

REVIEW

Role of Sp1 in insulin regulation of gene expression

S L-A Samson and N C W Wong

Libin Gene Therapy Unit and Diabetes and Endocrine Research Group, Departments of Medicine and Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

(Requests for offprints should be addressed to Norman C W Wong, Departments of Medicine and Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, Health Sciences Center, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1; Email: ncwong@ucalgary.ca)

Abstract

Sp1 is a ubiquitous nuclear factor that plays a key role in maintaining basal transcription of 'house-keeping' genes. However, recent evidence points to a more important function for Sp1 in mediating 'cross-talk' between selected signaling cascades to regulate the target genes that respond to these pathways. The role of Sp1 in mediating the actions of the peptide hormone insulin is of specific interest and serves as a model for detailing effects of intracellular signaling on Sp1 activity. This review summarizes studies suggesting that changes in Sp1 phosphorylation provide one potential mechanism for manipulating activity of this protein. A growing body of evidence reveals that the DNA binding and transcription activity of Sp1 may increase or decrease in response to changes in phosphorylation. This enables 'fine-tuning' of Sp1 activity for regulation of gene transcription. Several mechanisms exist by which Sp1 alters gene activity in response to insulin. These include independent Sp1 activity as well as collaboration or competition with other factors. This review points to an ever-increasing role for Sp1 in regulating the transcription of genes in response to extracellular signals such as insulin.

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Introduction

The peptide hormone, insulin, has a multitude of effects on cellular function. The response to insulin begins with hormone binding to a membrane receptor. This step triggers a cascade of intracellular events leading to a modification of protein activity, changes in gene regulation and cellular function. Many of the physiological effects of insulin arise from its ability to regulate the transcription of specific genes. The insulin responsive gene products are not limited to enzymes that mediate selected metabolic pathways, but also include transcription factors, hormones, oncogenes, and proteins of importance to the cell membrane and architecture. These widespread actions of insulin underlie its effects on cell function and growth (O'Brien & Granner 1996). The scope of this review deals with the role of transcription

factors, and specifically Sp1, that mediate the gene regulatory actions of insulin.

There exist many pathways by which insulin increases or represses gene activity. In some cases, well-characterized transcription factors have been shown to mediate insulin action in addition to their other known functions (reviewed by O'Brien & Granner 1996). For example, the insulin responsive sequences within the collagenase and malic enzyme (ME) promoters both contain a consensus AP1 binding sequence (TGA CTCA) (Streeper *et al.* 1998), an element which normally mediates a response to phorbol esters. However, in the context of surrounding gene-specific sequences, both AP1 motifs are able to confer an insulin response on a heterologous promoter, but only the collagenase site responds to phorbol esters (Streeper *et al.* 1998). Although the *c-fos* gene also contains an AP1 motif, it is a nearby serum response element (SRE) that is

essential for insulin induction of c-fos expression (Thompson *et al.* 1994, Jacob *et al.* 1995). With regard to insulin repression, the phosphoenolpyruvate carboxykinase (PEPCK) remains the model for negative regulation, and the developmentally important forkhead transcription factor, FKHR, has been implicated in this regard (reviewed by O'Brien *et al.* 2001).

In contrast to the multiple roles of the above mentioned transcription factors and their binding sites, the distal insulin responsive element (IRE-A) of the glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene appears to be regulated solely by insulin. The IRE-A binds multiple nuclear proteins in DNA binding studies (Nasrin *et al.* 1990, Alexander-Bridges *et al.* 1992a). The cDNA for one such protein, IRE-A binding protein (IRE-ABP), has been cloned using DNA affinity screening of an expression library with the IRE-A sequence (Nasrin *et al.* 1991). IRE-ABP is unique from known transcription factors and contains an HMG-box DNA binding domain similar to that in the testis determining factor, SRY (Nasrin *et al.* 1991, Alexander-Bridges *et al.* 1992b). However, although these reports implicate IRE-ABP, its true contribution to insulin-regulated gene transcription remains to be defined (Alexander-Bridges *et al.* 1992b). At this juncture, evidence points towards IRE-ABP involvement in sex-specific gene expression through opposing the binding of transcription factor CCAAT/enhancing binding protein α (C/EBP α) to overlapping recognition sites (Buggs *et al.* 1998).

In addition to the insulin responses reviewed above, a pattern is beginning to emerge from the study of an expanding group of insulin responsive genes that include fatty acid synthetase, leptin, ATP citrate-lyase, and apolipoprotein (Apo) A1. A common feature is the presence of GC-rich motifs within their insulin responsive regions. These motifs are consensus binding sites for the ubiquitous transcription factor Sp1 and related family members. The growing list of insulin-responsive genes that contain Sp1 binding sites prompted us to address this topic. Here, we review the current understanding of the role of Sp1 in basal and activated transcription. Our goal is to try and unravel the potential role of Sp1 as a mediator of signal transduction cascades required for insulin gene regulation.

Role of Sp1 in basal transcription

Sp1 was one of the first eukaryotic transactivators to be isolated. It was purified by fractionation of HeLa cell extracts and shown to activate *in vitro* transcription in a discriminate manner from the SV40 viral promoter (Dyan & Tjian 1983). These findings prompted the authors to speculate that Sp1 could be involved in the recognition and regulation of other cellular and viral genes (Dyan & Tjian 1983). This is truly an understatement because Sp1 is now recognized as the matriarch of a family of transcription factors that includes Sp2, Sp3, and Sp4 (Kadonaga *et al.* 1987, reviewed by Suske 1999). The ubiquitous Sp1 and Sp3 factors, and the neural cell-specific Sp4, bind to the 'GC box' sequence (GGGGCGGGG), a motif that is common to many viral and eukaryotic promoters. In contrast, Sp2 binds to the GT/CACCC sequence.

