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Cell Cycle-dependent Regulation of the *Skp2* Promoter by GA-binding Protein[1]

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ABSTRACT

Skp2 is the F-box protein component of an SCF-type ubiquitin ligase that interacts specifically with $p27^{Kip1}$ and thereby promotes its ubiquitylation and degradation. The abundance of Skp2 mRNA oscillates in a cell cycle-dependent manner, being maximal in S and G_2 phases. The regulation of *Skp2* transcription was investigated by cloning the promoter region of the mouse gene and determination of its activity in a luciferase reporter assay. Deletion analysis identified a minimal ~0.3-kb promoter region with marked transcriptional activity and a 105-bp essential sequence within this region. Electrophoretic mobility shift assays indicated the presence in nuclear extracts of proteins that bind to this sequence. Site-directed mutagenesis revealed that the core binding motif, CACTTCCG, which is similar to that of GA-binding protein (GABP), is essential for *Skp2* transcription. "Supershift" analysis indicated that the protein-probe complexes detected by electrophoretic mobility shift assays contain GABP. Endogenous GABP bound to *Skp2* promoter element in a cell cycle-dependent manner. Furthermore, overexpression of GABPβ increased *Skp2* promoter activity, and suppression of GABPα or GABPβ by a small interfering RNA resulted in the reduction of *Skp2* promoter activity. These data suggest that the cell cycle-dependent binding of GABP to the *Skp2* promoter plays an important role in the regulation of *Skp2* expression and cell cycle progression from G_1 to S phase.

INTRODUCTION

The ubiquitin-proteasome pathway of protein degradation plays an important role in control of the abundance of short-lived regulatory proteins (1, 2). Protein ubiquitylation is mediated by several components that act in concert (3, 4). A ubiquitin-activating enzyme (E1) with ATP as a substrate, catalyzes the formation of a thioester bond between itself and ubiquitin, and it then transfers the activated ubiquitin to a ubiquitin-conjugating enzyme (E2). Certain 17β-estradiol enzymes transfer ubiquitin directly to the protein substrate, whereas others require the participation of a third component, termed a ubiquitin ligase (E3), to achieve this effect.

SCF complex, an E3 ligase, consists of the invariable components Skp1, Cul1, and Rbx1 (Roc1/Hrt1) as well as a variable component, known as an F-box protein, that binds to Skp1 through its F-box motif and is responsible for substrate recognition (5–7). Skp2, which contains an F-box domain followed by leucine-rich repeats, was originally identified as a protein that interacts with the cyclin A-CDK2[4] complex (8). More recently, however, Skp2 has been implicated in the ubiquitin-mediated degradation of $p27^{Kip1}$ (9, 10). We have generated Skp2-deficient mice and shown that they exhibit cellular accumulation of $p27^{Kip1}$, a reduction in both body size and the size of individual organs, an increase in the mass of individual cells, polyploidy, and multiple centrosomes per cell (11). These observations suggest that SCF^{Skp2} is the principal ubiquitin ligase responsible for determination of the abundance of cell cycle regulatory proteins during progression of cells from G_1 to S phase. The expression of Skp2 has been shown recently to correlate inversely with that of $p27^{Kip1}$ and to define cells in S phase in human sarcomas and other cancers (12–17). Mice transgenic for Skp2 in the T-lymphocyte lineage also exhibit an increased susceptibility to the development of lymphoma (14). Thus, the deregulation of Skp2 expression may lead to carcinogenesis.

Although the abundance of the SCF^{Skp2} components Skp1, Cul1, and Rbx1 does not change substantially during the cell cycle, the amount of Skp2 varies in a cell cycle-dependent manner. Skp2 is first detectable at the transition between G_1 and S phases, accumulates during S and G_2 phases, and then decreases in abundance as cells proceed through M phase. Both transcriptional and post-translational regulation are thought to underlie these changes. Thus, the abundance of Skp2 mRNA is periodic in HeLa cells (8) and NIH 3T3 cells (this study), whereas the concentration of Skp2 is regulated mainly by ubiquitylation-mediated protein degradation (as well as by transcriptional control) in other cell types (18).

To explore the molecular basis for the cell cycle-dependent oscillation of Skp2 mRNA, we have now characterized the promoter region of *Skp2*. We have identified a minimal promoter region and a transcription factor, GABP (19–21), which binds to its core sequence. Our results suggest that the cell cycle-dependent binding of GABP regulates *Skp2* expression and cell cycle progression from G_1 to S phase.

