Growth/Cell Cycle Regulation of Sp1 Phosphorylation

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Sp1 sites can mediate growth/cell cycle induction of dihydrofolate reductase in late G₁ (Jensen, D. E., Black, A. R. Swick, A. G., and Azizkhan, J. C. (1997) J. Cell. Biochem. 67, 24–31). To investigate mechanisms underlying this induction, effects of serum stimulation on regulation of Sp1 were examined. In Balb/c 3T3 cells, serum stimulation did not affect Sp1 synthesis or the relative binding of Sp1 family members to DNA; however, it did result in a rapid, 2-fold increase in Sp1 levels and an ~3-fold increase in specific Sp1 phosphorylation in mid-G₁. In normal human diploid fibroblasts, serum stimulation also increased Sp1 phosphorylation in mid-G₁ but did not affect Sp1 levels. Therefore, Sp1 phosphorylation is regulated in a growth/cell cycle-dependent manner which correlates temporally with induction of dihydrofolate reductase transcription. Further studies revealed a kinase activity specifically associated with Sp1 in a growth-regulated manner. This activity is distinct from purified kinases previously shown to phosphorylate Sp1 in vitro and phosphorylates Sp1 between amino acids 612 and 678 in its C terminus, a region also phosphorylated in mid-G₁ in vivo. Therefore, this study indicates that phosphorylation of the C terminus of Sp1 may play a role in the cell cycle regulation of its transcriptional activity.

Expression of a large number of genes associated with DNA synthesis, such as dihydrofolate reductase (DHFR), is tightly regulated with cell growth and the cell cycle. Many of these genes have promoters which lack a TATAA element but contain binding sites for the transcription factors Sp1 and E2F (1). Although the role of E2F sites in growth/cell cycle regulation of transcription and the regulation of E2F by retinoblastoma protein (pRB) and related pocket proteins have been extensively characterized (see Refs. 2–4, for review), a role for Sp1 sites in growth/cell cycle regulation of transcription is only beginning to emerge (e.g. Refs. 5 and 6). We have determined that Sp1 and E2F sites have distinct roles in the growth/cell cycle regulation of the hamster DHFR promoter (6). Although complete repression of DHFR transcription in G₀ and early G₁ requires E2F sites, its induction in late G₁ is mediated by Sp1 sites. A direct role of Sp1-dependent transcription in growth regulation of transcription is supported by targeting of Sp1 by viral oncoproteins (e.g. Refs. 7–9), down-regulation of Sp1 expression, and/or DNA binding activity upon differentiation in some systems (10), and increased Sp1 expression during events associated with transformation (11).

Sp1 is a ubiquitous, 778-amino acid transcription factor that recognizes GC-rich sequences present in many promoters (see Refs. 1 and 2, for review). Although Sp1 has been viewed as a constitutive transcriptional activator which acts as a basal factor for TATAA-less promoters, an increasing number of studies indicate that Sp1-dependent transcription is regulated in response to a variety of signals. For example, in addition to their role in growth/cell cycle regulation of transcription, Sp1 sites are involved in induction of DHFR transcription in response to methotrexate (12), induction of CYP11A transcription in response to cyclic AMP (13), and transforming growth factor β induction of p15 (14).

Regulation of Sp1-dependent transcription could be effected by changes in Sp1 abundance, DNA binding activity, and/or transactivation activity. Sp1 is O-glycosylated and phosphorylated, and both of these modifications are likely to be important in its regulation. Phosphorylation has been implicated in changes in Sp1 binding and transcriptional activation (15–17), and changes in O-glycosylation alter the stability of Sp1 in vivo (18) and its interaction with other factors (19). An additional mechanism that could underlie the regulation of gene expression through Sp1 sites has come to light with the finding that Sp1 is a member of a multigene family. Of the four known members of the family, Sp2 does not recognize the same sequence as Sp1 and Sp4 expression is restricted to the brain. Sp3, on the other hand, is ubiquitously expressed and recognizes the same sequences as Sp1 but has a more complex transcriptional activity. Whereas Sp1 appears to be almost exclusively an activating transcription factor, Sp3 contains a transcriptionally repressive domain and can act as a transcriptional activator or repressor, dependent on the promoter and cell type (20–25). A role for Sp3 in regulation of Sp1 site-dependent transcription is seen in its mediation of p21 induction during keratinocyte differentiation (26).

To determine the mechanism(s) underlying growth/cell cycle-regulated induction of Sp1 site-dependent transcription, we characterized changes in Sp1 following serum stimulation of quiescent cells. Serum stimulation leads to an increase in Sp1 levels and phosphorylation, with the increased phosphorylation correlating with induction of Sp1-dependent activation of the DHFR promoter in mid-late G₁. We also determined that this change in phosphorylation is accompanied by changes in the association of Sp1 with a novel Sp1 kinase activity which phosphorylates the C terminus of Sp1.

