An N-terminal Region of Sp1 Targets Its Proteasome-dependent Degradation in Vitro*

(Received for publication, November 19, 1998, and in revised form, February 25, 1999)

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The transcription factor Sp1 is important for the expression of many cellular genes. Previously, it was shown that reduced O-glycosylation of Sp1 is associated with increased proteasome susceptibility. Sp1 undergoes proteasome-dependent degradation in cells stressed with glucose deprivation and adenylate cyclase activation, and this process is blocked in cells treated with glucosamine. In this study, using a reconstituted in vitro system, we identified the principal structural determinant in Sp1 that targets Sp1 for proteasome-dependent degradation. We found by using deletion analysis that the N-terminal 54 amino acids of Sp1 is required for Sp1 degradation. This element can act as an independent processing signal by directing degradation of an unrelated protein. Recognition of this Sp1 element by the proteasome-dependent system is saturable, and ubiquitination of this element is not required for recognition. Time course experiments revealed that Sp1 degradation is a two-step process. First, a discrete endoproteinolytic cleavage occurs downstream of the target region immediately C-terminal to Leu56. The Sp1 sequence C-terminal to the cleavage site is subsequently degraded, whereas the N-terminal peptide remains intact. The identification of this Sp1 degradation-targeting signal will facilitate the identification of the critical proteins involved in the control of Sp1 proteasome-dependent degradation and the role of O-GlcNAc in this process.

Sp1 is a ubiquitous transcription factor that is particularly important for the regulation of TATA-less genes that encode housekeeping proteins (1–4). Although Sp1 generally has been considered constitutively to regulate gene expression (5), its activity and cellular content have been shown to be regulated during development (6–9), cellular proliferation (10, 11), apoptosis (12), and other cellular processes (13–19). Most of this regulation occurs through either post-translational modifications of Sp1 or by alterations in the abundance of Sp1 protein. The principal known post-translational modifications of Sp1 are phosphorylation and glycosylation through the O-glycosylation of Sp1 function is in the control of Sp1 stability (25). By using a model peptide based on the Sp1 transcriptional activation domain, we previously showed that the hydrophobic interactions into which this peptide entered were blocked by O-GlcNAc modification of the peptide (24). The other potential role of O-GlcNAc in Sp1 function is in the control of Sp1 stability (25). We found that under the experimental conditions of glucose starvation and adenylate cyclase activation, the O-GlcNAc modification of most of the cellular proteins vanished. Sp1 was among the proteins so modified; however, Sp1 was the only transcription factor examined that was degraded by a protease that could be inhibited by specific inhibitors of the 26 S proteasome. Conversely, treatment of cells with glucose or glucosamine resulted in increased protein modification by O-GlcNAc and stabilization of Sp1 to proteolytic degradation. These observations have established a correlation between the glycosylation state of Sp1 and its ability to be proteolytically degraded. Furthermore, these results suggested that the degradation of Sp1 under these circumstances was relatively specifically targeted at Sp1. However, Sp1 is probably not the only protein whose stability is associated with the O-GlcNAc state. It has been shown that an O-glycosylated protein, p67, controls the phosphorylation and activity of elongation factor 2 in protein synthesis (26, 27). Under conditions of serum starvation, p67 is first deglycosylated and then degraded (28). Loss of p67 results in the phosphorylation of elongation factor 2 and the inhibition of protein synthesis. We have proposed that the O-GlcNAc state of Sp1 or perhaps other proteins may be involved in a mechanism that coordinates cellular nutritional status with growth and general protein synthesis.

That the proteasome-dependent degradation of Sp1 appears to be relatively selective is in concordance with evidence that transcription factor abundance or activity can be selectively regulated by proteolytic mechanisms (29–32). This selective protein degradation is usually accomplished by the 26 S proteasome (32, 33). How the proteasome recognizes its target is quite variable. In general, those proteins that are destined for degradation are first modified covalently by the attachment of multiple ubiquitin peptides, which, in turn, are recognized by the proteasome (31, 33–35). Furthermore, proteasome substrate proteins appear to be recognized through a variety of sequence motifs (31). The examples include the proline-glutamate/aspartate-serine-threonine (PEST) sequence in short lived proteins (36–41), the N-end rule for unstable proteins (42–44), the glycine-rich region (GRR) in NF-κB precursor

* This work was supported by a program grant from the Juvenile Diabetes Foundation International. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: O-GlcNAc, O-linked N-acetylglucosamine; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; GRR, glycine-rich region; GST, glutathione-S-transferase; LLeu, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; NC, newborn calf serum; NRR, normal rat kidney; PAGE, polyacrylamide gel electrophoresis; PEST, proline-glutamate/aspartate-serine-threonine; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate.

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p105 (45), the destruction box in mitotic cyclins (46, 47), and other motifs in various labile proteins (48–50). But how Sp1 is targeted to the proteasome-dependent degradation is still unknown.

