). The catalytic subunit in subclass 1a is subdivided into p110 alpha, beta and delta. The regulatory subunit maintains the catalytic subunit in a low-activity state in quiescent cells and mediates its activation through interactions between SH2 domains of the regulatory subunit and adaptor proteins such as the IRSs (Cantley, 2002). The single class Ib PI3K is the p110 gamma catalytic subunit bound with a p101 regulatory protein and mainly activated by heterotrimeric G protein based-signaling pathways. Direct binding of p110 to activated Ras plays an important role in the stimulation of PI3K in response to growth factor (Shields *et al.*, 2000), but the physiological significance of this interaction in insulin-mediated PI3K signaling is not entirely clear.

Following the recruitment of PI3K to the plasma membrane, the lipid kinase phosphorylates the 3-OH position of the inositol ring to generate PI3,4,5-triphosphate, PI(3,4)P2 and PI(3)P. The preferred substrate of class I PI3Ks appears to be PI(4,5)P2. These events occur within the first minute of insulin binding to its receptor and resulting lipid products then interact with a number of signaling proteins with PH domains, resulting in their membrane targeting and/or modulation of their enzyme activity.

The rapid increase in PI3,4,5-triphosphate concentration in response to insulin activates several protein kinases, such as phosphotidylinositide-dependent kinase 1 (PDK1), Akt, PKC isoforms and p70 S6 kinase (Vanhaesebroeck and Alessi, 2000). Among the PI3,4,5-triphosphate-dependent kinases, Akt has received much attention. Akt/PKB was identified as a protein kinase with high homology to PKA and PKC, and is the cellular homologue of the viral oncoprotein v-Akt. Akt is a 57 kDa serine/threonine kinase with a PH domain and the three known isoforms of Akt (Akt1, 2, 3) are widely expressed (Chan *et al.*, 1999).

Akt exists in the cytosol of unstimulated cells in a low-activity conformation. The activation of Akt 1 by insulin is accompanied by its phosphorylation on threonine-308 in the kinase domain (T-loop) and serine-473 in the C-terminal regulatory domain (hydrophobic motif). Activation of Akt and phosphorylation of both these residues are abolished by pretreatment of cells with PI3K inhibitors such as wortmannin and LY294002 (Alessi *et al.*, 1996). Upon activation of PI3K, association of PI3,4,5-triphosphate at the membrane brings Akt and PDK1 into proximity through their PH domains and facilitates phosphorylation of Akt at threonine-338 by PDK1 (Vanhaesebroeck and Alessi, 2000). The mechanism mediating serine-473 phosphorylation remains to be clarified.

p70 S6 kinase catalyzes the phosphorylation of the S6 protein, a component of the 40S subunit of eukaryotic ribosomes, and thus plays a role in protein synthesis (Jefferies *et al.*, 1997). The p70 S6 kinase participates in the translational control of mRNA transcripts that contain a polypyrimidine tract at their transcriptional start

site. Although these transcripts represent only 100 to 200 genes, most of these transcripts encode components of the translational apparatus. The initial step in p70 S6 kinase activation appears to involve a phosphorylation-induced conformational change in the C-terminal domain, revealing additional phosphorylation sites. Subsequently, phosphorylation of the newly exposed sites (threonine 229, 389 and serine 371) occurs, which is dependent on both PI3K and the mammalian target of rapamycin (mTOR), based on wortmannin and rapamycin sensitivity, respectively.

Although expression of a constitutively membrane-anchored and active Akt variant induces the activation of p70 S6 kinase (Kohn *et al.*, 1996), Akt does not appear to represent the immediate upstream effector of p70 S6 kinase. Conus *et al.* (1998) suggested that p70 S6 kinase activation could be achieved independent of Akt. Dufner *et al.* (1999) demonstrated that a constitutively active wortmannin-resistant form of Akt was sufficient to induce glycogen synthase kinase-3 and eIF4E-binding protein 1 phosphorylation, but not phosphorylation and activation of p70 S6 kinase. The data suggest that p70 S6 kinase activation by membrane-targeted forms of Akt may be an artifact of membrane localization and that Akt resides on a parallel PI3K-dependent signaling pathway to that described for p70 S6 kinase.

Recent findings indicate that atypical PKC isoforms ζ (rat) and λ (mouse) serve as downstream effectors for PI3K. Increased activity of PKCζ/λ results from PDK1-dependent phosphorylation of the catalytic domain, via threonine 410 in rat PKCζ and threonine 411 in mouse PKCλ, followed by autophosphorylation of threonine 560 in rat PKCζ and threonine 563 in mouse PKCλ (Standaert *et al.*, 1999). PI3,4,5-triphosphate may interact with the N-terminal lipid-binding domain of PKC to facilitate the interaction of threonine-410 with the catalytic site of PDK1 (Standaert *et al.*, 1999). PI3,4,5-triphosphate also stimulates autophosphorylation of PKCζ and relieves the autoinhibition exerted by the N-terminal pseudosubstrate sequence on the C-terminal catalytic domain of PKCζ (Standaert *et al.*, 1999, 2001). Insulin-stimulated glucose transport and protein synthesis are dependent on PI3K/PKCζ activity (Mendez *et al.*, 1997). The latter is consistent with the observation that dominant negative PKCζ antagonizes activation of the p70 S6 kinase (Romanelli *et al.*, 1999). However, it is not known whether PKCζ can directly phosphorylate p70 S6 kinase or which residue(s) is/are involved.