MATERIALS AND METHODS

Cell Culture and Synchronization—Balb/c 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), 10% calf serum (Colorado Serum Co.), penicillin/streptomycin (Life Technologies, Inc.) at 37 °C in a 10% CO₂ atmosphere (6). Normal human
diploid fibroblasts (NHDF) from Clonetics were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum (Life Technologies, Inc.), penicillin/streptomycin at 37 °C in a 10% CO2 atmosphere (27) and used at less than 30 doublings. Cells were synchronized by serum starvation in medium containing 0.5% serum for 24 h (Balb/c 3T3) or 0.2% serum for 16–72 h (NHDF), to re-enter the cell cycle by addition of serum to 10–20% (6, 27).

In Vivo Labeling and Western Blot Analysis—For [32P]methionine labeling, cells were rinsed with 20 ml HEPEs, pH 7.2, 150 mM NaCl and placed in methionine-free Dulbecco’s modified Eagle’s medium containing appropriate concentrations of dialyzed fetal bovine serum. After 90 min, medium was removed and cells were labeled in the same medium containing 7.5 μCi/ml [32P]methionine. [32P]PO4 labeling was carried out similarly except that cells were incubated in phosphate-free medium for 2 h prior to being labeled in phosphate-free medium containing 7.5–15 μCi/ml [32P]PO4 for 2 h. At the indicated times following serum stimulation, cells were rinsed with phosphate-buffered saline (135 mM NaCl, 4 mM KCl, 10 mM Na2PO4, pH 7.4) and lysed directly in boiling 10 mM Tris-HCl, pH 7.2, 1% SDS, reboiled and DNA was sheared. Following addition of 2.2 volumes of ice-cold 15 mM Tris-HCl, pH 7.2, 7.5 mM EDTA, 150 mM sodium fluoride, 230 mM NaCl, 1.5% Triton X-100, 0.75% Nonidet P-40, 100 mM β-glycerophosphate, 15 mM sodium pyrophosphate, 400 μM Na2VO4, 2 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 10 μg/ml aprotinin, particulate material was removed by centrifugation (13,000 × g, 10 min). Supernatants were precleared with normal rabbit serum and Protein A-Sepharose and Sp1 was immunoprecipitated with anti-Sp1 antibody (PEP2, Santa Cruz) and protein A-Sepharose. Immunoprecipitates were washed 4 times with RIPA (phosphate-buffered saline containing 1% (w/v) Ipegal CA, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% (w/v) SDS) and separated by 8% SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting with anti-Sp1 (PEP2) antibody and anti-rabbit horseradish peroxidase secondary antibody as prescribed by the manufacturers (Santa Cruz and Promega). Detection utilized the Pierce Supersignal system.

Electrophoretic Mobility Shift Assay of Sp1 Family Members—Balb/c 3T3 cells were harvested at the indicated times following serum stimulation and nuclear extracts were prepared as described (28). Electrophoretic mobility shift assay of binding to a double-stranded [32P]-labeled probe corresponding to the first Sp1 site in the hamster DHFR promoter was performed as described (28, 29). Assays were conducted with equal amounts of nuclear extract protein from serum-starved and serum-stimulated extracts. Specificity of binding was confirmed by competition with a 50-fold excess of unlabeled probe.

Analysis of ATP Pools—Balb/c 3T3 cells were labeled with [32P]PO4, washed with phosphate-buffered saline, and harvested by scraping in 1 M formic acid. Non-acid soluble material was removed by centrifugation and free [32P]PO4 was removed and samples were neutralized as described (30). Nucleotides were separated on a 31-cm linear gradient of 0.1 M potassium dihydrogen phosphate, pH 6.0, 8 mM tetrabutylammonium, 0.1 M potassium dihydrogen phosphate, pH 6.0, 8 mM tetrabutylammonium, 30% methanol at a flow rate of 1.5 ml/min (31). Eluted nucleotides were detected by their absorbance at 254 nm using a Perkin-Elmer UV95 uv/visible in-line detector and radioactivity was detected by counting Cerenkov radiation with an INUS Systems Inc. β-RAM in-line radiation detector. Levels of ATP in samples were estimated by comparison of peak height with that obtained with unlabeled standard ATP. Relative specific activity of ATP was determined by dividing radioactive peak height by absorbance peak height.

Phosphoamino Acid Analysis—Sp1 was immunoprecipitated from serum-stimulated [32P]PO4-labeled cells, separated by 8% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The region of the membrane containing the [32P]-labeled Sp1 (as determined by autoradiography) was excised and phosphoamino acid analysis was performed by two-dimensional thin layer electrophoresis in the presence of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards as described (32).