The Sp isoforms belong to an extended family of transcription regulators, the mammalian Sp/XKLF or 'Kruppel-like' factors. The Sp/XKLF proteins share homology with others found in *Drosophila melanogaster*, *Caenorhabditis elegans*, and yeast (reviewed by Phillipson & Suske 1999, Turner & Crossley 1999). Proteins belonging to this extended family bind similar GC-rich motifs because they have homologous DNA binding domains that contain three contiguous carboxyl-terminal Cys₂His₂ zinc fingers (Fig. 1). However, Sp1 has unique features that include the combination of transcriptional activation domains comprised of two glutamine-rich domains A and B, a weakly basic domain, and a carboxyl-terminal D domain (Fig. 1; Kadonaga *et al.* 1987, 1988, Courey & Tjian 1988, Pascal & Tjian 1991, Phillipson & Suske 1999). Sp1 may form oligomers through interaction of domains A and B resulting in a tetrameric configuration. These complexes may facilitate DNA looping to juxtapose distant binding sites to the proximal promoter (Mastrangelo *et al.* 1991, Matsushita *et al.* 1998). Domain D interactions of Sp1 enable even higher order complexes, such as the stacking of Sp1 tetramers (Matsushita *et al.* 1998). These interactions appear to facilitate the synergistic transcriptional activation that is observed among Sp1 sites in a single promoter or enhancer.

Since the initial discovery of Sp1, it has generally been defined as a 'basal' transcription factor. It is

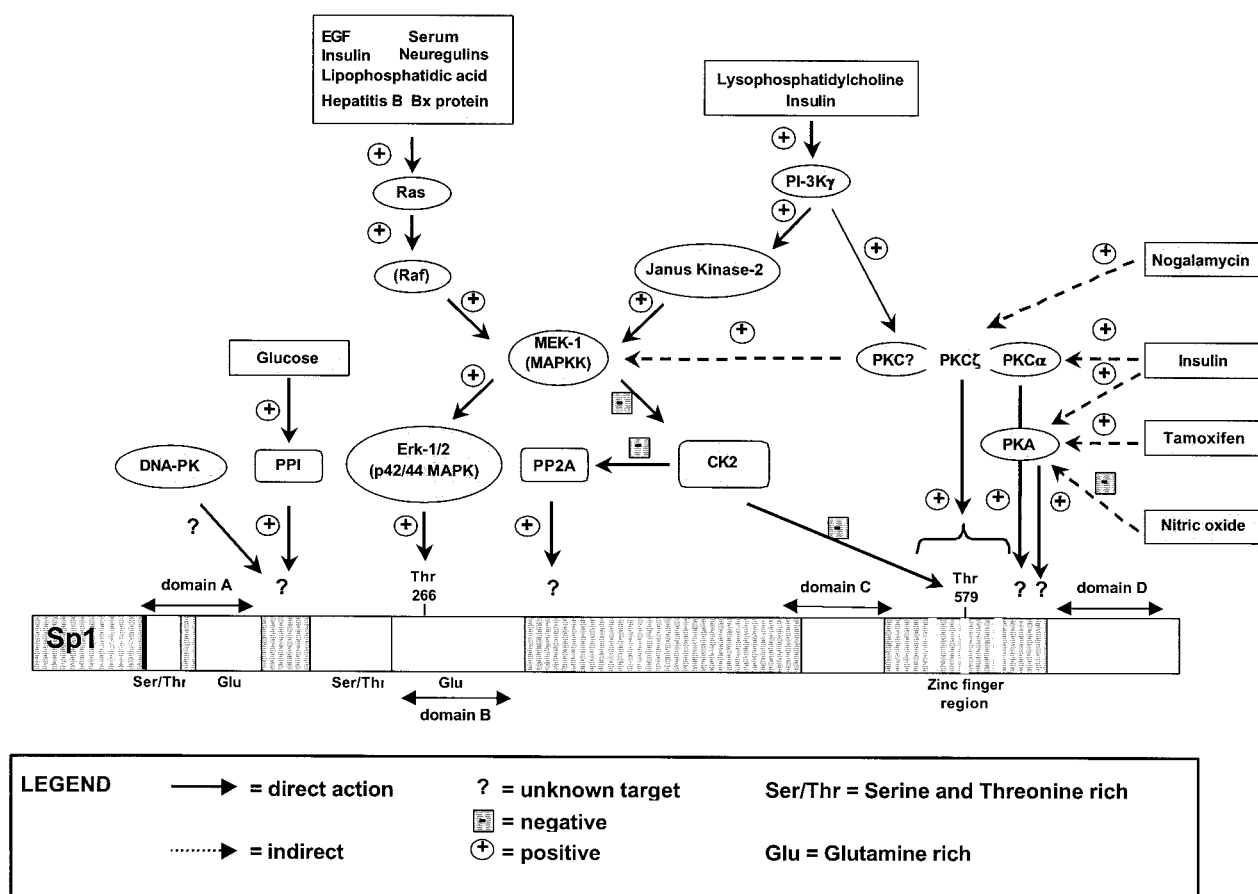


Figure 1 Sp1 is a target of signal transduction pathways. A schematic diagram of Sp1 transcription factor is presented with labeled functional domains. The signal transduction pathways known to directly or indirectly modify Sp1 activity are shown. See references in text and Table 1. MEK-1, MAP/ERK kinase 1; MAPK, mitogen activated kinase; MAPKK, mitogen activated kinase kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PKA, protein kinase A; PKC, protein kinase C; PI-3K, phosphatidylinositol-3 kinase; CK2, casein kinase 2; DNA-PK, DNA protein kinase.