In this study, we established an in vitro system to analyze Sp1 degradation. This system allowed the identification of the N-terminal 54 amino acids of Sp1 as the major domain that targets Sp1 for proteasome-dependent degradation. We also showed that Sp1 degradation is a two-step process. An endo-proteolytic cleavage occurs downstream of this target region, followed by the degradation of the C-terminal portion. This target domain in Sp1 is both necessary and sufficient for the targeting of this transcription factor for the endocleavage, and this domain does not need to be ubiquitinated for this function. The elucidation of this Sp1 target domain will facilitate studies that connect protein O-glycosylation with the control of Sp1 abundance.

EXPERIMENTAL PROCEDURES

Materials—L-LnL (N-acetyl-L-leucyl-L-leucyl-L-norleucinal) and forskolin were purchased from Sigma. Lactacystin was purchased from Dr. J. Corey (Harvard University, Boston, MA), and clasto-lactacystin β-lactone was purchased from Calbiochem. The above drugs were dissolved in dimethyl sulfoxide (Me2SO) and stored at −20 °C for later use. Glutathione-Sepharose and reduced free glutathione were purchased from Amersham Pharmacia Biotech. Glucosamine, ATP, AMP-PNP, N-ethylmaleimide, hemin, 2,4-dinitrophenol, 2-deoxyglucose, phospho-creatine, creatine phosphokinase, and thymbin were purchased from Sigma. Monoclonal anti-GST antibody was purchased from Sigma. Polyclonal anti-Gal4 DNA binding domain (amino acids 1–147) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antiserum to Sp1 (3517) was raised against the C-terminal portion of Sp1 as described previously (11).

Cell Culture—Normal rat kidney (NRK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum (NCS), nonessential amino acids (Life Technologies, Inc.), 100 mM gentamicin (Sigma), and 50 μg/ml gentamicin (Sigma) at 37 °C in a humidified incubator with 7.0% CO2. For stimulation of the cells, exponentially growing cells were seeded at 2.5 × 106 cells per 100-mm dish at approximately 30% confluence. After overnight incubation, the cell culture medium was changed to glucose-free DMEM containing 10% NCS, and the incubation was continued for an additional 20 h. The cells were then treated with 5 μM glucosamine or 100 μM forskolin as indicated in glucose-free DMEM containing 10% NCS, and the incubation was continued for an additional 24 h. Lactacystin and L-LnL were added to the cells at a concentration of 20 and 50 μM, respectively, 24 h prior to harvest. BSC40 cells were grown in DMEM with 10% NCS, 100 μg/ml penicillin, and 50 μg/ml gentamicin at 37 °C in a humidified incubator with 7.0% CO2 (51). For stimulation of the cells, growing cells were washed by ice-cold phosphate-buffered saline twice and then subjected to enhanced chemiluminescence detection (Amerham Pharmacia Biotech) for 1 h at room temperature, and then subjected to enhanced chemiluminescence detection (Amerham Pharmacia Biotech).

Vaccinia Virus Expression and Purification of Recombinant GST Fusion Proteins—The full-length human Sp1 cDNA (kindly provided by Dr. James Kadonaga) was cloned into the pTM3GST vector, and a recombinant vaccinia virus was generated as described previously (25, 52). The cDNAs encoding various fragments of Sp1 were obtained by restriction enzyme digestion or PCR amplification using high fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA) and cloned into the pTM3GST vector. The resulting plasmids (pTM3GST-Sp1 fragment) were transfected into BSC40 cells by electroporation at 250 V and 500 microfarads. After overnight recovery, the cells were infected with recombinant vaccinia virus VTF7-3 containing the T7 RNA polymerase coding sequence (53). At 24 h after infection, whole cell extract was prepared by freezing and thawing the cells three times in the extraction buffer (20 mM Tris (pH 7.5), 0.5 mM NaCl, 0.5% Nonidet P-40, 1 mM MgCl2, 0.5 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM leupeptin, 1 μM aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). Glutathione-Sepharose beads were incubated with the whole cell extract for 30 min at 4 °C. The glutathione beads were then collected and washed three times in the extraction buffer. The fusion protein bound to the glutathione beads were either eluted with 20 mM reduced free glutathione or cleaved from GST with 4 units of thrombin per mg of fusion protein.