Analysis of Proteolytic Activity—Balb/c 3T3 cells were transfected with 20 μg of DNA (5 μg of reporter constructs and 10 μg of Flag-Sp1 constructs) by the calcium phosphate co-precipitation method and chloramphenical acetyltransferase assay was assayed as described (6). Luciferase activity was assayed 24 h after serum stimulation using the Promega Luciferase Assay System. Correction for differences in transfection efficiency between precipitates used the activity of a co-transfected Rous sarcoma virus long-term repeat-chloramphenical acetyltransferase construct which is unaffected by Sp1 overexpression. DHFR3E2F and 5XSp1L53MLP constructs and adenovirus major late promoter constructs were as described (6, 27) except where the chloramphenical acetyltransferase cDNA was replaced with that of firefly luciferase.

In Vitro Kinase Assays—NHDF, serum-starved or serum-stimulated, were extracted in 5 volumes of 100 mM HEPEs, pH 7.4, 500 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 5 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and freeze/thawed 3 times. Particular matter was removed by centrifugation (13,000 × g, 10 min) and extracts were stored at −90 °C. For immunoprecipitation, extracts were diluted 20-fold in HEPEs, pH 7.4, 0.625% Nonidet P-40, 10 μM leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and precleared with normal rabbit serum and Protein A-Sepharose. Sp1 was then immunoprecipitated with anti-Sp1 and Protein A-Sepharose and the beads were washed 4 × 5 min in 20 ml HEPEs, pH 7.4, 100 mM KCl, 1 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA. Where indicated, an equal weight of PEP2 pellet (Santa Cruz) was added to the extracts prior to the addition of anti-Sp1 (PEP2) antibody. For direct kinase assay of the immunoprecipitates, kinase reactions were carried out in 50 μl of kinase buffer (125 mM HEPEs, pH 7.4, 1% (w/v) glycerol, 10 mM NaCl, 1 mM MgCl2, 0.5 mM MnCl2 containing 10 μCi of [γ-32P]ATP (3000–6000 Ci/mmol) for 30 min at 37 °C. Phosphorylated proteins were analyzed by 8% SDS-PAGE and autoradiography. For 1 mM NaCl extraction of immunoprecipitates, were eluted from Sp1 immunoprecipitates with 32PO4, followed by immunoprecipitation of Sp1 and SDS-PAGE/autoradiography and Western blotting. Serum stimulation, cells were rinsed with phosphate-buffered saline and labeled for 1 h with 32PO4, followed by immunoprecipitation of Sp1 and SDS-PAGE/autoradiography and Western blotting. Serum stimulation, cells were rinsed with phosphate-buffered saline and labeled for 1 h with 32PO4, followed by immunoprecipitation of Sp1 and SDS-PAGE/autoradiography and Western blotting.

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RESULTS

Comparison of Sp1 from G0 and G2 Balb/c 3T3 Cells—To elucidate mechanisms underlying the growth/cell cycle regulation of Sp1-dependent transcription, changes in various aspects of Sp1 regulation were monitored in Balb/c 3T3 cells and in these cells following 8 h of serum stimulation (i.e. cells in G0 and late G1, respectively (6)). Levels of expression, rate of synthesis, and phosphorylation of Sp1 were examined by labeling equal numbers of cells with [32P]methionine or [32PO4] followed by immunoprecipitation of Sp1 and SDS-PAGE/autoradiography and Western blotting. Serum stimula-
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Fig. 1. Analysis of differences in Sp1 regulation in G₀ and G₁ cells. Equal numbers of Balb/c 3T3 cells were serum-starved for 32 h (G₀) or serum-starved for 24 and refed with 20% serum for 8 h (G₁). A, Western blot analysis of Sp1 levels in extracts of G₀ or G₁ cells. Arrowsheads indicate the migration of molecular weight standards (203, 116, 83, and 48 kDa). Lines show the migration of phosphorylated (upper) and non-phosphorylated (lower) Sp1. B, G₀ and G₁ cells were labeled with [³²P]methionine for 2 h prior to harvest and extraction. Sp1 was immunoprecipitated from cellular extracts and the levels of ³²P incorporation into (synthesis of) Sp1 were determined by 8% SDS-PAGE and autoradiography. C, G₀ and G₁ cells were labeled with ³²PO₄ for 2 h prior to harvest and the levels of ³²P labeling of Sp1 were determined as for ³²S incorporation. D, electrophoretic mobility shift assay of Sp1 site binding material in nuclear extracts from G₀ and G₁ cells. The ³²P-labeled probe was a double-stranded oligonucleotide corresponding to the first GC box in the hamster DHFR promoter. The arrowhead and arrows indicate the migration of Sp1 and Sp3 containing complexes, respectively, as shown by others (e.g. Ref. 24) and confirmed by separate antibody supershift assays. Specificity of binding was confirmed by competition with unlabeled probe (not shown).