believed to have a key role in maintaining expression of ‘house-keeping’ genes that lack a TATA-box. The TATA-box binds a protein complex called TFIID. This complex contains the TATA-box binding protein, TBP, and associated factors called TAFs. In the absence of a TATA-box, Sp1 facilitates binding of TFIID to the promoter, which, in turn, recruits RNA polymerase II (Pol II) transcription machinery (Blake *et al.* 1990, Smale *et al.* 1990, Javahery *et al.* 1994, Kaufmann & Smale 1994). Sp1 physically interacts with TFIID through TAFs, specifically human TAF_{II}130, TAF_{II}55 and *Drosophila* TAF_{II}110 (Hoey *et al.* 1993, Gill *et al.* 1994, Chiang & Roeder 1995, Tanese *et al.* 1996). An additional 9 sub-unit co-factor, CRSP, separate from TAFs, also appears

to be essential for Sp1 activation of Pol II transcription (Ryu *et al.* 1999).

Sp1 may also play a role in maintenance of housekeeping genes by preventing gene silencing by DNA methylation. Multiple GC boxes have been found in the CpG-rich regulatory regions of a number of constitutively expressed genes. These motifs are likely the binding sites of a nuclear factor *in vivo*, presumably Sp1 (Gardiner-Garden & Frommer 1987, Pfeifer *et al.* 1990, Macleod *et al.* 1994). The removal of the Sp1 motifs exposes these regulatory regions to DNA methylation (Macleod *et al.* 1994). Further support that Sp1 protects the DNA from being methylated comes from the insertion of ectopic Sp1 sites upstream of methylated β-globin regulatory sequences

(Brandeis *et al.* 1994). This manipulation results in demethylation of the surrounding sequences (Brandeis *et al.* 1994). However, Sp1 gene knockouts in mice have shown that the methylation-free status of the APRT, DHFR, and ApoA1 genes is maintained without Sp1, although overlapping contributions of other Sp family members may provide an explanation for this observation (Marin *et al.* 1997).

In addition to the participation of Sp1 in basal transcription, there is evidence now emerging that shows the interplay among Sp proteins in modulating cell-type-specific gene expression. Both Sp1 and its relative, Sp3, are ubiquitous proteins that bind the same motif. Therefore, it is reasonable to expect they also compete for binding to promoters containing GC-boxes. In fact, these two factors have been shown to display differential activity, including parallel or opposing transcription effects depending on the promoter (Conn *et al.* 1996, Kennett *et al.* 1997, Hata *et al.* 1998, Hoppe & Francone 1998, Nielsen *et al.* 1998, Rajakumar *et al.* 1998, Yajima *et al.* 1998, Merchant *et al.* 1999, Vines & Weigert 2000). Cell-type-specific levels of Sp1 and Sp3 variants have the potential to be an important mode of regulating the contribution of Sp1 to promoter activity. Also, numerous Sp1/Sp3 experiments have been performed in the Sp-null background of *Drosophila* cells, so that cell-type-specific differences in the abundance of Sp-family proteins cannot be responsible for their differential activity. It is more likely that inherent features of the promoter will prove to be important (Rajakumar *et al.* 1998).

Similar to the interplay between Sp1 and Sp3, the Sp proteins functionally interact with the early growth response factor, Egr-1, leading to either opposing or parallel actions. Egr-1 is an immediate-early gene product that also binds to a GC-rich consensus GCGC(G/T)GGCG (Cao *et al.* 1993). Phorbol esters or serum stimulation of cells increases the synthesis and DNA binding activity of Egr-1. Increased abundance of Egr-1 displaces Sp1 bound to an overlapping sequence (Cao *et al.* 1993). Similar opposing Sp1/Egr-1 interactions are also found in the human tumor necrosis factor alpha (TNF- α), human interleukin 2, platelet derived growth factor (PDGF) genes and several other promoters (Kramer *et al.* 1994, Khachigian *et al.* 1995, Skerka *et al.* 1995). With other promoters, Egr-1 and Sp1 may cooperate rather than oppose

each other's action. For example, such interactions are found for regulation of the human interleukin-2 receptor β -chain promoter (Lin & Leonard 1997) and the 5-lipoxygenase promoter (Silverman *et al.* 1997). Others (Huang *et al.* 1997) have defined the promoter architecture that enables Sp1 and Egr-1 to work in a cooperative or opposing fashion. These investigators noted that if the Sp1 binding site overlaps an Egr-1 site, then increasing abundance of Egr-1 displaces Sp1 from the DNA leading to a repression of Sp1-activated transcription. In contrast, juxtaposition of the sites without overlap yields a promoter where Egr-1 cooperates with Sp1.

There are studies, too numerous to discuss here, that report the functional and physical co-operation of Sp1 with other transcription factors. Examples include cell-type-specific regulators, such as octamer transcription factor Oct-1 (Janson & Pettersson 1990), oncogenes, such as c-Myc (Kyo *et al.* 2000), and nuclear receptors for estrogens or androgens (Krishnan *et al.* 1994, Lu *et al.* 2000, Salvatori *et al.* 2000). The interpretation of such studies could be that a basal level of Sp1 activity co-operates with induced factors to contribute to activation of gene expression. However, current evidence suggests that the activity of Sp1 is neither basal nor static. Sp1 does not wait in anticipation to aid the actions of newly induced activators. Instead, Sp1 is believed to mediate gene-specific responses to a variety of signals in the cellular environment independent of co-operative interactions with 'inducible' transcription factors. The key question to be answered is how the actions of this ubiquitous transcription factor are regulated, thus allowing it to mediate specific cell-type and temporal patterns of gene expression. Part of the answer lies in the unfolding story of Sp1 post-translational modifications which give it the ability to respond to signal transduction cascades independent of other transcription factors.