Nuclear Extract Preparation and the Reconstituted Degradation Assay—Nuclear extract was prepared as described (54). Approximately 106 cells were washed with ice-cold phosphate-buffered saline twice and scraped into 6 ml of cold phosphate-buffered saline. The cells were collected by centrifugation for 3 min at 2000 × g at 4 °C. The cells were resuspended in 1 ml of ice-cold buffer A (10 mM HEPES-KOH (pH 7.9 at 4 °C), 3 mM MgCl2, 10 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, and 1 mM PMSF) and allowed to stand on ice for 10 min. The cells were gently vortexed for 10 s and centrifuged for 15 s at 16,000 × g to pellet nuclei. The supernatant was transferred to another tube, and the pellets were resuspended in 80 μl of buffer C (20 mM HEPES (pH 7.9 at 4 °C), 20% glycerol, 1.5 mM MgCl2, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF). The suspension was intermittently homogenized by pipetting over a period of 30 min incubation on ice and then centrifuged for 15 min at 16,000 × g at 4 °C. The resulting supernatant was collected as nuclear extract (containing 50 μg of protein) in the presence or absence of 330 μM L-LnL. The mixing reaction was performed in a volume of 12 μl in 20 mM HEPES (pH 7.9), 250 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 9% glycerol, 1 mM DTT, and 1 mM PMSF and was incubated on ice or room temperature for 2 h or as indicated. The proteins in the reaction mixture were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blot analysis using anti-GST antibody. In the experiment involved clasto-lactacytin β-lactone, nuclear extract was preincubated with β-lactone for 25 min at room temperature prior to the addition of GSTSp1 protein. To test the ATP dependence of the degradation assay, the indicated reagents were added at the following final concentrations: 2 mM ATP, 5 or 20 mM AMP-PNP, 5 mM MgCl2, 5 mM NEM, and 100 μM hemin. The ATP-regenerating system consisted of 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase (55, 56). These reagents were added to the nuclear extract, and the mixture was incubated at 30 °C for 20 min. Purified GSTSp1 protein was added to the preincubated mixture, and the incubation was continued at 30 °C for another 60 min. The reaction mixture was then subjected to Western blot analysis using anti-GST antibody.

Western Blot Analysis—The samples were resolved by SDS-PAGE on 8 or 10% gels, transferred to ECL-Hybrid nitrocellulose membranes (Amersham Pharmacia Biotech), and blotted with a rabbit antibody overnight at 4 °C with the secondary antibody (anti-rabbit or mouse, immunoglobulin, horseradish peroxidase-linked whole antibody from donkeys, Amersham Pharmacia Biotech) for 1 h at room temperature, and then subjected to enhanced chemiluminescence detection (Amerham Pharmacia Biotech).

Mass Spectroscopy—The vaccinia virus-expressed GST-SpV or GST-Sp for cleaved and immobilized on glutathione-Sepharose beads. The beads were mixed with differently treated NRK nuclear extracts for 1 h at room temperature. The beads were then extensively washed and cleaved with thrombin to release the Sp1 fragment from GST; 5 μM guanidine was applied to elute all the components into the solution. The resulting solution was subjected to mass spectroscopy analysis. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer was carried out on a Perspective Biosystems (Framingham, MA) Voyager Elite MALDI-TOF mass spectrometer. Samples were mixed with a saturated solution of a-cyano-4-hydroxycinnamic acid in a water/acetonitrile (50:50) mixture acidified with 0.1% trifluoroacetic acid. A 1-μl aliquot of the sample was spotted onto the gold plate target. Ionization of the sample was accomplished with a nitrogen laser operated at 337 nm. A delayed extraction method was used in the determination of molecular mass. Measurement of ion flight times through the drift region of the mass spectrometer was carried out with a Tektronix (Beaverton, OR) model TDS784A oscilloscope. The instrument was calibrated with external molecular weight standards.

RESULTS

In Vitro Reconstituted Degradation System—To map the domain of Sp1 protein that targets its degradation, we established an in vitro reconstituted system for Sp1 degradation. Our previous studies had shown that when NRK cells were glucosamine-starved and stimulated with forskolin, Sp1 protein was rapidly degraded in intact cells, and the degradation was inhibited by the proteasome inhibitor lactacystin and L-LnL, but not by a cysteine protease inhibitor, E64-D (25). Sp1 protein was not degraded when the cells were glucose-starved alone, or treated with glucosamine, or cultured in normal glucose-containing medium (5 mM). Furthermore, we showed, by using mixing experiments, that intact Sp1 in nuclear extract from
glucosamine-treated cells could be degraded by nuclear extract from cells previously treated with glucose starvation and forskolin. Based on these observations, we established an Sp1 degradation assay using purified GSTSp1 that had been expressed recombinantly in a vaccinia virus-based protein expression system. This GSTSp1 protein was mixed with nuclear extract from differently treated NRK cells. The mixture was incubated on ice for 2 h before protein separation on SDS-PAGE. Western blot analysis using anti-GST monoclonal antibody was performed to detect the degradation of GSTSp1. As shown in Fig. 1A, GSTSp1 was not degraded by nuclear extract from cells that had been glucosamine-treated (Fig. 1A, lane 1), whereas GSTSp1 was degraded by extract from glucose-starved and forskolin-stimulated cells (Fig. 1A, lane 2). A degradation product, termed GSTSpX (Fig. 1A, lane 2), was detected with the GST antibody and is discussed later. The degradation of GSTSp1 was inhibited by the addition to the reaction mixture of the proteasome inhibitor, LLnL, suggesting that this degradation was proteasome-dependent (Fig. 1A, lane 3). As negative controls, vaccinia virus-expressed GST, GST-epidermal growth factor receptor extracellular domain, and GST-SpE (SpE contains domain B, a glutamine-rich activation domain of Sp1 (24)) were not degraded under the same conditions (Fig. 1A, lanes 4–12). Similarly, we also observed that GST-Sp1 was not degraded by nuclear extract from the cells that had been glucose-starved alone or cultured in normal glucose-containing medium (data not shown). The degradation of GSTSp1 in vitro has been correlated with the degradation of Sp1 in vivo.