Ras/Raf/MEK/ERK

Many RTKs including the insulin receptor and grow factor receptor, are known to activate intracellular protein serine/threonine kinases, termed MAPKs, which phosphorylate various cellular targets in a proline-directed manner, including transcription factors and other kinases. The MAPK family consists of subfamilies with multiple members (Fig. 2): these include ERK1/2, JNKs/SAPKs, p38 MAPKs and ERK5. Each MAPK is a member of a three-protein kinase cascade; a MAPK kinase kinase (MKKK) phosphorylates a MKK which subsequently phosphorylates the MAPK. Of the various MAPKs, the ERK1/2 subfamily was the first to be characterized. The basic arrangement of the ERK signal cascade includes the Ras, Raf (MKKK), MEK1/2 (MKK) and ERK1/2 (MAPK) (Fig. 2).

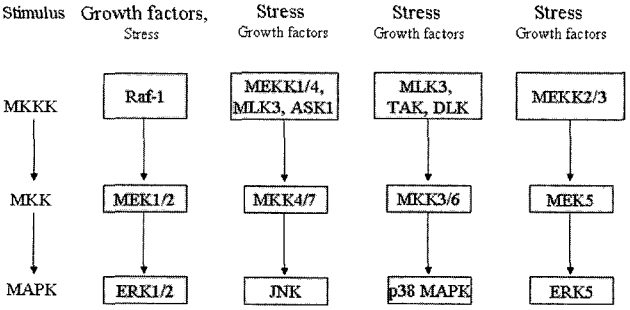

Fig. 2. MAPK signaling cascades.

Mammalian cells contain three different Ras genes that give rise to four Ras small GTPases, H-Ras, N-Ras, K_A-Ras and K_B-Ras, which are key regulators of signal transduction pathways controlling cell proliferation, differentiation, survival and apoptosis (Chong *et al.*, 2003). In response to a great variety of extracellular stimuli, including hormones, growth factors, cell-extracellular matrix interactions and oxidative stress, Ras proteins are activated through the GDP/GTP nucleotide exchange factor SOS, which induces the exchange of GDP for GTP, and thereby converts Ras to its active form. Ras cycles between the inactive GDP-bound and active GTP-bound states through the controlled activity of GTP nucleotide exchange factors and GTPase activating proteins. Upon activation of insulin receptor through agonist binding, the link between RTKs and Ras is provided by the GTP exchange factor SOS that exists in a complex with the adaptor protein Grb2 in the cytosol. Phosphorylated tyrosine residues in insulin receptor are docking sites for Grb2. In addition, the interaction between Grb2/SOS and the receptors can be mediated by the adaptor protein Shc, which becomes tyrosine phosphorylated when recruited to the cytosolic domains of the activated receptors. This process brings SOS to the plasma membrane in close proximity to Ras where it can promote GDP/GTP exchange. GTP-bound activated Ras recruits and activates three main classes

of effector proteins, Raf kinases, PI3K and RalGDS (Shields *et al.*, 2000).

Three genes encode for the Raf family of serine/threonine kinases found in mammalian cells, A-Raf, B-Raf and Raf-1 (c-Raf). The large majority of studies regarding the role of Raf in ERK activation have been performed with Raf-1. In resting cells, Raf-1 is located in the cytosol and is stabilized by a 14-3-3 scaffold protein dimer binding to phosphorylated serines 259 and 621, which are phosphorylated in resting cells (Tzivion *et al.*, 1998). The binding of Raf to Ras and translocation to the plasma membrane can displace 14-3-3 from phosphoserine 259, which makes it accessible to dephosphorylation and activation by protein phosphatase 2A (PP2A) (Kubicek *et al.*, 2002), although the role of dephosphorylation of serine 259 in Raf-1 activation was recently challenged (Light *et al.*, 2002).

The activation of Raf-1 is required for the subsequent multistep events to occur at the plasma membrane following the relief from autoinhibition. Agonists such as insulin and growth factors stimulate the phosphorylation of several residues, including serine 338, tyrosine 341, tyrosine 491 and serine 494 (Mason *et al.*, 1999). Phosphorylation at serine 338 and tyrosine 341 is a critical step for Raf activation (Catling *et al.*, 1995) and serine 338 phosphorylation appears to be a good qualitative indicator of Raf-1 activation.

MEK1 (MKK1) and MEK2 (MKK2) contain a proline-rich sequence necessary for the interaction of MEK with Raf-1 (Catling *et al.*, 1995). MEKs are phosphorylated by Raf-1 on two serine residues (serine 217, 221), which are necessary for full activation. MEK1 and MEK2 activate ERK1 (p44 MAPK) and ERK2 (p42 MAPK) via phosphorylation of a threonine-glutamate-tyrosine-motif in the activation loop. ERK is a proline-directed serine/threonine kinase at the end of this pathway with more than 50 identified substrates, including transcription factors, MAPK-activated protein kinase-2, and the p90 ribosomal S6 kinase (Lewis *et al.*, 1998). ERK activation has traditionally been associated primarily with cell proliferation.

The stress-activated protein kinases (SAPKs) such as JNK, p38 MAPK and ERK5 are slightly activated by insulin but vigorously activated by stress signals (UV irradiation, heat- or cold-shock, osmotic stress, mechanical shear stress, oxidative stress), cytokines and G-protein-coupled receptor agonists (Kyriakis and Avruch, 2001). The SAPKs are involved in the regulation of growth arrest, apoptosis and proliferation. The SAPKs are activated through a similar kinase cascade as ERK, although some different mechanisms have been noted. MKK4/SEK1 and MKK7 phosphorylate and activate JNK, whereas p38 MAPK is activated by MKK3 and MKK6. At the level of the MKKK, many kinases activating either or both JNK and p38 MAPK have been identified by overexpression or dominant-negative experiments (Hagemann and Blank, 2001).