Sp1 as a target of signal transduction cascades

A topic of rising interest is the potential participation of Sp1 as an effector of signal transduction cascades. Soon after Sp1 was isolated and purified, it was shown to be modified by phosphorylation of serine and threonine residues

(Jackson *et al.* 1990). Despite the fact that these reports were published more than a decade ago, investigation of the role of post-translational modifications in regulating Sp1 activity has not been exploited until recently. In addition to phosphorylation, Sp1 also has multiple potential *O*-glycosylation sites that may be modified by *N*-acetylglucosamine residues (Jackson & Tjian 1988). This modification appears to protect Sp1 from proteasome degradation (Han & Kudlow 1997).

Recent studies show that Sp1 phosphorylation is not a constitutive modification and is altered in response to extracellular stimuli through a variety of signal transduction pathways (Fig. 1). The primary sequence of Sp1 contains consensus phosphorylation sites for numerous kinases including calmodulin kinases (CamKs), casein kinases (CK) 1 and 2, protein kinases (PK) A and C, and mitogen activated protein kinases Erk 1 and 2 (MAPK) (Kreegipuu *et al.* 1999, Merchant *et al.* 1999, Zheng *et al.* 2001). At this juncture, the volume of literature on Sp1 regulation by phosphorylation continues to grow. Unfortunately, so does the complexity surrounding the possible signaling pathways involved, and the effects of phosphorylation on Sp1 binding and transcription activity. It is not difficult to imagine that different kinases and phosphatases could target selected motifs and thus alter Sp1 phosphorylation in a cell-type-specific manner. This could lead to differential effects that may be positive or negative on transcriptional activity, DNA binding, as well as interactions with other activators and/or the transcription machinery. Table 1 summarizes much of the current literature on Sp1 as a target of cellular kinases and phosphatases, and effects on DNA binding. This table also notes effects on transcription activity, but the evidence is indirect and gathered using inhibitors or co-expressed kinases and phosphatases, rather than *in vitro* transcription systems. Figure 1 provides a schematic summary of the pathways that have been shown to converge on Sp1.

Evidence supporting the role of PKC in Sp1 regulation comes from the positive effect of phorbol esters, and presumed PKC activation, on Sp1. Although studies suggest that the effect of phorbol esters may be at the level of increased synthesis (D'Angelo *et al.* 1996, Ries *et al.* 1998, Langmann *et al.* 1999, Tanaka *et al.* 2000, Noe *et al.* 2001), our

laboratory showed that Sp1 binding and transcription activity in human HepG2 cells is increased by phorbol esters without requiring Sp1 synthesis (Zheng *et al.* 2000, 2001). Similarly, Kang-Park *et al.* (2001) have shown Sp1 phosphorylation and activation by PKC α co-transfection in HepG2 cells. However, only atypical PKC ζ has been shown to physically interact with and phosphorylate Sp1 *in vitro*, with no apparent modification by other PKC isoforms (Pal *et al.* 1998, 2001, Garcia *et al.* 2000, Rafty & Khachigian 2001).

Sp1 may also be directly modified *in vitro* by DNA-PK (Jackson *et al.* 1990), PKA (Rohlf *et al.* 1997), and Erk 2 (Merchant *et al.* 1999) with a resultant increase in DNA binding activity demonstrated for the latter two kinases. PKA also appears to have a positive effect on Sp1 activity in transfection (Ahlgren *et al.* 1999, Wang *et al.* 1999a, Zheng *et al.* 2000). Casein kinase 2 (CK2) is also known to phosphorylate Sp1 *in vitro*, but this modification has a negative effect on DNA binding due to modification of threonine-579, a residue located in the second zinc finger of Sp1 (Armstrong *et al.* 1997, Zhang & Kim 1997, Wang *et al.* 1999b).

There is also evidence that CK2 has an indirect role in regulating Sp1 phosphorylation, through known interactions of CK2 with protein phosphatase 2A (PP2A) (Cieslik *et al.* 1998, 1999). PP2A forms stable complexes with a long list of protein kinases *in vitro* (Millward *et al.* 1999) including the catalytic alpha subunit of CK2 (reviewed by Allende & Allende 1998). Cieslik *et al.* (1998, 1999, 2000) have teased out the pathway by which lysophosphatidylcholine (LPC) induces endothelial nitric-oxide synthase (eNOS) gene transcription. The positive effect of LPC is dependent on Sp1, and is blocked by okadaic acid treatment of cells in culture or nuclear extracts *in vitro*, implicating PP2A (Cieslik *et al.* 1998, 1999). CK2 phosphorylates PP2A, resulting in down-regulation of PP2A activity (Cieslik *et al.* 1999). This decrease in PP2A activity is an indirect path to augmenting Sp1 phosphorylation, decreasing its activity (Cieslik *et al.* 1998, 1999). These authors provide further data to support a model by which LPC treatment of cells results in activation of a cascade of phosphatidylinositol 3-kinase (PI-3K γ), Janus kinase (Jak2), mitogen activated protein kinase kinase (MEK-1/MAPKK), and finally Erk-1 and Erk-2 (Fig. 1; Cieslik *et al.* 1999). Erk-1/2, in turn, phosphorylate and inhibit CK2 so that it cannot inactivate PP2A,