Table I
Specific activity of ATP pools in ³²PO₄-labeled Balb/c 3T3 cells

| Cells          | ATP pools | Relative ATP specific activity |
|----------------|-----------|-------------------------------|
|                | G₀        | G₁                           | G₀        | G₁             | n mole/10⁶ cells | arbitrary units |
| Balb/c 3T3     | 10.06 ± 0.40 | 17.75 ± 3.86           | 1.00 ± 0.02 | 1.30 ± 0.43   | 3.86 ± 0.09     | 0.40 ± 0.09     |
| NHDF           | 3.6 ± 0.16 | 4.24 ± 0.69                | 1.0 ± 0.11 | 0.97 ± 0.09    |

Sp3 do not change significantly following serum stimulation (Fig. 1D, arrowhead and arrows). Therefore, of the parameters tested, only changes in Sp1 levels and/or phosphorylation could account for the induction of Sp1-dependent transcription seen upon serum stimulation.

Time Course for Serum Induction of Sp1 Levels and Phosphorylation—Since serum induction of Sp1-dependent transcription from the DHFR promoter occurred in late G₁ (6), Sp1 levels and phosphorylation were assessed in Balb/c 3T3 cells at various times following serum stimulation. As seen in Fig. 2A (lower panel), induction of Sp1 levels was apparent by 2 h and was essentially maximal by 4–6 h following serum stimulation. In keeping with the increased levels of Sp1, ³²P incorporation into Sp1 also increase by 2 h; however, a clear difference between the timing of the increase in Sp1 levels and its increased phosphorylation became apparent when the changes in Sp1 levels were taken into account (Fig. 2B). Following serum-stimulation, specific Sp1 phosphorylation changed little for the first 4 h, but increased to maximal levels by 6–8 h. Since S-phase occurs 10–12 h following serum stimulation of these cells, Sp1 phosphorylation is induced in mid-G₁ and, therefore, occurs concomitant with or slightly precedes the induction of Sp1-dependent DHFR transcription (6). The delayed induction of DHFR following serum stimulation.
would indicate that it is relatively insensitive to Sp1 levels. This is supported by experiments in which overexpression of Sp1 had little (1.34 ± 0.44-fold, n = 4) effect on DHFRΔE2F-luciferase activity in serum-stimulated cells, whereas it led to a 2.89 ± 0.69-fold induction of transcription from a promoter containing 5 Sp1 sites upstream of a TATAA box (5XSp1Δ53MLP). The higher sensitivity of the latter promoter to Sp1 levels is consistent with its more rapid induction following serum stimulation of Balb/c 3T3 cells (6).

To determine if growth regulation of Sp1 phosphorylation is unique to Balb/c 3T3 cells, a similar experiment to that in Fig. 2A was performed using NHDF which enter S-phase 10–14 h after serum stimulation (27). These cells were chosen because (a) they represent primary human cells, and (b) Sp1 levels do not change significantly during G0 to S phase transition in these cells (Ref. 27 and Fig. 3A). Serum stimulation of NHDF led to an increase in Sp1 phosphorylation (2.4 ± 0.4-fold, n = 4) while neither Sp1 levels (G0/G1 levels = 1.2 ± 0.1, n = 7) nor the specific activity of ATP pools was affected (Fig. 3A, Table I). Thus, induction of Sp1 phosphorylation in response to growth stimulation is not cell type-specific and can be seen in the absence of significant changes in Sp1 protein levels. Induction of Sp1 phosphorylation was also delayed following serum stimulation of NHDF, with increased phosphorylation first seen 6–8 h following addition of serum.

The vast majority of Sp1 phosphorylation is on serine in cycling HeLa cells (35). To determine if this was the case following serum stimulation of serum-starved cells, phosphoamino acid analysis was performed on 32P-labeled Sp1 from late G1 cells. Since only phosphoserine was detected (Fig. 3B), the increased phosphorylation of Sp1 seen in mid- to late G1 must be due to serine phosphorylation.