METHODS FOR DETERMINATION OF INSULIN SIGNALING

Methods for examination of protein kinase activity and phosphorylation

Phosphorylation plays an essential role in the regulation of most protein kinases. Phosphorylation of specific residues is a major determinant of protein kinase activity. For example, the activation of Akt by insulin is accompanied by phosphorylation on threonine 308 in the kinase domain and serine 473 in the C-terminal regulatory domain. Activation of Akt and phosphorylation of both these residues are abolished by treatment with the PI3K inhibitors, wortmannin or LY294002, prior to stimulation with an agonist such as insulin.

Since first reported by Ross *et al.* (1981) antibodies reactive with phospho-residues (e.g. phosphotyrosine, phosphoserine and phosphothreonine) have become invaluable tools for isolating phosphorylated proteins and examining phosphorylation states. Phospho-specific antibodies for many protein kinases have been developed and these antibodies can be used for immunoblot analysis, immunoprecipitation, immunocytochemistry and flow cytometry. In general, immunoblot analysis, co-immunoprecipitation and kinase activity assays are the most frequently used methods for examination of protein kinase activation. Phospho-specific antibodies against a number of kinases and receptors are commercially available, and are used in standard immunoblotting procedures. If phospho-specific antibodies are not available, the kinase or receptor can be immunoprecipitated followed by immunoblotting with anti-phospho tyrosine/serine/threonine antibodies.

Protein kinase activation usually occurs within a few minutes of agonist binding to a receptor (e.g. insulin and growth factors), suggesting that the phosphorylation state is dynamically regulated. Thus, inhibition of phosphatase activity is very important during preparation of cell lysates. Treatment of cells with phosphatase inhibitors may result in activation of kinases. Generally, cell lysis buffer contains phosphatase inhibitors such as sodium orthovanadate, sodium fluoride, ethylenebis(oxyethylenenitrilo)tetraacetic acid and okadaic acid, to prevent dephosphorylation of protein kinases and other phosphoproteins. In immunoblot analysis, Laemmli sample buffer that contains SDS and dithiothreitol can be

used directly for making cell lysates.

For protein kinase assays, phospho-specific antibodies to protein kinases have been used for selectively immunoprecipitating activated protein kinases from cell lysates. This method depends on the availability of a specific immunoprecipitating antibody that does not interfere with the kinase activity, but many of these are commercially available. Protein kinase activity can be assayed by incorporation of phosphate from ATP into a synthetic peptide substrate based on the sequences of the phosphorylation sites on the target substrate protein. Many protein kinases phosphorylate the short peptide substrate with kinetic parameters similar to those of the native target proteins. In some cases, however, protein kinases that recognize or require an aspect of three-dimensional structure for their target, in addition to the primary sequence, will phosphorylate synthetic peptides poorly or not at all. Kinases that fall into this class must be assayed using the native protein target as substrate, or at least an expressed domain of the substrate that contains the requisite recognition features. Most protein kinase assays using synthetic peptides utilize radioactive ATP, resulting in a radiolabelled phospho-peptide that can be quantified by scintillation counting. Recently, non-radioactive kinase assays employing phospho-specific antibodies to the substrate protein have been developed and allow detection and quantification of kinase activity following immunoprecipitation of an active kinase.

We examined the insulin- and mechanical stress-activation of protein kinases involved in the PI3K and MAPK signaling pathways using phospho-specific antibodies in primary cultured rat hepatocytes (Fig. 3; Kim *et al.*, 2005). Insulin (10 nM) resulted in a substantial increase (>10-fold) in the phosphorylation of Akt (Ser473) relative to corresponding untreated cells at all time points examined (Fig. 3A). Phosphorylation of p70S6K (Thr421, Ser424) was increased ~1.7-fold within 10 min and the maximal increase of 7.5-fold was observed at 3 h (Fig. 3B). These results demonstrate that insulin results in a marked and prolonged activation of Akt and p70S6K, downstream kinases of PI3K, in primary cultured rat hepatocytes. Treatment of hepatocytes with 10 nM insulin also increased the phosphorylation of ERK (Thr202, Tyr204) ~3-fold within 5 min and this increase declined after 10 min with a return toward basal levels by 30 min (Fig. 3C). ERK phosphorylation was increased again 3 h following initiation of insulin treatment and remained elevated through 24 h. Insulin-mediated elevation of p38MAPK phosphorylation (Thr180, Tyr182) was observed 1 h after insulin addition and was elevated maximally at 3 h (Fig 3D). Phosphorylation of JNK

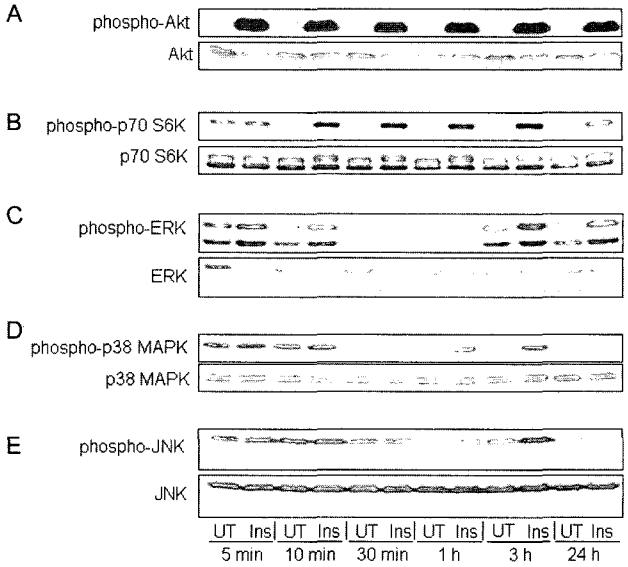

Fig. 3. Insulin-mediated phosphorylation of Akt (A), p70S6K (B), ERK (C), p38 MAPK (D) and JNK (E) in primary cultured rat hepatocytes. Hepatocytes were cultured in the presence (Ins) or absence (UT) of insulin for the indicated times (Reprinted from Kim, S.K., Woodcroft, K.J., Khadadadeh, S.S. and Novak, R.F., *J. Pharmacol. Exp. Ther.*, **311**, 99-108, 2004a, with permission from ASPET).