Table 1 Post-translational modifiers of Sp1

Modifier	Cell Type	Promoter	Inducer	Modification	Sp1 binding	Transfection Activity	Reference
DNA-PK	SV40 infected CV1-L/HeLa cells	SV40 intergenic control region	HIV type 1 TAT protein	Phosphorylation (Ser/Thr; N-terminus Ser-131)	No effect	Increased	Jackson <i>et al.</i> (1990) Chun <i>et al.</i> (1998)
PKA	HL-60/HL-60 AR leukemia cells	SV40 promoter	Inhibited by 8-Cl-cAMP & RpcAMP[S]	Phosphorylation (Thr-366)	Increased by PKA <i>in vitro</i>	Increased by co-transfected PKA	Rohlf <i>et al.</i> (1997)
	Human H295R/mouse Y1 adrenocortical cells	P450 CYP11A	Forskolin/8-CPT-cAMP ACTH	N.D.	No effect	Increased by co-transfected PKA	Ahlgren <i>et al.</i> (1999)
	Human hepatoblastoma HepG2 cells	Apolipoprotein A1	Forskolin phorbol dibutyrate	N.D.	Increased	Increased	Zheng <i>et al.</i> (2000)
	Human H358 estrogen receptor negative lung cancer cells	p21Cip1/Waf1 cyclin kinase	Tamoxifen/forskolin/dibutyryl cAMP	N.D.	No effect	Increased in transfection	Lee <i>et al.</i> (2000)
PKC α	Human hepatoblastoma HepG2 cells	IGF-II promoter 4	Hepatitis B virus X protein	Phosphorylation by co-transfected PKC α and Erk 2	Increased	Increased	Kang-Park <i>et al.</i> (2001)
Atypical PKC ζ	Human renal cell carcinoma 768-0	VEGF	None	Phosphorylation in zinc finger region	Increased by <i>in vitro</i> PKC ζ	Decreased by dominant negative PKC ζ mutant	Pal <i>et al.</i> (1998) Garcia <i>et al.</i> (2000)
	Rat pup aortic smooth muscle cells WKY12-22	PDGF B-chain	Nogalamycin	Phosphorylation (Thr 410)	Increased	Decreased by dominant negative PKC ζ mutant	Raffy & Khachigian (2001) Kavurma <i>et al.</i> (2001)
PP2A	Human ECV-304 umbilical vein endothelial cells	Endothelial nitric oxide synthase	Lysophosphatidylcholine	Dephosphorylation	Decreased by okadaic acid	Increased in transfection	Cieslik <i>et al.</i> (1998, 1999)
PP1	Y10/L8057 megakaryocyte cells	Cyclin D3	Mpl ligand/MGDF	Dephosphorylation <i>in vitro</i> by PP1	Increased by <i>in vitro</i> PP1	Decreased by okadaic acid	Wang <i>et al.</i> (1999b)
	Mouse 30A5 pre-adipocytes	Acetyl-CoA carboxylase promoter II	Glucose	Dephosphorylation <i>in vitro</i> by PP1	Decreased by okadaic acid	N.D.	Daniel <i>et al.</i> (1996)

Table 1 Continued

	Cell Type	Promoter	Inducer	Modification	Sp1 binding	Transfection Activity	Reference
CK2	Rat liver & K562 cells	RCE of the DBP promoter	Terminal differentiation	Phosphorylation (C-terminal D domain Ser/Thr-579 in second zinc finger)	Decreased by <i>in vitro</i> CK2	N.D.	Armstrong <i>et al.</i> (1997) Leggett <i>et al.</i> (1995)
p44 MAPK (Erk2)	Human gastric carcinoma AGS cells	Gastrin EGF responsive element	EGF	Phosphorylation	Increased by <i>in vitro</i> Erk2	Increased by Ras or Erk2 co-expression	Merchant <i>et al.</i> (1999)
	Human hepatoblastoma HepG2 cells	IGF-II promoter 4	Hepatitis B virus X protein	Phosphorylation	Increased by co-transfected Erk2	Increased	Kang-Park <i>et al.</i> (2001)
	Human hepatoblastoma HepG2 cells	Apolipoprotein A1	Insulin/EGF	Phosphorylation (Thr-266 in second glutamine rich domain)	N.D.	Decreased by dominant negative Ras	Zheng <i>et al.</i> (2001)
Ca ²⁺ /CamK II and IV	Mouse AtT-20/16-16 pituitary tumor cells	RCE of c-fos	Depolarization with K ⁺	N.D.	No effect	Increased by CamK II/IV co-transfection	Sohm <i>et al.</i> (1999)
60 kDa kinase	P-19 teratocarcinoma cells	Acetylcholine receptor ϵ subunit	Neuregulin (Neu differentiation factor)	Phosphorylation <i>in vitro</i>	Increased	N.D.	Alroy <i>et al.</i> (1999)
'Sp1 kinase'	Mouse Balbc & 3T3 cells	DHFR	Serum	Phosphorylation <i>in vitro</i> (Ser a.a. 612-678)	No effect	Increased	Black <i>et al.</i> (1999)
MEK 1 dependent 'Sp1 kinase'	Human AGS gastric adenocarcinoma cells	Gastrin EGF responsive element	EGF	Phosphorylation (Ser/Thr a.a. 1-530)	Increased <i>in vitro</i>	Increased	Chupreta <i>et al.</i> (2000)

ACTH, adrenocorticotropin; CK2, casein kinase 2; CamK, calmodulin kinase; DBP, D-site binding protein; DHFR, dihydrofolate reductase; DNA-PK, dsDNA dependent protein kinase; EGF, epidermal growth factor; IGF-II, insulin-like growth factor II; MEK, MAP/ERK kinase; MGDF, PEG-rHuMGDF (truncated Mpl ligand thrombopoietin); PKA, protein-kinase A; PP2A, protein-phosphatase 2A; PP1, protein phosphatase 1; PDGF, platelet-derived growth factor; VEGF, vascular permeability/endothelial growth factor; RCE, retinoblastoma control element; N.D., not determined.

thus freeing PP2A to activate Sp1 (Fig. 1; Cieslik *et al.* 1999).