Identification of a Growth-regulated Sp1-associated Kinase Activity—It has been proposed that association of kinases with their substrate proteins can be an important factor for their specificity and activity (36); therefore, the possibility that increased Sp1 phosphorylation could be due to its association with a cellular kinase was investigated. Sp1 was immunoprecipitated from extracts of G0 or G1 cells and the immunoprecipitates were subjected to an in vitro kinase reaction. As seen in Fig. 4A, kinase activity immunoprecipitated with Sp1. Furthermore, the kinase activity associated with Sp1 was 2–3-fold higher in G1 cells than in G0 cells. Similar results were obtained when Sp1 was immunoprecipitated from whole cell extracts (Fig. 4A) or nuclear extracts and were seen with extracts from NHDF (Fig. 4) and Balb/c 3T3 cells.3 In these reactions, although a phosphorylated band corresponding in size to Sp1 was sometimes seen (Fig. 4A, arrow), this was not the major phosphoprotein detected. The low level of Sp1 phosphorylation is not unexpected since (a) antibody, rather than

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3 S. Pajovic and J. C. Azizkhan, unpublished data.

A. R. Black and J. C. Azizkhan, unpublished data.
were extensively washed and extracted with 1M NaCl (kinase reactions were performed with the immunoprecipitates which allowed by 8% SDS-PAGE/autoradiography (presence of no antibody (No antibody in the antibody preparations used for immunoprecipitation and possibly Sp1-associated proteins. Nevertheless, the data clearly demonstrate that a kinase activity does co-immunoprecipitate with Sp1 and that this activity is growth-regulated.

To determine if the Sp1-associated kinase activity could phosphorylate Sp1, it was eluted from Sp1 immunoprecipitates and used in an in vitro kinase reaction containing bacterially expressed glutathione S-transferase Sp1 fusion protein (GST-Sp1). The 1 M NaCl extraction eluted the majority of the kinase activity from the immunoprecipitates (Fig. 4A) and the eluted kinase was able to phosphorylate GST-Sp1 in a growth-regulated manner (Fig. 4B). The specificity of the association of this Sp1 kinase activity with Sp1 was confirmed, since very little phosphorylation of GST-Sp1 could be detected when a blocking peptide (recognized by the anti-Sp1 antibody) was added to the cell extracts prior to immunoprecipitation of Sp1, and no activity was detected when anti-Sp1 antibody was omitted from the immunoprecipitation (Fig. 4C). Phosphorylation did not occur in the GST portion of the fusion protein since the activity was unable to phosphorylate GST (data not shown) or some of the GST-Sp1 truncation mutants tested below (Fig. 6). Phosphorylation of Sp1 by a contaminating bacterial kinase can also be excluded since kinase reactions performed in the absence of eluate from Sp1 immunoprecipitations revealed no phosphorylation of GST-Sp1 (see Fig. 6A, lane 1). In these reactions, a band which migrated below GST-Sp1 at ~80 kDa could be seen; this band was seen to varying extents in different preparations of GST proteins and is presumably due to a bacterial kinase.

The Sp1-associated Kinase Activity Requires Protein Kinase A (PKA) (17). Therefore, various substrates and inhibitors were used to determine if the Sp1-associated kinase activity corresponded to any of these enzymes. CK2 can utilize GTP as a phosphate donor essentially as efficiently as ATP (39). However, GTP was a very poor phosphate donor for the Sp1-associated kinase (Fig. 5, lanes 1 and 2); therefore it is unlikely to represent CK2. DNA-dependent protein kinase requires Sp1 and the kinase to be bound to DNA (38). Since disruption of protein-DNA interaction with 50 μg/ml ethidium bromide (EtBr) did not affect Sp1 phosphorylation (Fig. 5, lane 3), the Sp1-associated kinase activity cannot be due to DNA-dependent protein kinase. Finally, the ability of staurosporine to inhibit the Sp1-associated kinase activity was tested. Staurosporine is a competitive inhibitor of ATP binding to a number of kinases and has a $K_i$ for PKA of 5 nM (41). Although stauro-

![Figure 4. Association of Sp1 with a kinase activity.](image)

In vitro kinase assays of the Sp1-associated kinase activity were carried out as described in the legend to Fig. 4B, except that [γ-32P]GTP was used as a phosphate donor in lane 1 (GTP); reactions shown in other lanes used [γ-32P]ATP as a phosphate donor. Ethidium bromide (EtBr) or staurosporine (Staur.) were added to reactions at the indicated final concentration prior to addition of the eluate from Sp1 immunoprecipitations.

![Figure 5. Characterization of the Sp1-associated kinase activity.](image)
sporine was able to inhibit the Sp1-associated kinase activity (Fig. 5, lanes 4 and 5), the IC$_{50}$ of this inhibition was 40–50 nm under conditions where the IC$_{50}$ for PKA would be <10 nm; therefore, the Sp1-associated kinase activity also differs from PKA. Inhibition of the activity by 50 nm staurosporine further argues against the kinase being CK2 which has a $K_i$ of 6 μM for staurosporine (41). Thus, the growth-regulated Sp1-associated kinase activity differs from kinases known to phosphorylate Sp1 and presumably represents a novel Sp1 kinase.