Given that LLnL is also a calpain inhibitor (57, 58), we tested whether the degradation of GSTSp1 in this reconstituted system had other features characteristic of the 26 S proteasome-dependent degradation (56). As shown in the left panel of Fig. 1B, the addition of the nonhydrolyzable ATP analog, AMP-PNP, to the reaction mixture at 5 and 20 mM concentrations blocked the degradation of GSTSp1 (Fig. 1B, lanes 2–4). Furthermore, we also prepared ATP-depleted nuclear extract. NRK cells were glucose-starved and stimulated with forskolin. At 2 h before cell harvest, 2,4-dinitrophenol and 2-deoxyglucose were added to the culture medium to deplete the cellular ATP. As shown in Fig. 1B, lane 5, GSTSp1 was not degraded by this nuclear extract. However, when 2 mM ATP and MgCl2 were added to the extract, degradation of GSTSp1 was restored (lane 8). The addition of Mg2+ alone or Mg2+ with AMP-PNP did not stimulate the degradation (lanes 7 and 9). Together, these data demonstrate that the degradation of Sp1 in this reconstituted system is ATP-dependent. As shown in lanes 10 and 11, the addition of 5 mM NEM or 100 mM hemin to the reaction inhibited the degradation of GSTSp1, showing that the degradation of GSTSp1 in this system was sensitive to NEM and hemin. The ATP dependence and the sensitivity to hemin and NEM mixture, and the reaction continued to incubate at 30 °C for another 60 min, followed by Western blot analysis with anti-GST antibody. Right panel, the state of the endogenous Sp1 in the NRK nuclear extracts used for the experiments shown in the left panel was determined by Western blot analysis with anti-Sp1 antibody. C, proteasome-specific inhibitors inhibit the degradation of GSTSp1. Left panel, GSTSp1 was mixed with nuclear extract from glucosamine-treated (lane 1), glucose-starved, and forskolin-treated (lane 2), forskolin plus 20 μM lactacystin (lane 3) or 50 μM LLnL-treated (lane 4) cells. The reaction mixture was incubated for 30 min at room temperature, followed by Western blot analysis with anti-GST antibody. Right panel, the nuclear extract (50 μg) from glucose-starved and forskolin-treated cells was preincubated with 1 μM MeSO (lane 2), 100 μM β-lactone (lane 3), or 330 μM β-lactone (lane 4) in a total volume of 11 μl for 25 min at room temperature. GSTSp1 (10 ng) was then added to the preincubation mixture, and the incubation was continued for additional 30 min at room temperature, followed by Western blot analysis with anti-GST antibody.
are all features of the 26 S proteasome and suggest that the in vitro degradation of GSTSp1 is proteasome-dependent. The right panel shows a Western blot analysis using anti-Sp1 antibody to determine the content of endogenous Sp1 in the nuclear extract used in the reconstituted assay. The endogenous Sp1 protein level in the nuclear extract confirms our previous observation that the treatments of the cells resulted in the respective preservation or loss of Sp1 in vivo.

To confirm further the degradation of GSTSp1 in this reconstituted system is proteasome-dependent, we made use of the proteasome-specific inhibitor, lactacystin. Lactacystin has been known to inhibit specifically the 20 S and 26 S proteasome activity without inhibiting any other protease yet tested, including the serine proteases trypsin and chymotrypsin and the cysteine proteases papain, calpain I and II, and cathepsin B (59–62). In aqueous solution, lactacystin undergoes spontaneous hydrolysis to form an intermediate clasto-lactacystin β-lactone that is the sole species to interact with the proteasome and inhibit its activity (63–65). As shown in the left panel of Fig. 1C, GSTSp1 was degraded by the nuclear extract prepared from glucose-starved and forskolin-treated cells (lane 2). However, GSTSp1 was not degraded by the nuclear extract from the cells that had been treated with forskolin plus 20 μM lactacystin or 50 μM LLnL (Fig. 1C, left panel, lanes 3 and 4). Furthermore, we tested if the active lactacystin derivative β-lactone can directly inhibit GSTSp1 degradation in vitro. As shown in the right panel of Fig. 1C, the addition of increasing amounts of β-lactone to the in vitro reaction mixture blocked the degradation of GSTSp1 in a dose-dependent manner (lanes 3 and 4). However, the in vitro treatment of nuclear extract with lactacystin at a similar concentration failed to block significantly GSTSp1 degradation (data not shown). In our reaction system, the transient accumulation of β-lactone could be blocked by the large amounts of glutathione from GSTSp1 preparation (59, 64, 65). Nevertheless, our results strongly suggest that GSTSp1 degradation in the in vitro reconstituted system is proteasome-dependent, and this system can mimic what happens in the living cells.