Similarly, the glucose regulation of the acetyl-CoA carboxylase PII promoter also appears to be modulated by interplay between CK2 and cellular protein phosphatases (Daniel & Kim 1996, Daniel *et al.* 1996, Zhang & Kim 1997). In pancreatic β -cells, glucose induction of Sp1 is abrogated by expression of the CK2 catalytic subunit or by treatment with okadaic acid or immunodepletion to decrease PP1 activity (Zhang & Kim 1997).

Other groups also have demonstrated the importance of cellular protein phosphatases in the regulation of Sp1 activity. However, there is difficulty in reconciling some contradictory observations. *In vitro*, treatment of Sp1 with calf intestinal phosphatase (Leggett *et al.* 1995, Alroy *et al.* 1999, Chupreta *et al.* 2000, Merchant *et al.* 1999, Ray *et al.* 1999, Noe *et al.* 2001), potato acid phosphatase (Kumar & Butler 1998), or PP2A (Rolff *et al.* 1997) decreases DNA binding activity. Others have shown that alkaline phosphatase (Boreilini *et al.* 1990) or PP1 (Daniel *et al.* 1996, Armstrong *et al.* 1997, Wang *et al.* 1999b) increases binding. Obviously, some of these differences could be explained by differing experimental approaches, such as omission of phosphatase inhibitors during nuclear extract preparation (Edmead *et al.* 1999). Also, it is possible that the amount and location of phosphorylated residues could differ among cell types and the methods of extract preparation. For example, hyperphosphorylated Sp1 could be dephosphorylated to a more active state, while in other experiments, the baseline phosphorylation may be lower such that further phosphorylation decreases activity. What appears to be most consistent is the observation that *in vivo* treatment with okadaic acid, a cellular phosphatase inhibitor, increases Sp1 activity in the majority of reports (Daniel *et al.* 1996, Armstrong *et al.* 1997, Cieslik *et al.* 1998, 1999, Alroy *et al.* 1999, Wang *et al.* 1999b). Exceptions are the HIV long terminal repeats (LTR) and the serum amyloid A gene where okadaic acid treatment is reported to increase Sp1 DNA binding and transcription activity (Vlach *et al.* 1995, Ray *et al.* 1999).

It is clear from the preceding discussion that the regulation of Sp1 phosphorylation and activity is complex. Sp1 is regulated by a number of different signal transduction pathways (Fig. 1). Beyond inducible transcription, evidence for the impor-

tance of Sp1 in the regulation of the cell cycle is mounting (reviewed by Black *et al.* 2001). Sp1 phosphorylation is known to be dynamic throughout the cell cycle (Leggett *et al.* 1995, Black *et al.* 1999) and Sp1 can mediate a response to mitogenic signals, such as serum, through Ras-pathway activation (Miltenberger *et al.* 1995, Kumar & Butler 1998, Black *et al.* 1999, Spencer & Misra 1999). Furthermore, it is involved in the regulation of cell cycle proteins, such as multiple cyclin-dependent kinase inhibitors (Kivinen *et al.* 1999, Pagliucia *et al.* 2000, Wang *et al.* 2000), cyclin-dependent kinase-2 (Tikoo *et al.* 2000), and cyclin D3 (Wang *et al.* 1999b), and is itself regulated by cyclin activity (Shao & Robbins 1995). Recent experiments also implicate Sp1 as an essential player in activation of apoptotic events (Kavurma *et al.* 2001). The intimate role of Sp1 in mediating growth and environmental signals makes it an attractive candidate as a final target for the peptide hormone, insulin.

Sp1: regulation by insulin

Sp1 binding sites are found in the insulin-responsive regions of numerous genes, as summarized in Table 2. This finding points to the participation of Sp1 in the insulin regulation of gene activity. The role of Sp1 in insulin regulation is not uniform and is highly dependent on the inherent characteristics of each promoter. A survey of the insulin-responsive promoters listed in Table 2 reveals three different mechanisms by which Sp1 mediates insulin action. First, Sp1 acts alone in mediating the effects of insulin. Secondly, Sp1 binding sites may be closely juxtaposed to those of other insulin-responsive transcription factors, thus suggesting a co-operative interaction is required for insulin induction. Thirdly, Sp1 binding to an insulin-responsive promoter may lead to basal activity, but dissociation of Sp1 from this site permits the actions of another factor or factors to modulate gene activity in response to insulin.

Sp1 alone mediates insulin action

The plasminogen activator inhibitor type 1 (PAI-1) gene is up-regulated by insulin, with over twofold induction in Hep G2 cell transfection experiments (Banfi *et al.* 2001). Using signaling pathway

Table 2 Insulin responsive motifs that contain Sp1 sites (underlined)