Consistent with the physiological relevance of the Sp1-associated kinase activity, treatment of serum-stimulated cells with 100 nm staurosporine for 6 h led to a 58% ($\pm 5\%$, n = 5) reduction in activity of DHFR. Thus, the growth-regulated Sp1-associated kinase activity differs from kinases known to phosphorylate Sp1 and presumably represents a novel Sp1 kinase.

DISCUSSION

To elucidate possible mechanisms underlying the serum induction of Sp1 site-dependent transcription, we have examined the levels, synthesis, phosphorylation, and DNA binding of Sp1. Serum stimulation of Balb/c 3T3 cells leads to an increase in Sp1 levels and phosphorylation, while neither Sp1 synthesis nor its specific DNA binding activity change. The increase in Sp1 phosphorylation was greater than that of protein, indicating that it results, at least in part, from an increase in the level of phosphorylation of individual Sp1 molecules. This was confirmed in NIHDF where increases in Sp1 phosphorylation were seen with no significant increase in protein levels. The ratios of the different Sp1 site binding complexes were not affected by serum stimulation, indicating that the induction of transcription is not simply due to changes in the relative binding of stimulatory (Sp1) and inhibitory (Sp3) factors to the Sp1 sites. Therefore, of the parameters tested, only changes in Sp1 levels and phosphorylation could account for the growth-related changes in Sp1-dependent transcription.

Analysis of the kinetics of these events revealed that induction of Sp1 protein levels occurs rapidly following serum stimulation of Balb/c 3T3 cells (Fig. 2A), whereas the increase in phosphorylation is delayed (Figs. 2B and 3). Previously, we have reported that the kinetics of serum induction of Sp1-dependent transcription is promoter dependent in Balb/c 3T3 cells (6), occurring either with delayed kinetics (the DHFR promoter) or rapidly (5XSp1L53MLP) following serum stimulation. In vivo footprinting data demonstrating that the DHFR promoter is constitutively occupied during the G1–S phase transition (42) suggest that Sp1 is not limiting at this promoter. The finding that the 5XSp1L53MLP promoter shows greater sensitivity to Sp1 overexpression than the DHFR promoter (see “Results”) supports this notion and indicates that Sp1 levels are more limiting at the more rapidly induced 5XSp1L53MLP promoter. This leads to a model for differential regulation of promoter activity by Sp1: at promoters where Sp1 levels are limiting, the rapid induction of Sp1 levels by serum would lead to rapid induction of transcription, whereas with promoters which are regulated by Sp1 phosphorylation and where Sp1 levels are not limiting, induction would be delayed.

While the current study was ongoing, it was reported that serum stimulation of CHO K1 cells leads to a delayed induction of Sp1 levels and that this change correlates with the increase in Sp1-dependent transcription of DHFR (43). The discrepancy between this study and our findings presumably resides in the different cell types and/or promoters used (the studies in Chinese hamster ovary cells used a truncated DHFR promoter containing only one Sp1 site which has shown very low activity in other studies (28)). Although the possible contribution of phosphorylation to the induction seen in Chinese hamster ovary cells was not addressed, it appears that Sp1-dependent transcription may be regulated at multiple levels dependent on the promoter and cell type studied. This is supported by the finding that Sp1 sites play a greater role in growth regulation of the rep3 promoter than the DHFR promoter in NIH 3T3 cells (5), and that Sp1 levels and/or binding activity do not change following serum stimulation of NIH 3T3 cells or human fibroblasts (8, 27, 44, 45).

Although not examined here, another potential mechanism for regulation of Sp1 is through O-glycosylation which has been shown to play a role in the association of Sp1 with other factors (19) and in regulation of its degradation (18). Our findings are suggestive of a possible role for $O$-glycosylation since Sp1 levels change in Balb/c 3T3 cells without concomitant changes in Sp1 synthesis, indicating that its levels may be regulated by changes in degradation.