(Thr183, Tyr185) was altered in a time-dependent manner in response to 10 nM insulin (Fig. 3E). Addition of insulin caused a maximal ~3-fold increase in JNK phosphorylation at 3 h. Interestingly 24 h exposure to 10 nM insulin markedly inhibited the basal phosphorylation of JNK. Thus, all MAPKs monitored in this study were phosphorylated in response to insulin.

Chemical inhibitors of protein kinases

A widely used approach for examining the role of a kinase or kinase family in a cell signaling pathway is the pharmacological inhibition of the kinase (Fig. 4). In order to elucidate the signaling function of individual protein kinases and phosphatases, inhibitors should be potent, highly specific and cell-membrane permeable. Many inhibitors of protein kinases and phosphatases have been developed as therapy for diseases such as cancer, inflammation and diabetes. The vast majority of protein kinase inhibitors have been designed to target the ATP-binding site of protein kinases (Fabbro *et al.*, 2002). Expectations for inhibitor specificity were initially poor because the number of protein kinases encoded in the human genome is estimated to be in excess of 500, and the significant number of other cellular enzymes that use ATP further complicates the issue. Nevertheless, several ATP-binding site directed protein kinase inhibitors have been developed with a high degree of

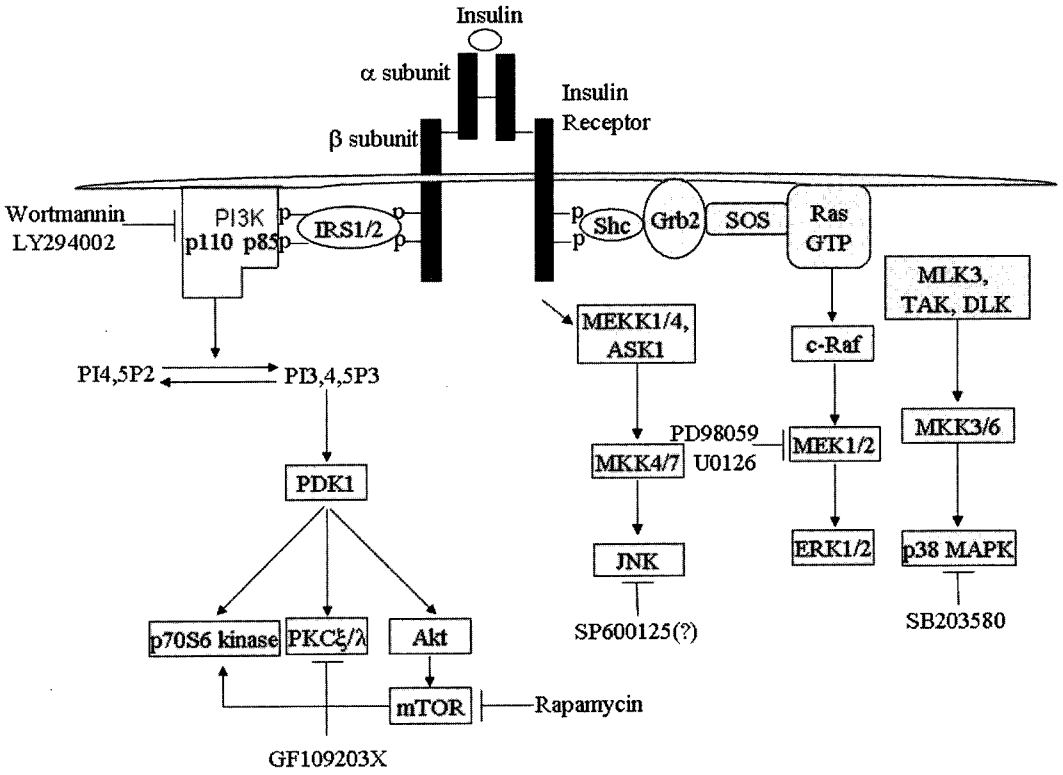

Fig. 4. Kinase inhibitors acting on insulin signaling pathways.

selectivity. Based on the numerous structures of complexes with ATP, it is clear that the ATP-binding cleft has regions that are not occupied by ATP and these regions show structural diversity among kinases (Toledo *et al.*, 1999). In general these inhibitors are added into cells prior to agonist addition. For longer periods of treatment (e.g. 24 to 48 h), the inhibitor may need to be replenished since the inhibitor half-life may be limited.