MATERIALS AND METHODS

Plasmids. Mouse *Skp2*-pGL hybrids for luciferase assays were constructed from pGL2-basic (Promega). To generate pGL2–2275, we excised the DNA fragment corresponding to nts −2275 to +94 (numbered relative to the transcription initiation site) of the 5′ flanking sequence of *Skp2* from pBlue-2.4k (11) with the use of *Apa*I. The fragment was purified by electrophoresis, rendered blunt-ended, and cloned into the *Sma*I site of pGL2-basic. The plasmid pGL2–318 was constructed from pGL2–2275 by removing the fragment comprising nt −2275 to m319 of *Skp2* with the use of *Hind*III. For the construction of pGL2 −207, pGL2 −102, and pGL2 + 6, the DNA fragments comprising nts −207 to +94, −102 to +94, and +6 to +94, respectively, of *Skp2* were amplified by PCR with pBlue-2.4k as the template, sense primers that correspond to nts −207 to −184 (5′-AGTCAATCGGCTGACATTTC-CCAG-3′), −102 to −79 (5′-GAGTTGTGGGTATCTGGAGGGTTG-3′), and +6 to +29 (5′-AAGGCGGCTGCTGGAGAGCGGGAT-3′), and an antisense primer corresponding to nts +71 to +94 (5′-GGGCCCCAGCAGTT-GGACCCGCGA). A *Xho*I linker was incorporated at the 5′ end of the sense primers and a *Hind*III linker at the 3′ end of the antisense primer. The PCR products were digested with *Xho*I and *Hind*III, and then subcloned into the *Xho*I-*Hind*III site of pGL2-basic.

To generate pBlue −207/−103, we amplified the DNA fragment comprising nts −207 to −103 of *Skp2* by PCR with pBlue-2.4k as the template, a sense primer corresponding to nts −207 to −188 (5′-AGTCAATCGGCTGA-

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[4] The abbreviations used are: CDK, cyclin-dependent kinase; GABP, GA-binding protein; nt, nucleotide; EMSA, electrophoretic mobility shift assay; GFP, green fluorescence protein; ChIP, chromatin immunoprecipitation; MAPK, mitogen-activated protein kinase.

CATTTC-3′), and an antisense primer corresponding to nts −122 to −103 (5′-CCTGCGGCGCCGGGCGGGAT-3′). An *Eco*RI linker was incorporated at the 5′ end of the sense primer and a *Not*I linker at the 3′ end of the antisense primer. The PCR product was digested with *Eco*RI and *Not*I, and then subcloned into the corresponding sites of pBluescriptII SK+ (Stratagene).

The mutated constructs pGL2 −207M1, pGL2 −207M2, and pGL2 −207M3 were generated with the use of a Quickchange mutagenesis kit (Stratagene) and the primers M1QC+ (5′-GAGCTCCACCCTCATCCGCCTGTCC-3′), M1QC− (5′-GGACAGGCGGATGAGGGTGGAGCTC-3′), M2QC+ (5′-GAGCTCCACCCATAACCGCCTGTCCTC-3′), M2QC− (5′-GAGGACAGGCGGTTATGGGTGGAGCTC-3′), M3QC+ (5′-CCACCCACTTCAGCCTGCCTGTCC-3′), and M3QC− (5′-GGACAGGCAGGCTGAAGTGGGTGG-3′). The sequences of the mutated constructs were verified by DNA sequence analysis (Amersham Pharmacia Biotech).

The expression vectors pCAGGS-GABPα and pCAGGS-GABPβ were generated as described previously (22).

Northern Blot Analysis. The Northern blot analysis was performed as described previously (23).

Cell Culture and Cell Cycle Analysis. Mouse NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum (Life Technologies, Inc.). Human HeLa and T98G cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The growth of NIH 3T3 and T98G cells was arrested by incubation for ~72 h in DMEM without serum; the cells were subsequently stimulated by the addition of calf serum or fetal bovine serum, respectively, to a final concentration of 10%.

For cell cycle analysis, single-cell suspensions of NIH 3T3 or T98G cells were fixed in 70% ethanol overnight at −20°C and then stained with propidium iodide (5 μg/ml). The cells were then analyzed with a FACSCalibur flow cytometer and with Cell Quest and Modifit LT software (Becton Dickinson).