The N-terminal Region of Sp1 Contains a Target Domain for Its Degradation—The reconstituted degradation system allowed us to map the domain of Sp1 protein that targets its degradation. The domain structure of Sp1 has been well characterized. As shown in Fig. 2A, the glutamine-rich domains in the N-terminal half of the protein confer transactivation. The C-terminal zinc finger domain binds DNA (66). We made a series of N- and C-terminal deletion fragments of Sp1 that are termed SpN (amino acids 81–621 of Sp1), SpBS (amino acids 245–621), SpBC (amino acids 345–621), SpZD (amino acids 621–778), SpBCD (amino acids 245–778), SpV (amino acids 1–81), and SpK (amino acids 1–54), as shown in Fig. 2A. These fragments of Sp1 were expressed as GST fusion protein using the vaccinia virus expression system and were subjected to the reconstituted degradation assay with nuclear extract prepared either from cells treated with glucosamine (inactive extract) or cell treated with glucose deprivation and forskolin (active extract). Sp1 protein, the deletion Sp1 fragments that were used in the following studies, and the amino acid sequence of the N-terminal 81 amino acids of Sp1. B, vaccinia virus-expressed GST fusion proteins containing full-length Sp1 or Sp1 deletion fragments were applied to the in vitro reconstituted degradation system as described in Fig. 1. The reaction mixture was subjected to Western blot analysis with anti-GST antibody. C, the N-terminal 81 amino acids of Sp1 (SpV) contain the target domain for its degradation. GST-Sp1 and GST-SpN were used as controls. The reaction mixture was subjected to Western blot analysis with anti-GST antibody.
tract). LLnL was added to the active extract to block proteasome activity. Fig. 2B, lanes 1, 4, 7, 10, 13, and 16, shows the effect of the inactive extract (labeled GlcN) on the various GST fusion proteins and therefore indicates the amount of the input GST-Sp1 fusion protein. Similarly, the lanes labeled LLnL show the effect of proteasome blockade on the active extract and also indicate the input GST-Sp1 fusion protein level. As shown in Fig. 2B, lanes 1–3, full-length GST-Sp1 was almost completely degraded by active nuclear extract, producing a degradation product, GSTSpX, of about 35 kDa. All the other Sp1 domains (SpN, SpBS, SpBC, Sp2D, and Sp2CD) were degraded by the active extract to a considerably lesser degree than the full-length Sp1, although some degradation fragments were produced (lanes 4–18). These data suggest that there may be multiple structural elements that are responsible for Sp1 degradation, but the major one does not lie in the five domains shown in Fig. 2B. By elimination, the major targeting domain appears to reside in the N-terminal 81 amino acids of Sp1. As shown in Fig. 2A, this N-terminal region of Sp1 contains a segment from amino acid 12 to 46 that is glycine-rich, with 14 glycine residues in this 34-amino acid region. This glycine-rich feature is reminiscent of the glycine-rich region (GRR, it contains 15 glycines from amino acids 372–394 of p105) in NF-κB precursor p105 that serves as a proteasome processing signal for this transcription factor (45). To determine whether this N-terminal region of Sp1 can also serve as a proteasome-dependent processing signal, we made GST fusion proteins that contain only the first 81 amino acids (GSTSpV) or the first 54 amino acids (GSTSpK). As shown in Fig. 2C, GSTSpV was degraded by the forskolin-treated nuclear extract, and from it was generated the same 35-kDa GSTSpX degradation product as from full-length Sp1 (Fig. 2C, lane 8). Because GSTSpK is so close in size to the degradation product, GSTSpX, it was impossible to determine whether GSTSpK was degraded. These data are compatible with the notion that SpV contains a target for Sp1 degradation, perhaps the GRR-like domain.

**The Target Domain of Sp1 Directs the Degradation of a Heterologous Protein**—To determine whether the N-terminal 81 amino acids of Sp1 (SpV) can act as an independent signal for Sp1 processing, we constructed a fusion protein, GST-SpV-Gal4, in which SpV was fused to the C-terminal of GST and the N-terminal of yeast Gal4 DNA-binding domain (amino acids 1–147) (Fig. 3A). We also constructed GST-SpK-Gal4 to determine whether the N-terminal 54 amino acids of Sp1 contain the degradation signal. These fusion proteins were then subjected to the in vitro degradation assay. As shown in Fig. 3B, GSTSpVGal4 was degraded by active extract and produced a similarly sized degradation product, GSTSpX, as GSTSp1 (Fig. 3B, lane 5). GSTSpKGal4 was also degraded and produced the same size degradation product as GSTSp1 (Fig. 3B, lane 8). The two heterologous proteins were expressed as a doublet that may be due to posttranslational modifications of Gal4 (67). As specificity controls, GSTGal4 or GSTSpEGal4 (SpE contains amino acids 424–521 of Sp1) were not degraded under the same condition, although the Gal4 portion of the molecule appears to have been inefficiently cleaved to produce a larger GST fusion product. These data indicate that the first 54 amino acids of Sp1 contain a sequence that can confer processing to a heterologous protein, in this case a segment of Gal4. This N-terminal element of Sp1 therefore appears both necessary as the main target domain for Sp1 degradation and sufficient as an independent processing signal for an unrelated protein.