The inhibitors PD98059 and U0126 bind to the inactive form of MEK, preventing its activation by Raf-1 and other upstream activators (Alessi *et al.*, 1995). These inhibitors do not compete with ATP and do not inhibit the phosphorylation of MEK, and thus are likely to have a distinct binding site on MEK. Quantitative evaluation of the steady state kinetics of MEK inhibition by these compounds shows that U0126 had more affinity than PD98059 (Favata *et al.*, 1998). In a comparison of multiple kinase inhibitors, the MEK1 and MEK2 inhibitors appeared to be the most specific kinase inhibitors tested (Davies *et al.*, 2000). But both of these inhibitors have recently been shown to inhibit activation of the ERK5 pathway through direct effects on MEK5 (Karihaloo *et al.*, 2001). It is recommended that PD98059 or U0126 be added to cells at a concentration of 50~100 μM or 5~25 μM, respectively.

SB203580 and SB202190, a class of pyridinyl imidazoles, are relatively specific inhibitors of p38 MAPK alpha and beta, but not p38 MAPK gamma and delta, at a concentration of 10 M. However, these inhibitors were reported to inhibit the activation of PDK1 and its downstream effectors, including Akt and p70 S6 kinase (Wang *et al.*, 2001) although PDK1 activity remained unaffected by *in vitro* incubation with SB203580 or SB202190 (Davies *et al.*, 2000). We have found that in primary cultured rat hepatocytes, these p38 MAPK inhibitors failed to affect insulin-mediated Akt phosphorylation (Kim *et al.*, 2003a). These compounds bind the ATP-binding cleft of the low-activity p38 MAPK which weakly binds ATP (Frantz *et al.*, 1998). As a consequence of binding the unphosphorylated form, these inhibitors appear to interfere with the activation of p38 MAPK. Generally, SB203580 and SB202190 completely inhibit p38 MAPK at a concentration of 10 μM.

SP600125, an anthrapyrazolone inhibitor of JNK1, JNK2 and JNK3, has been reported to inhibit JNKs through a reversible ATP-competition. A number of studies have reported that the compound prevents the expression of several anti-inflammatory genes in cell-based assays and the activation of AP1 in synoviocytes (Bennett *et al.*, 2001). The inhibitor is starting to be used more widely as a JNK inhibitor. However, Bain *et al.* (2003) recently reported that SP600125 was a rel-

atively weak inhibitor of JNK isoforms and also inhibited other protein kinases with similar or greater potency. Care must be used therefore when employing this inhibitor and in the interpretation of resulting data. For inhibition of JNKs, SP600126 has been used at a concentration of 10~25 μM.

SU6656 (Blake *et al.*, 2000) and the related pyrazolopyrimidine, PP1 (Hanke *et al.*, 1996) were developed as inhibitors of the Src family of enzymes. PP1 was originally described as a selective, ATP-competitive inhibitor of Src family kinases and has been widely used to investigate the contribution of Src kinases to a number of biological functions (Hanke *et al.*, 1996). It is recommended that SU6656 be added to cells at a concentration of 1~5 μM.

Rapamycin, a potent immunosuppressant, rapidly inactivates p70 S6 kinase and prevents the activation of this kinase by all known agonists (Kuo *et al.*, 1992). Rapamycin binds to the immunophilin FK506 binding protein 12, and the resultant complex interacts with the protein kinase mTOR/FKBP 12-rapamycin-associated protein, thereby inhibiting it. This leads to the dephosphorylation and inactivation of the p70 S6 kinase. Generally, rapamycin completely inhibits p70 S6 kinase at a concentration of 100 nM.

GF109203X (bisindolylmaleimide I; G?6850) and Ro-31-8220 are bisindolylmaleimides which differ from each other in two functional groups and are analogues of staurosporine (Toullec *et al.*, 1991; Davis *et al.*, 1989). These inhibitors, which compete for the ATP binding site on PKC, have approximately 100-fold selectivity for PKC over PKA. They are both potent inhibitors of the alpha, beta and gamma isoforms of PKC with IC50 values in the nanomolar range *in vitro*. However, micromolar concentrations of GF109203X are required to inhibit atypical PKCs (Martiny-Baron *et al.*, 1993). These classes of compounds may also have the ability to selectivity inhibit PKC isoforms. G?6976, another staurosporine-related compound, inhibits alpha- and beta1-PKCs when utilized at nanomolar concentrations, but fails to inhibit delta-, epsilon- and zeta-PKC isoform (Martiny-Baron *et al.*, 1993). It is recommended that GF109203X be added to cells at a concentration of 1~10 μM.