Gene promoter	No. of Sp1 sites	Sequence	Other nearby factors	Cells	Inducer	Fold induction	Reference
GADPH	1	-471-AACTTTCCCGCCTCTCAGCCTTTGAAAAG-436	IRE-ABP	3T3 adipocytes	1000 mU insulin 16 h	4	Nasrin <i>et al.</i> (1990)
Apolipoprotein A1	1	-419-ACCTTTGAGGGGGGATGTGAGT-388	None known	Human Hep G2 hepatoblastoma	100 mU/l insulin 24 h	2	Murao <i>et al.</i> (1988), Zheng <i>et al.</i> (2001)
Fatty acid synthase	2	-57-GTGGCCGGGGGATGGCCGGC-35	Ebox (USF) CATGTG	Rat hepatocyte	20 mM glucose/0.1 μM insulin 48 h	1-5	Fukada <i>et al.</i> (1997b), Fukada & Iritani (1999)
		-332 to +1		3T3-L1 pre-adipocytes/H4IIE hepatoma	10 nM insulin 48 h	3	Moustaid <i>et al.</i> (1993)
Rat leptin	1	-101-GGGGGGAGTTGGCGCTC-83	None known	Rat hepatocytes	20 mM glucose/0.1 μM insulin 48 h	2	Fukada & Iritani (1999)
Insulin-like growth factor I region V	1	+140-GCCTCATTTCCTGCCCAATT	IRBP binding ATTATT	Rat hepatocyte/H4IIE hepatoma	1 μM insulin 24-48 h	1-5	Kaytor <i>et al.</i> (2001a, 2001b)
Calmodulin	3	-393 to +1	Upstream	H4IIE hepatoma	10 000 μU/ml	1-5	Solomon <i>et al.</i> (1997), Pan <i>et al.</i> (2001)
Rat ATP citrate-lyase	1	-64-TGATGGGGGGGGAGGAGCCCG-41	None known	Rat hepatocytes	20 mM glucose/0.1 μM insulin 48 h	2	Fukada <i>et al.</i> (1996, 1997a)
Human insulin receptor gene	4	-618-GGGGGGGGGGGACCGGGCGG-93	None known	Rat hepatocytes	20 mM glucose/0.1 μM insulin 48 h	2	Fukada <i>et al.</i> (2001)
Plasminogen activator inhibitor type 1	1	-93-CCCAGCCAGTGAGTGGGTGGGGCTGGAACA-62	None known	Human Hep G2 hepatoblastoma	100 nM insulin 16 h	3-5	Banfi <i>et al.</i> (2001)
Rat malic enzyme	1	-175-CCCCCGCCCTCCCGCA-156	Egr-1 (IRF)	Rat H35 hepatoma	N.A.	N.A.	Garcia-Jiménez <i>et al.</i> (1994), Barroso & Santisteban (1999)

GADPH, glyceraldehyde 3-phosphate dehydrogenase; USF, upstream stimulating factor; IRE-ABP, insulin responsive element-A binding protein; Egr-1, early growth response factor-1; IRBP, insulin receptor binding protein; IRF, insulin response factor; N.A., not applicable.

inhibitors, it appears that insulin induction is Ras independent, but is mediated instead through a cascade involving phosphatidylinositol (PI) 3-kinase, PKC, and MAP/ERK (MEK) kinase. Insulin induces nuclear protein binding to three different regions of the PAI-1 promoter, all of which contain Sp1 binding sites. However, only the most proximal sequence (−93/−62) binds Sp1 specifically, and this binding is induced by insulin. A tetramer of this region confers a significant insulin response on a minimal promoter suggesting that Sp1 may mediate the response to insulin in the context of immediately surrounding PAI-1 promoter sequences.

Insulin regulation of rat apolipoprotein (Apo) A1 gene expression provides another example of Sp1 independently mediating the actions of insulin. Studies from our laboratory have shown that insulin induces, but glucose inhibits, the activity of the apoA1 promoter in transfected Hep G2 cells (Murao *et al.* 1998). A mutant promoter lacking an intact insulin responsive core element, IRCE (−411 to −404), cannot respond to insulin. Importantly, Sp1 specifically binds the IRCE motif in gel mobility assays (Zheng *et al.* 2000). Insulin action is mediated by pathways involving the participation of PI-3K, PKC and MAP/ERK kinases, similar to the PAI-1 promoter presented above (Zheng *et al.* 2001). Co-transfection of Sp1 along with the ApoA1 IRCE reporter construct augments the actions of insulin. Further, the use of antisense RNA to lower the amount of Sp1 protein in the cell attenuates the insulin response.

In other promoters, Sp1 may oppose the effects of insulin. In rat hepatocytes, the fatty acid synthase (FAS) promoter is induced by insulin together with glucose, while it is inhibited by polyunsaturated fatty acids (PUFA) (Fukada *et al.* 1997*b*). The FAS response is mediated by a motif within a −57/−35 fragment with 70% homology to the GC box (Fukada *et al.* 1997*b*). A second group has also identified this region (−68/60) as essential for insulin/glucose regulation in adipocytes (Moustaid *et al.* 1993, 1994). Sp1 binds this sequence in a specific manner as demonstrated by competition studies and supershifts with Sp1 antibodies. Interestingly, over-expression of Sp1 in rat hepatocytes leads to a decrease in FAS-promoter activity, suggesting that it plays a negative transcriptional role at this site (Fukada *et al.* 1997*b*).

In a similar fashion, this group has studied other insulin-regulated genes such as leptin, ATP citrate-lyase (ACL), and the insulin receptor (IR), to extend their model of Sp1 action (Table 1). Insulin and glucose induction of the leptin and ACL promoters only require a single proximal Sp1 site (Fukada *et al.* 1997*a*, Fukada & Iritani 1999), as with the FAS promoter, while the response of the IR promoter is dependent on four closely apposed Sp1 binding sites (Fukada *et al.* 2001). Sp1 and Sp3 bind specifically to the ACL, leptin and IR sites as demonstrated in antibody supershift experiments (Fukada *et al.* 1997*a*, 2001, Fukada & Iritani 1999). As with the FAS promoter, Sp1 co-expression inhibits transfection activity.