**Exogenous Target Domain Competitively Blocks Sp1 Degradation**—To determine whether the processing of Sp1 is a saturable process, we loaded the reconstituted degradation assay with excess SpV to determine the effect on holo-Sp1 degradation. As shown in Fig. 4 lanes 3–5, the addition of increasing amounts of GSTSpV inhibited the degradation of GSTSp1. This inhibition was specific in that the addition of large amounts of GST alone did not alter GSTSp1 degradation (lanes 6–8). These data show that exogenous target domain SpV can competitively block the degradation of holo-Sp1. Our results suggest that the factors required for Sp1 degradation are saturable by the target domain.

**Ubiquitination of the Target Domain Is Not Required for Sp1 Processing**—To determine whether ubiquitination is required for recognition of the N-terminal target domain of Sp1, we mutated the only two potential ubiquitination sites in this domain (the first 81 amino acids). The lysine residues at positions 9 and 12 were mutated to alanine residues by site-directed mutagenesis. The resulting mutant, GST-Sp1mKK, was expressed, purified, and subjected to the in vitro degradation assay described previously. As shown in Fig. 5, GSTSp1mKK was degraded (lanes 3–5) as efficiently by the active extract as did wild-type Sp1, and the processing generated the same size product as wild-type GSTSp1. The proteasome inhibitor, LLnL, also inhibited the degradation of this mutant Sp1 (lane 5). This result suggests that ubiquitination of the first 81 amino acids of Sp1 is not required for its recognition by the proteasome-dependent system. Our data do not exclude the possibility that another region of Sp1 is modified by ubiquitination in the process of Sp1 degradation.
antibody, a new band, termed Sp1a, was detected at about the 89-kDa position (Fig. 6B, lanes 2–7). Sp1a is smaller than Sp1 by about 6 kDa, suggesting that the GSTSp1 is cleaved at a position corresponding to about 6 kDa from the N terminus of the Sp1 portion of the fusion protein.

During this time course experiment, Sp1a first accumulated, reaching a maximum level between 5 and 25 min of incubation in the activated extract (Fig. 6B, lanes 2 and 4). The level of Sp1a then decreased at longer incubation times, suggesting that the Sp1a band was first produced and then degraded (Fig. 6B, lanes 5–7). In contrast, the GST-SpX remnant peptide is not degraded. The addition of proteasome inhibitor, LLLnL, inhibited the initial endocleavage step (Fig. 6, A and B, lane 8).

To determine further the role of the first 81 amino acids of Sp1 in this degradation process, we performed the same time course experiment using the heterologous protein substrate, GSTSpVGal4. As shown in Fig. 6C, the GSTSpVGal4 substrate was also endocleaved, generating the N-terminal peptide GSTSpX, which accumulated with increasing incubation time (lanes 2–6). We reblotted the same membrane with anti-Gal4 DNA-binding domain polyclonal antibody to detect the C-terminal Gal4 peptide after the endocleavage. As shown in Fig. 6D, the level of Gal4 peptide also accumulated with increasing incubation time (lanes 2–6), suggesting that the Gal4 peptide was not subsequently degraded like Sp1a. The proteasome inhibitor, LLLnL, still blocked the initial endocleavage step (Fig. 6, C and D, lane 7). These results suggest that Sp1 degradation is a two-step process as follows: an endoproteolytic cleavage followed by the degradation of the C-terminal portion and that the N-terminal 81 amino acids of Sp1 only direct the endocleavage step, and additional signals may be necessary for the further degradation of the C-terminal portion of Sp1.

**Mapping of the Endocleavage Site of Sp1 Degradation**—To confirm the notion that Sp1 is endoproteolytically cleaved and to determine the endocleavage site of Sp1, we used mass spectroscopy to determine the molecular mass of the degradation product SpX. Since the amino acid sequence of Sp1 is known, it was possible to determine the last amino acid in SpX from a precision determination of its molecular mass. Because GSTSpV degradation produces the same size product as GSTSp1, and because the protein expression system yields higher quantities of GSTSpV, GSTSpV was used as the starting substrate for the generation of GSTSpX. The GSTSpX was generated by exposure of GSTSpV to activated extract. The GSTSpX was repurified and cleaved with thrombin at the thrombin cleavage site encoded in the expression vector at the C terminus of GST. The upper panel of Fig. 7A shows a mass spectroscopic analysis of GSTSpV that had been exposed to inactive extract made from glucosamine-stimulated cells. Three peaks were detected. The 9,661.97-Da peak has a mass corresponding to SpV; the 26,126-Da peak corresponds to GST; and the 35,791.8-Da peak corresponds to GSTSpV that failed to be cleaved by thrombin. The lower panel of Fig. 7A shows the analysis of GSTSpV that had been exposed to activated extract. Again, three major peaks were detected, indicating that the GSTSpV was processed to completion, producing the degradation product GSTSpX. The SpX corresponds to the 5,961.92-Da peak. The other peaks represent GST and GSTSpX uncleaved by thrombin.