Wortmannin and LY294002 are cell-permeable inhibi-

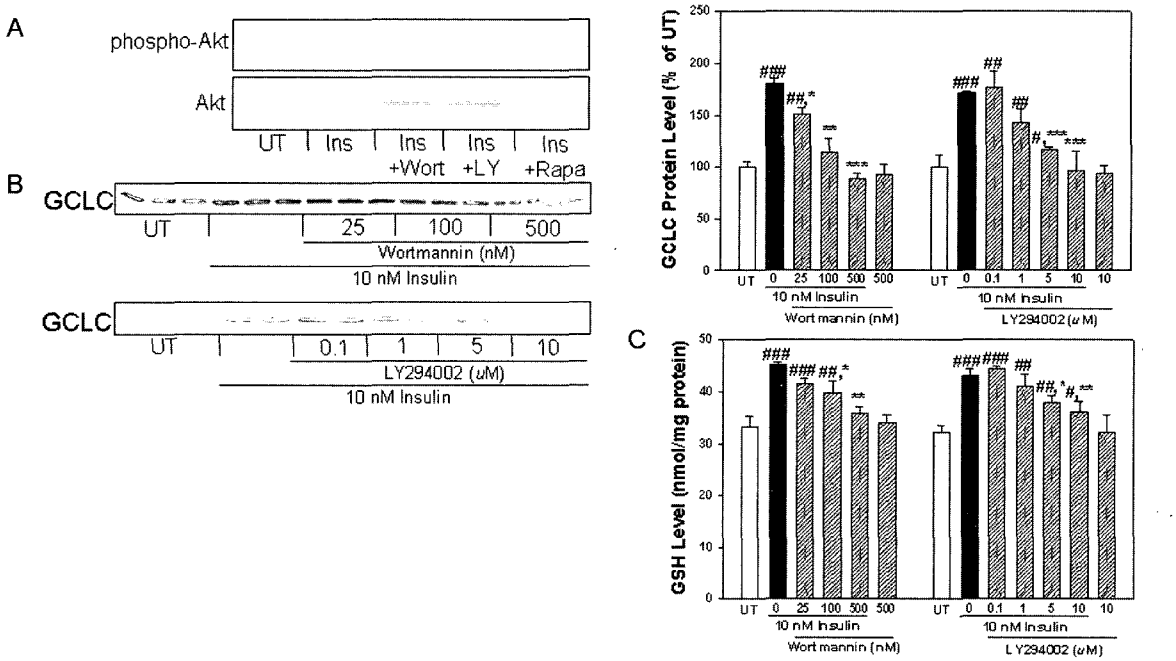

Fig. 5. Effect of wortmannin or LY294002 on the insulin-mediated increase in Akt phosphorylation (A), GCLC protein (B) and GSH (C) levels in primary cultured rat hepatocytes. A: hepatocytes were treated with wortmannin (500 nM), LY294002 (10 μM) or rapamycin (1 μM) for 1.5 h before addition of 10 nM insulin for 30 min. B and C: hepatocytes were treated with wortmannin or LY294002 for 1.5 h before addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Data are means ± SD of 3-6 preparations of cell lysates from a single hepatocyte preparation. $^{\#,\#\#,\#\#\#}$ Significantly different than levels monitored in untreated hepatocytes, $p<0.05$, $p<0.01$ or $p<0.001$, respectively. *,**,*** Significantly different than levels monitored in hepatocytes treated with insulin only, $p<0.05$, $p<0.01$ or $p<0.001$, respectively (Reprinted from Kim, S.K., Woodcroft, K.J., Khadadadeh, S.S. and Novak, R.F., *J. Pharmacol. Exp. Ther.*, **311**, 99-108, 2004a, with permission from ASPET).

tors of PI3K (Vlahos *et al.*, 1994; Stein, 2001). Wortmannin, an irreversible inhibitor, alkylates a lysine residue at the putative ATP binding site of p110 alpha of PI3K and LY294002 is a pure competitive inhibitor of ATP. It is recommended that wortmannin or LY294002 be added to cells at a concentration of 100~500 nM or 10~20 μM, respectively. At higher concentrations, wortmannin inhibits a number of other kinases, including the class 2 PI3K (Izzard *et al.*, 1999). If a longer incubation time is required, LY294002 is the inhibitor of choice rather than wortmannin, because of its higher stability in aqueous solution.

For longer treatment durations in highly metabolically competent cells, such as primary hepatocytes, the concentrations of protein kinase inhibitor recommended above may not be sufficient to inhibit each target protein kinase activity. Thus, higher concentrations of most of these inhibitors are often required to offset metabolism of the inhibitor, and care must therefore be exercised in the interpretation of these data.

In primary cultured rat hepatocytes, wortmannin and LY294002, but not rapamycin effectively inhibit both basal and insulin-mediated Akt phosphorylation (Kim *et al.*, 2003a; Fig. 5A). We have utilized these inhibitors to demonstrate that PI3K plays an obligatory role in the insulin-mediated induction of glutathione synthesis (Fig. 5B and C), microsomal epoxide hydrolase (Kim *et al.*, 2003a) and alpha-class glutathione S-transferase (Kim *et al.*, unpublished data), and the insulin-mediated suppression of CYP2E1 mRNA expression (Woodcroft *et al.*, 2002). Also we have utilized MAPK inhibitors and PI3K/p70 S6 Kinase/PKC inhibitors to determine acetoacetate-mediated signaling pathways involved in regulation of CYP2E1 expression (Abdelmegeed *et al.*, 2004, 2005).

Dominant negative protein kinase constructs

The activity of a protein kinase can be interfered with by expression of a dominant negative mutant. The generation of dominant negative mutants involves the design of an inactive form of the protein that can sequester interacting proteins. Some knowledge of the mechanism of regulation or function of the protein of interest is helpful when designing these molecules. In general, the activity of protein kinases requires the phosphorylation of specific residues for activation and the binding of ATP to a conserved protein motif for phosphorylation of effector proteins. Thus, the point mutation of the phosphorylation site or ATP-binding region can produce an inactivated kinase or kinase-dead mutant, respectively. Overexpression of an inactive form of the kinase may act as a dominant negative by sequestering interacting proteins or cofactors and thus inhibiting the activity of the endogenous wild-type kinase. Many protein kinases are inactive in resting cells and this basal inhibition is achieved by interaction with a regulatory protein or an inhibitory domain within the same polypeptide. Thus, in some cases, overexpression of a regulatory protein or an inhibitory domain can reduce or inhibit the ability of the pathway to stimulate the endogenous protein. Similarly, overexpression of a pseudosubstrate domain that can bind the enzyme but cannot be converted to product can often result in inhibition of signaling, as it will compete against the endogenous substrate (House and Kemp, 1987).