In keeping with the serum stimulation of the phosphorylation of Sp1, we have observed an Sp1-associated kinase activity, whose interaction with Sp1 is growth-regulated. Although rigorous proof that the Sp1-associated kinase activity represents a physiologically relevant Sp1 kinase will require identification of the kinase(s) involved and determination of the
effects of its inhibition in vivo (46) (and is, therefore, beyond the scope of the present study), several factors argue in favor of its physiological relevance. The level of the activity is growth-regulated, indicating that either the activity and/or association with Sp1 of the relevant kinase(s) is likewise growth-regulated. Furthermore, at least the majority of the activity is specifically associated with Sp1 (Fig. 4B). The specificity of the interaction is reinforced by the finding that this activity binds strongly to FIG. 6. Region of Sp1 phosphorylated by the Sp1-associated kinase activity. A, in vitro labeling of GST-Sp1 truncation mutants. Autoradiogram and Coomassie-stained 10% SDS-PAGE gel of in vitro kinase assays of the Sp1-associated kinase activity carried out as in the legend to Fig. 4B. In lane 1 (1–778, no eluate), kinase reactions were carried out with GST-Sp1 but without addition of eluate from Sp1 immunoprecipitations. The substrates in the other kinase reactions (which contained eluate from Sp1 immunoprecipitations) were full-length GST-Sp1 (1–778) or various truncation mutants of Sp1 fused to GST. The numbers above the lanes indicate the amino acids of Sp1 present in these fusion proteins. The arrowhead indicates the migration of a phosphoprotein due to nonspecific phosphorylation. B, in vivo labeling of Sp1 truncation mutants. Flag-tagged, full-length Sp1 (Flag-Sp1), and Flag-tagged truncation mutants containing the N-terminal 612 (Flag-Sp1(1–612)) or C-terminal 167 (Flag-Sp1(612–778)) amino acids of Sp1 were transfected into Balb/c 3T3 cells prior to serum starvation for 24 h. Cells were then serum stimulated for 9 h with 32P labeling for 2 h prior to harvest. Flag-tagged proteins were immunoprecipitated from cell extracts with anti-Flag antibody and 32P incorporation into the proteins was determined by 8% (Flag-Sp1 and Flag-Sp1(1–612)) or 15% (Flag-Sp1(612–778)) SDS-PAGE and autoradiography. For clarity, panels show only the region of the autoradiogram where the fusion proteins migrate. With the exception of material trapped at the top of the 15% gel, no other bands were apparent on the autoradiograms. C, amino acid sequence of the C-terminal region of Sp1. Underlined regions delineate the zinc fingers of Sp1, while the bold letters show the region phosphorylated by the Sp1-associated kinase in the in vitro assays. The 3 serines in the labeled region are shown in bold italics.
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GST-Sp1 (it remains bound following extensive washing of glutathione-Sepharose-bound GST-Sp1 which had been preincubated with eluate from Sp1 immunoprecipitates\(^2\)). Finally, the Sp1-associated kinase activity phosphorylates Sp1 in its C-terminal domain, a region phosphorylated in late G\(_1\) cells in \(\text{vivo}\).

Although these studies have identified an Sp1-associated kinase that is likely to be involved in regulation of its transcription, the identity of the kinase is unknown at present. Sp1 has been shown to be phosphorylated \(\text{in vitro}\) by CK2, PKA, and DNA-dependent protein kinase; however, the Sp1-associated kinase differs from these kinases in its ability to utilize GTP and its sensitivity to staurosporine and ethidium bromide, respectively. Although it is possible that the Sp1-associated kinase represents more than one kinase (it phosphorylates at least two sites on Sp1 \(\text{in vitro}\), the low level of its GTP usage, minimal inhibition by low concentration of staurosporine, and insensitivity to ethidium bromide indicate that, if it contains any of the above kinases, they represent only a small proportion of the total activity. During the preparation of this manuscript, it was reported that DNA-dependent protein kinase leads to an increase in Sp1 activity through phosphorylation of serine 131 in the N terminus of Sp1 (47). Although this phosphorylation could be enhanced by HIV-TAT, it remained dependent on the presence of DNA. Since the Sp1-associated kinase did not phosphorylate the N terminus of Sp1 and was insensitive to disruption of protein-DNA complexes by ethidium bromide, these findings further emphasize that the kinase identified here is distinct from DNA-dependent protein kinase. Therefore, the Sp1-associated kinase activity identified here contains a novel growth-regulated Sp1 kinase(s).