From the molecular mass determination of SpX, it can be derived that the endocleavage occurred between Leu<sup>66</sup> and Leu<sup>57</sup> of Sp1 (Fig. 7B). Subsequently, the GSTSpX remnant from GSTSp1 was similarly analyzed, and the endocleavage site was mapped to the same leucine residues (data not shown). These results confirm the notion that Sp1 is endoproteolytically cleaved at a site just downstream of the GRR-like sequence.

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**Fig. 4.** Exogenous target domain competitively blocks Sp1 degradation. Increasing amounts of vaccinia virus-expressed GSTSpV or GST were mixed with forskolin-treated nuclear extract (NE) as indicated for 15 min at room temperature. After preincubation, GSTSp1 substrate was added into the reaction for additional 30 min at room temperature. The reaction mixture was subjected to Western blot analysis with anti-GST monoclonal antibody.

**Fig. 5.** Ubiquitination of the N-terminal 81 amino acids of Sp1 is not required for Sp1 processing. GSTSp1mKK, in which the lysine residues at the positions 9 and 12 of Sp1 were mutated to alanine residues, was applied to the reconstituted degradation assay as described in Fig. 1. Wild-type GSTSp1 was used as a control. Western blot analysis with anti-GST antibody was performed. For, forskolin.

The Degradation of Sp1 Is a Two-step Process—To understand better the process of Sp1 degradation, we performed a time course experiment using GSTSp1 as the substrate in the reconstituted system. GSTSp1 protein was exposed to the activated nuclear extract for different incubation times, and the activated extract was subjected to Western blotting with anti-Sp1 DNA-binding domain polyclonal antibody to detect the C-terminal Sp1 remnant and Sp1a in this degradation process, we performed the same time course experiment using the heterologous protein substrate, GSTSpVGal4. As shown in Fig. 6C, the GSTSpVGal4 substrate was also endocleaved, generating the N-terminal peptide GSTSpX, which accumulated with increasing incubation time (lanes 2–6). We reblotted the same membrane with anti-Gal4 DNA-binding domain polyclonal antibody to detect the C-terminal Gal4 peptide after the endocleavage. As shown in Fig. 6D, the level of Gal4 peptide also accumulated with increasing incubation time (lanes 2–6), suggesting that the Gal4 peptide was not subsequently degraded like Sp1a. The proteasome inhibitor, LLLnL, still blocked the initial endocleavage step (Fig. 6, C and D, lane 7). These results suggest that Sp1 degradation is a two-step process as follows: an endoproteolytic cleavage followed by the degradation of the C-terminal portion and that the N-terminal 81 amino acids of Sp1 only direct the endocleavage step, and additional signals may be necessary for the further degradation of the C-terminal portion of Sp1.
DISCUSSION

The transcription factor Sp1 is particularly important in the transcriptional regulation of TATA-less genes, including growth factors, receptors, enzyme involved in DNA synthesis, critical regulators of the cell cycle, and other regulatory proteins (4, 5). Previously, we provided evidence that Sp1 is degraded in vivo by the proteasome system in cells exposed to the stress of glucose deprivation and cAMP accumulation (25). The cAMP signal is often associated with nutrient deprivation in lower eukaryotes, whereas in mammalian cells, cAMP accumulation results in cell cycle arrest at G1 phase (68–70). Thus, the Sp1 degradation under the experimental conditions may reflect the extremes of Sp1 regulation during physiological processes.

In this study, we show that the degradation of recombinant Sp1 can be accomplished in vitro by using nuclear extract from cells treated by the same nutritional stress. This reconstituted system also appears to degrade Sp1 through an ATP-dependent system that has features characteristic of the proteasome. The use of this reconstituted system allowed us to define the principal structural determinant in Sp1 that directs its degradation.