DNA constructs encoding inactive kinase mutants must be transported through the cell membrane and into the nucleus, in order to inhibit signaling pathways through their expression. There are several well-established techniques that allow transient transfection of recombinant DNA into cells in culture. These methods generally involve the permeabilization of cell membranes by chemical or electrical means, or the use of viral constructs that can recognize specific receptors on the cell surface, resulting in cellular uptake. A variety of viral systems, including adenoviruses and retroviruses, have become available for transporting recombinant DNA into cells (Yeh and Perricaudet, 1997). The DNA can either be incorporated into the viral genome or be chemically linked to the exterior of the virion. After transfection of adenovirus into a mammalian cell, viral production may be monitored with green fluorescent protein (GFP), which is encoded by a gene incorporated into the viral backbone (He *et al.*, 1998).

A dominant negative kinase-dead mutant of Akt1 that has a point mutation (K179M) resulting in loss of kinase activity was expressed by adenoviral infection of primary cultured hepatocytes. Immunoblot analysis of hepatocytes infected with adenovirus containing a dominant negative kinase-dead mutant of Akt (AdV-Akt) indicated expression of a higher molecular weight Akt protein in addition to the endogenous Akt owing to the additional Myc/His tags on the adenovirally introduced Akt (Fig. 6A). To confirm that the overexpressed dominant negative Akt construct was functional, Akt activity was examined in hepatocytes treated with 10 nM insulin for 30 min (Fig. 6B). Akt kinase activity was increased ~10-fold in response to 10 nM insulin and this increase was inhibited ~65% by AdV-Akt. Overexpression of dominant negative Akt in hepatocytes resulted in a decline in the insulin-mediated increase in GCLC protein and GSH levels, suggesting that Akt is involved in the insulin-mediated increases in GSH synthesis (Fig. 6C and D).

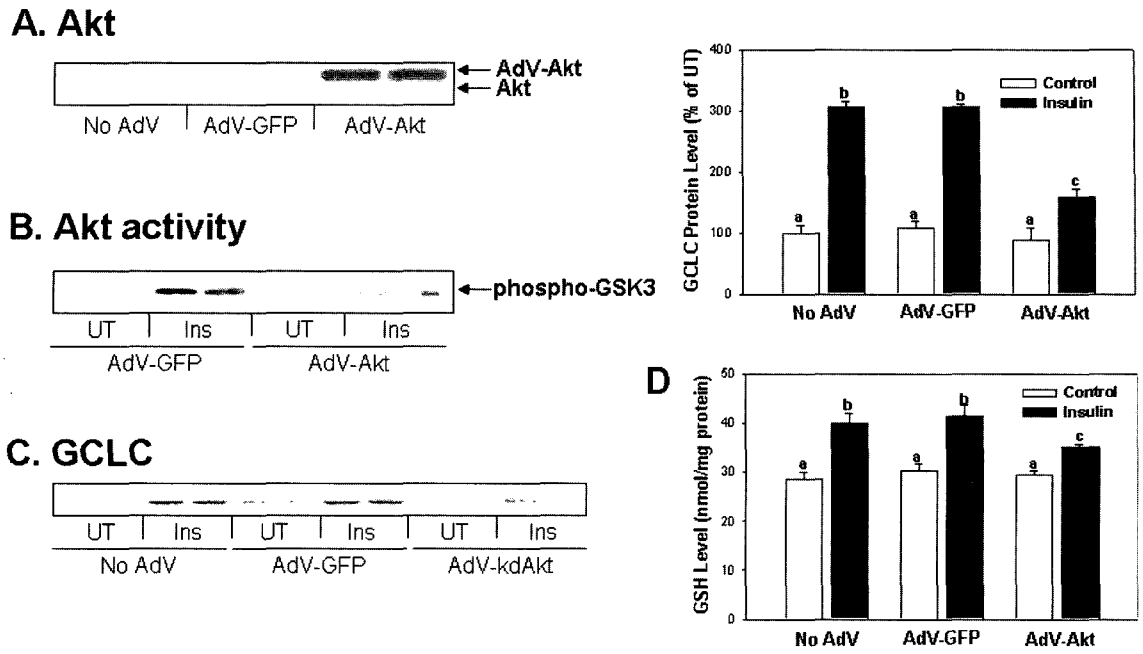

Fig. 6. Effect of dominant negative Akt expression on insulin-mediated GCLC protein and GSH levels in primary cultured rat hepatocytes. A: hepatocytes were infected with 150 MOI AdV-Akt or 15 MOI AdV-GFP and 24 h later, cells were harvested for determination of Akt protein level. B: Following 24 h infection with AdV-Akt or AdV-GFP, hepatocytes were treated with 10 nM insulin for 30 min and assayed for Akt activity. C and D: 24 h after infection with AdV-Akt or AdV-GFP, hepatocytes were treated with 10 nM insulin for 2 days. Data are means ± SD of 3-4 preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, $p<0.05$ (Reprinted from Kim, S.K., Woodcroft, K.J., Khadadadeh, S.S. and Novak, R.F., *J. Pharmacol. Exp. Ther.*, **311**, 99-108, 2004a, with permission from ASPET).

siRNA

In 1998, Fire *et al.* (1998) described a new technology that was based on the silencing of specific genes by double-stranded RNA (dsRNA) and termed RNA interference (RNAi). RNAi consists of the presentation of a triggering dsRNA that is subsequently processed into 21 to 25 base-pair small interfering RNAs (siRNAs) through the action of the Dicer enzyme (RNase III endonuclease) (Zamore *et al.*, 2000). siRNAs with 2-nucleotide 3-end overhangs are then incorporated into a multi-subunit RNA-induced silencing complex, which targets their complementary RNA transcript for enzymatic degradation (Elbashir *et al.*, 2001b). The siRNA-induced degradation of mRNA is highly sequence specific, to the extent that even a 1-2 nucleotide difference in the targeting recognition sequence hampers RNAi function.