Consistent with the data obtained \(\text{in vitro}\), it is also unlikely that CK2, PKA, or DNA-dependent protein kinase are directly responsible for the increased phosphorylation of Sp1 in G\(_1\) \(\text{in vivo}\). Phosphorylation of Sp1 by CK2 leads to a reduction in Sp1 DNA binding and occurs on threonine (15, 37). Therefore, since G\(_1\) phosphorylation of Sp1 is on serine and no differences in DNA binding were observed between G\(_0\) and G\(_1\) cells, CK2 is unlikely to be directly responsible for the growth/cell cycle-regulated phosphorylation of Sp1. Although PKA activation leads to activation of Sp1 site-dependent transcription at a number of promoters, its precise role in this phenomenon is unclear. PKA phosphorylates Sp1 \(\text{in vitro}\) and this phosphorylation leads to increased Sp1 DNA binding (17). In contrast, activation of PKA \(\text{in vivo}\) leads to enhanced Sp1 site-dependent transcription of the \(\text{sgk}\) promoter without changes in Sp1 levels or DNA binding activity (48), and enhanced binding to Sp1 sites at the NF-L promoter following PKA activation is due to a factor other than Sp1 (49). The increased DNA binding induced by PKA and the presence of only threonine (Thr\(^{366}\)) in the PKA consensus site in Sp1 preclude it from being the kinase responsible for the G\(_1\) phosphorylation seen here. DNA-dependent protein kinase requires DNA ends for activity and has been implicated in responses to DNA damage (50); therefore, it is unlikely to be relevant to the G\(_1\) phosphorylation of Sp1. The involvement of kinases other than DNA-dependent kinase in phosphorylation of Sp1 is supported by its phosphorylation in G\(_1\), SCID mouse fibroblasts\(^2\) which lack DNA-dependent kinase activity (51). Thus, in keeping with the presence of Sp1 sites in multiple promoters, Sp1 is phosphorylated at multiple sites by multiple kinases which are likely to be important in regulation of its activity in response to diverse signals. Ongoing studies are aimed at identifying the Sp1-associated kinase(s) and determining the precise physiological roles of its phosphorylation of Sp1.

Sp1 is multiply phosphorylated and migrates as a doublet on SDS-PAGE due to different mobilities of the phosphorylated and unphosphorylated forms (35) (Fig. 1A). Interestingly, serum stimulation did not change the ratio of these forms in either Balb/c 3T3 cells or NHDF (Figs. 1–3 and Ref. 27). Therefore, the enhanced phosphorylation of Sp1 in mid-late G\(_1\) presumably reflects increased phosphorylation of only one or a small subset of the Sp1 phosphorylation sites. In keeping with this, the Sp1-associated kinase phosphorylated a limited region of Sp1 within its C terminus (Fig. 6). Furthermore, in addition to phosphorylation of the N terminus of Sp1, our studies revealed phosphorylation in the C-terminal 167 amino acids of Sp1 in G\(_1\) cells \(\text{in vivo}\). Unfortunately, due to low expression of transfected proteins in serum-starved cells, it was not possible to determine how phosphorylation of this fragment is related to the up-regulation of Sp1 phosphorylation in G\(_1\). Interestingly, while studies in asynchronously growing HeLa cells readily detected phosphorylation of the N-terminal portion of Sp1, they failed to detect significant phosphorylation of the C-terminal domain (35). Although this discrepancy may reflect cell type differences, together with the phosphorylation of the C terminus of Sp1 by a growth-regulated, Sp1-associated kinase activity, it further argues that the C terminus of Sp1 is preferentially phosphorylated in G\(_1\) cells.

The region of Sp1 phosphorylated \(\text{in vitro}\) lies in the N-terminal half of the DNA-binding domain of the protein. This region contains only 3 serines (Fig. 5C); however, due to the relaxed kinase substrate specificity observed \(\text{in vitro}\), it is not possible to say which of these are physiological sites of phosphorylation. Nonetheless, these studies delineate the region of Sp1 which can be phosphorylated by the Sp1-associated kinase activity. The N-terminal zinc finger contributes weakly to the high affinity binding of Sp1 to DNA (52), which may account for the finding that enhanced Sp1 phosphorylation in G\(_1\) does not correlate with significant changes in the specific DNA binding activity of Sp1.

Lack of correlation between DHFR transcription and increased levels or DNA binding of Sp1 indicate that the enhanced Sp1 activity is likely to involve alterations in its association with other proteins. With regard to enhanced phosphorylation of Sp1 in G\(_1\), it is noteworthy that the C terminus of Sp1 has been implicated in its interaction with other transcription factors. This region of Sp1 is involved in synergistic activation with sterol regulatory element-binding protein (53, 54) and mediates its interaction with TAF\(_{\text{p55}}\) (55) and with both YY1 and E2F1 (27, 56), two factors which can mediate serum induction and cell cycle regulation of transcription (2, 57). Indeed, the region of Sp1 that interacts with E2F1 maps precisely to the region phosphorylated by the Sp1-associated kinase activity.\(^4\) Since interaction with YY1 or E2F1 can affect Sp1-dependent transcription in the absence of their direct binding to DNA (27, 56), modulation of these interactions by phosphorylation of Sp1 in mid-late G\(_1\) could mediate changes in Sp1 site-dependent transcription. Sp1 has also been shown to physically or functionally interact with other cell cycle regulatory proteins such as retinoblastoma protein and p107 (58–61). Current efforts are directed at determining how mutation of this C-terminal region of Sp1 affects its interaction with other factors and the growth/cell cycle regulation of Sp1-dependent transcription.

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\(^4\) L-W. Guo, A. R. Black, and J. C. Azizkhan, unpublished data.