We identified the N-terminal 54 amino acids as the major determinant that directs Sp1 to a two-step processing through a proteasome-dependent mechanism. The endocleavage step appears to be an integral part of proteasome processing. For the degradation of Sp1, this first step is blocked by LLnL, hemin, and NEM and is ATP-dependent, all features of the 26 S proteasome (71, 72). More directly, the endocleavage step is also blocked in nuclear extract from cells treated in vivo with proteasome-specific inhibitor lactacystin and is blocked in vitro by treatment of nuclear extract with the active β-lactone derivative of lactacystin. Blockade of the first endocleavage step results in blockade of the subsequent degradation of the protein, and Sp1 fragments lacking the N-terminal 51 residues containing the target region and endocleavage site are rela-
the N-terminal region of Sp1 appears necessary for efficient proteasome-dependent processing. This N-terminal sequence also appears to be sufficient for the endocleavage, although it is not sufficient for degradation of the C-terminal fragment that is generated by the cleavage. That is, this element, when fused to a heterologous protein, can confer endoproteolytic processing to the fusion protein. However, the fragment of Gal4 liberated by the endocleavage was not subsequently degraded. This result suggests that the segment of protein C-terminal to the target region must have properties that allow its further degradation by the proteasome system. Of note, Sp1 fragments lacking the N-terminal target region could be degraded, albeit much less efficiently than proteins containing that target domain. A PEST sequence has been identified between amino acids 487 and 508 of Sp1 (10). Whether this PEST motif plays a role in the further degradation of the C-terminal portion of Sp1 remains to be elucidated. Furthermore, the endocleavage of Sp1 is a saturable process, in that excessive amounts of the N-terminal Sp1 element result in the blockade of holo-Sp1 processing and degradation. This saturability suggests that the Sp1 N terminus is specifically recognized by the proteasome-dependent system. Finally, mutation of the only two lysine residues in the N-terminal domain of Sp1 did not affect the degradation event, implying that the N-terminal target sequence in Sp1 need not be ubiquitinated. However, we have not entirely ruled out the possibility that the remaining C-terminal portion of Sp1 is ubiquitinated. Although ubiquitination is usually a requirement for proteasome degradation, the degradation of ornithine decarboxylase and c-Jun has been shown to be proteasome-dependent but ubiquitin-independent (73, 74). We have identified this N-terminal region of Sp1 as a target for proteasome-dependent degradation; however, the mechanism by which it targets to the proteasome remains unclear.

In this N-terminal target domain of Sp1, there is a glycine-rich-like region that has several similarities to the glycine-rich region (GRR) in NF-κB p105 that directs the proteasome processing of this NF-κB precursor (45). The GRR in p105 directs a similar two-step processing to this protein as follows: a GRR-dependent endoproteolytic cleavage, followed by the degradation of the C-terminal portion that is the inhibitory subunit of p105. The N-terminal portion liberated by the cleavage remains intact and is the active form of NF-κB subunit p50. However, for p105, the degradation event occurs in the cytosol, and the net result is an activation of this transcription factor. In contrast, the two-step processing of Sp1 appears to occur in the nucleus and results in the disposal of Sp1. Interestingly, the short segment of Sp1 that is at the N-terminal of the GRR does not appear to be degraded. Whether this residual fragment plays a role in the cell is unclear, but there is some prior evidence that this N-terminal region of Sp1 can be bound by cellular factors that have an effect on Sp1 function (75). For both Sp1 and p105, the endoproteolytic cleavage occurs at a position C-terminal to the GRR, and the amino acid residues at the site of cleavage are not important determinants of the cleavage. Rather, it appears that the spacing between the GRR and the cleavage site is the major determinant of the cleavage site. As evidence for this notion, we mapped the exact site of cleavage in Sp1 and found it immediately C-terminal to Leu56, 10 residues downstream of the GRR. We also showed that the Sp1-derived fusion protein GSTSpKGal4, which contains only the first 54 amino acids of Sp1 and, therefore, lacks the leucine residues found at the cleavage site in the native protein, is efficiently cleaved at a similar position C-terminal to the GRR that was observed for GSTSp1. Similarly, for p105, the endoproteolytic cleavage occurs downstream of the GRR at a fixed
position relative to the GRR that does not depend on specific downstream sequences.

Taken together, these results suggest that the N-terminal region of Sp1 is a primary recognition site for proteasome-dependent degradation (Fig. 8). This recognition results first in an endocleavage that generates two fragments. For both Sp1 and p105, the C-terminal fragment is further degraded, leaving the N-terminal fragment intact. The further degradation of the C-terminal fragment by the proteasome system appears to require properties in addition to the GRR. This two-step process provides an additional level of control of protein fate. It allows for the partial degradation of target proteins and the generation of remnants that have altered biological functions.

Previously, we correlated the glycosylation state of Sp1 with resistance to proteasome-dependent degradation (25). This correlation derived from our observation that glucose starvation combined with adenylate cyclase activation resulted in the rapid deglycosylation not only of Sp1 but multiple intracellular proteins, and this deglycosylation preceded the proteasome degradation. Furthermore, glucosamine treatment of the cells resulted in hyperglycosylation of proteins and reduced degradation. Our identification of a target domain in Sp1 that directs proteasome-dependent degradation will facilitate the identification of the critical proteins involved in the control of Sp1 degradation and the role of O-GlcNAc in this process.

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