In contrast to siRNAs, small temporal RNA (stRNA) molecules, which represent a large group of small transcripts called micro RNAs, mediate gene suppression by inhibiting translation of target mRNA (McManus and Sharp, 2002). In common with siRNAs, Dicer is also involved in the processing of the 21-23-nucleotide stRNAs from ~70-nucleotide stable hairpin precursors (Grishok *et al.*, 2001). But stRNAs are stem-loops that are processed into an imperfect complementary dsRNA that inhibit protein translation of an imperfectly matched target sequence which is almost invariably located at the 3 untranslated region of the target mRNA (Dykxhoorn *et al.*, 2003).

In mammalian somatic cells, dsRNAs longer than 30 nucleotides activate an antiviral defense mechanism that includes the production of interferon and activation of dsRNA-dependent protein kinase, resulting in inhibition of protein synthesis initiation and stimulation of apoptosis (Williams, 1997). One mechanism for dealing with these nonspecific dsRNA responses is to create dsRNA triggers of <30 base-pairs in length. Both siRNA and stRNA are long enough to induce sequence-specific suppression, but short enough to evade the host defense response. Although the use of siRNAs to silence genes in vertebrate cells was only reported a few years ago, the emerging literature indicates that most vertebrate genes can be studied with this technology.

Several laboratories demonstrated that synthesized dsRNAs induced sequence-specific gene silencing when transiently transfected into mammalian cells (Elbashir *et al.*, 2001a). Factors which could ultimately limit the usefulness of siRNAs include a relatively short and tran-

sient period of activity. The longevity of silencing is dependent on abundance of mRNA and protein, stability of protein, the half-life of the silencing complex and cell division rate. Generally the siRNA directs rapid reduction in mRNA levels that is readily observed in 18 hours or less and siRNA-mediated RNAi lasts for 3-5 days for most cell lines (McManus *et al.*, 2002a).

Recently a number of studies have reported the success of using RNA polymerase III promoters, such as U6 or H1, to direct *in vivo* synthesis of functional siRNAs (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). These siRNAs have been expressed in two ways. In the first case, hairpin constructs are expressed from a single RNA polymerase III promoter. The resulting RNAs are predicted to form hairpins containing 19- to 29-nucleotide stems that match target sequences precisely, three- or nine-nucleotide loops and 3 overhangs of four or fewer uridines. It is believed that these hairpin RNAs are processed by Dicer to active siRNAs *in vivo* (Paddison *et al.*, 2002). In the second case, coding and non-coding strands of a potential siRNA are driven from separate promoters and the expressed transcripts anneal in the cell nucleus. The hairpin siRNA strategy appears to inhibit gene expression more efficiently than the duplex siRNAs expressed from two separate plasmids (Paddison *et al.*, 2002). The use of a plasmid-based RNA polymerase III promoter system to intracellularly produce siRNAs could allow for a longer period of expression as compared with exogenously added siRNAs.

An alternative approach to prolong siRNA-mediated inhibition of gene expression is the introduction of modified nucleotides into chemically synthesized RNA. Amarzguioui *et al.* (2003) reported that siRNA generally tolerated mutations in the 5' end, while the 3' end exhibited low tolerance. An siRNA with two 2-O-methyl RNA nucleotides at the 5' end and four methylated monomers at the 3' end was as active as its unmodified counterpart and led to a prolonged silencing effect in cell culture (Amarzguioui *et al.*, 2003).

The effectiveness of a siRNA is likely to be determined by the accessibility of its target sequence in the intended substrate. It has been suggested that the first 50-100 nucleotides of a mRNA sequence, downstream of the translation initiation sequence, should be used to target a gene and that 5 or 3 untranslated regions, as well highly conserved domains (i.e. catalytic, ligand binding, etc.), should be avoided, as they are likely to contain regulatory protein binding sites (Sui *et al.*, 2002). However, successful gene inhibition has been reported for siRNAs targeting various sequences, including the 3 untranslated regions (McManus *et al.*, 2002b). There are no reliable ways to predict or identify the ideal sequence for a siRNA. However, targeting different regions of a given mRNA might give different results (McManus *et al.*, 2002a). Generally, siRNAs become susceptible to RNase H; therefore, the degree of the RNase H sensitivity of a given probe reflects the RNase H accessibility of the chosen sites. In practical terms, it might be just as easy to construct and test several siRNAs.

CONCLUSION

Our laboratory has used phospho-specific antibodies and chemical inhibitors of protein kinases to define the signaling pathways involved in insulin- and glucagon-mediated regulation of several drug metabolizing enzymes. But small molecular chemical inhibitors of protein kinases used for this purpose in many studies have been reported to have specificity problems, although many chemicals have been considered to be reasonably selective inhibitors for each target protein kinase. As with all pharmacological tools, interpretation of experiments with these protein kinase inhibitors requires caution. It is advisable to conduct experiments with at least two pharmacologically distinct inhibitors wherever possible. Dominant negative kinase constructs allow for more kinase-specific inhibition. Recently, RNAi methods have opened new opportunities for investigators to study cell signaling pathways by leading to a highly specific mRNA degradation. Furthermore, retrovirus or adenovirus vectors have been developed for use in carrying dominant negative kinase constructs or siRNA-expressing DNA templates into cells in order to mediate gene-specific silencing in cells or animals. Thus, RNAi using siRNAs to silence specific genes is a very promising method for determination of cell signaling pathways involved in protein expression in response to hormones and growth factors.

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