It is possible that Sp1 truly mediates a negative effect on the insulin response of the ACL, IR, leptin, and FAS promoters. Sp1 binding to the ACL promoter site is decreased in liver extracts from rats fed a high carbohydrate diet compared with fasting (Fukada *et al.* 1997*a*). The investigators hypothesize that this result represents an inhibition of Sp1 activity due to the higher insulin levels in the carbohydrate-fed rats. Further, there is evidence that induction of the FAS promoter by insulin involves an E-box motif at −65, which overlaps the Sp1 site (Wang & Sul 1998). However, the ACL, leptin, and IR promoters do not contain an E-box in their insulin responsive regions. An alternative explanation is that the observed inhibition of transfected promoter activity by co-expressed Sp1 is due to supra-physiological levels of Sp1 in the cell, such that other factors required for the transcription machinery are titrated out and unavailable for transcription. This would be similar to the phenomenon of 'squenching' which was first described for *in vitro* transcription systems (Ptashne & Gann 1990).

Sp1 acts co-operatively with other insulin-regulated factors

In contrast to the apparently independent actions of Sp1 in the preceding section, other insulin-responsive regions appear to require Sp1 activity in concert with additional insulin-responsive factors. Kaytor *et al.* (2001*a,b*) have investigated the sequences that mediate an insulin response of the insulin-like growth factor (IGF)-I promoter. Insulin induction of IGF-I from the third transcription start site, IS3, is dependent on 'region V'. This site

mediates differential liver-specific expression in diabetic and control rats. Region V is a 24 bp motif comprised of both AT- and GC-rich domains at opposite ends. Mobility shift assays show that the 3' GC-rich end of region V binds Sp1 or Sp3. In contrast, the AT-rich end binds a second factor, insulin responsive binding protein (IRBP). IRBP binding is activated two- to sixfold by insulin treatment in a dose-responsive manner. IRBP binding to region V is possible following mutations to the GC-rich end of the element, suggesting that Sp1 is not required for binding. However, efficient Sp1 interactions with the GC-rich sequence appear to depend on IRBP, as both Sp1 DNA binding and transcription activity decrease with mutation of the IRBP binding site (Kaytor *et al.* 2001*b*). Moreover, Sp1 binding is enhanced by insulin induction of extracts when the IRBP site is intact. Importantly, spacing between the sites is critical to transcription activation in transfection experiments, suggesting that a true synergistic interaction occurs between Sp1 and IRBP. Based on their results, the authors hypothesize that the IRBP works in concert with Sp1 to mediate insulin induction.

Sp1 may also interact with more distally placed factors for an insulin response. The rat calmodulin (CaM) I gene responds to insulin dependent on Sp1 (Solomon *et al.* 1997). CaM I promoter contains two upstream Sp1 motifs at -100 and -70 and one downstream at +50 relative to the transcription start site. In the Sp1 null background of *Drosophila* SL2 cells, Sp1 co-transfection with the CaM promoter results in basal expression of the gene. However, stimulation with insulin leads to a 1-5-fold induction in promoter activity that is not present in control cells lacking Sp1. Co-transfection of Sp1 or the long form of Sp3 is required to allow a response to insulin, suggesting that Sp1 is essential in this setting (Solomon *et al.* 1997, Pan *et al.* 2001). The effect of insulin is not due to Sp1 alone, because deletion of sequences upstream to the Sp1 sites abolishes insulin induction. This finding suggests that Sp1 interacts with other upstream factors common between mammals and *Drosophila* to mediate insulin effects. Further studies reported by the same group found that Sp1 and Sp3 protein accumulation was increased approximately threefold in response to insulin treatment. Exposure to TNF- α , a factor that antagonizes insulin action, caused a decrease in Sp1 levels to half that seen in control cells (Pan *et al.* 2001). It is

interesting to note that, in these studies, lower levels of Sp1 were present in diabetic rats and these levels were restored with insulin treatment (Pan *et al.* 2001).

Other promoters have the potential to be regulated in a similar fashion, with the Sp1 motif co-operating with a second factor for insulin regulation. The rat pyruvate carboxylase gene product is an important component of the gluconeogenic and lipogenic pathways. This gene is alternatively transcribed from two promoters. Both promoters contain similar overlapping IRE and Sp1 sites reminiscent of the GAPDH IRE-A region. In spite of the similar elements, in COS-1 cell transfection studies, the proximal promoter is inhibited 50% by insulin, while the distal promoter is unaffected (Jitrapakdee *et al.* 1997). This suggests that additional promoter elements are involved in regulation of the response to insulin. Confirmation of the involvement of the IRE-A-like element for insulin regulation requires further study.

Displacement of Sp1 mediates insulin action

ME is an important lipogenic enzyme that is controlled by insulin. The ME promoter is GC-rich and contains numerous Sp1 motifs (Moroika *et al.* 1988, Garcia-Jimenez *et al.* 1994). Transcription factor AP-1 has previously been shown to play a role in mediating insulin regulation of the ME promoter (Streeper *et al.* 1998). However, Barroso & Santisteban (1999) have studied one of the GC-rich sites resembling the IRE-A motif of the GAPDH promoter, called IRE-II (-175 to -156). IRE-II binds Sp1 and Sp3 constitutively, while Egr-1 binds to an overlapping recognition site, increasing in response to insulin. In transfection experiments, Egr-1 expression inhibited the response to insulin by the ME promoter. Further, increasing amounts of Egr-1 displaced Sp1 from its binding site. Overall, these experiments suggest that the ME promoter initially is inhibited in response to insulin through displacement of Sp1 or Sp3 by Egr-1 at the IRE-II site.

Summary

It is clear that Sp1 is much more than a basal transcription factor. Sp1 activity is modified by its phosphorylation state in response to a multitude

of signals, implicating Sp1 as a key mediator of 'cross-talk' between signaling pathways and gene transcription. The finding that Sp1 binding sites are common to many insulin responsive promoter regions suggests that it is a major factor in mediating the effects of insulin. However, its role appears to be particularly diverse, with positive or negative effects depending on the promoter.

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