Luciferase Reporter Assays. HeLa cells or NIH 3T3 cells(1×10^5/well of a six-well plate) were transiently transfected with 2 μg of luciferase construct and 0.1 μg of pRL-Tk or pRL-CMV plasmid DNA (Promega) with the use of the FUGENE6 transfection reagent (Roche). Transfection was performed with at least three different batches of each reporter plasmid. Where indicated, cells were transfected with pCAGGS-GABPα or pCAGGS-GABPβ in addition to 1 μg of pGL2 −207 and 0.1 μg of pRL-Tk. After incubation for 48 h, the cells were harvested with Passive Lysis Buffer (Promega), and luciferase activities of cell extracts were measured with the use of the Dual luciferase assay system (Promega). Luciferase activity was expressed as the ratio of LucF activity: LucR activity.

Preparation of Nuclear Extracts, *in Vitro* Translation, and EMSA Analysis. Nuclear extracts of HeLa and T98G cells were prepared as described previously (24), and portions were stored at −80°C. *In vitro* translated proteins were prepared by using 1 μg of pET3d plasmids containing each cDNA for GABPα, GABPβ, or GABPγ in the TNT T7-coupled reticulocyte lysate system (Promega). EMSA analysis was performed as described previously (25). For "supershift" analysis, 1 μg of antibodies to GABPα, to GABPβ/γ (20), or to Elk-1 (Santa Cruz Biotechnology) was added to the reaction mixture. For generation of the −207/−103, −207/−177, and −177/−103 probes, the corresponding DNA fragments were excised from pBlue −207/−103 by double digestion with *Eco*RI and *Not*I, with *Eco*RI and *Sac*I, or with *Sac*I and *Not*I, respectively, and were purified by electrophoresis on a 1.5% agarose gel. The oligonucleotides −175/−153+ (5′-GGAGCTCCACCCACTTCCGCCTG-3′), −175/−153− (5′-GGCAGGCGGAAGTGGGTGGAGCT-3′), −152/−126+ (5′-GGTGTCCTCCTCCTCCTCCTCCTCCT-3′), −152/−126− (5′-GGAGGAGGAGGAGGAGGAGGAGG AGGACA-3′), −125/−103+ (5′-GGTCAATCCCGCCCGGCGCCGCAGG-3′), −125/−103− (5′-GGCCTGCGGCGCCGGGCGGGATTGA-3′), NF-Y+ (5′-GGAGACCGTACGTGATTGGTTAATCTCTT-3′), and NF-Y− (5′-GGAAGAGATTAACCAATCACGTACGGTCT-3′), were chemically synthesized; the corresponding (+) and (−) oligonucleotides were annealed and labeled by filling in of the guanine-containing 5′ overhangs with the use of the Klenow fragment (New England Biolabs) in the presence of [α-^{32}P]dCTP (Amersham Biosciences).

Small interfering RNA (siRNA) Transfections. RNA interference was performed as described previously (26). GABPα was targeted with the sequence AAAGCAGAGTGCACAGAAGAA, GABPβ was targeted with the sequence AAATGGAGCTCCCTTTACTAC, and the GFP was targeted with the sequence AAGCTGACCCTGAAGTTCATC. Searches of the human genome database (BLAST) were carried out to ensure the sequences would not target other gene transcripts.

ChIPs. The ChIP was performed with ChIP assay kit (Upstate), and the primers 5′-hSKP2ChIP (5′-AAAATCCGTCTACAGTCCAG-3′) and 3′-hSKP2ChIP (5′-CCTTCGAGATACCCACAACC-3′). The 127-bp PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

RESULTS

Identification of a Positive Regulatory Element in the 5′ Flanking Region of *Skp2*. We first confirmed the cell-cycle dependence of Skp2 mRNA abundance by Northern blot analysis of synchronized cell populations (Fig. 1*A*). NIH 3T3 cells were synchronized in G_0 phase by serum deprivation and then released from growth arrest by reexposure to serum. Cell synchronicity was monitored by flow cytometry (Fig. 1*B*) in parallel with Northern analysis. The abundance of Skp2 mRNA was low in G_0 phase, increased as cells entered mid-G_1 phase, and peaked during S phase. Similar results were obtained with HeLa cells and T98G cells, although, consistent with previous observations (18), the basal amount of Skp2 mRNA in G_0 phase was greater in T98G cells than in the other cell types (data not shown).

We sequenced the 5′ flanking region of mouse *Skp2* (Fig. 2). To identify the transcriptional initiation site of the gene, we performed 5′-RACE (data not shown). Although a TATA box-like sequence is not apparent in the transcriptional initiation region, three GC boxes (Sp1 binding sites) are present upstream of the start site. In addition, potential binding sites for GABP (also known as E4TF1 or NRF-2; Refs. 20, 27, 28), Taf-1 (29), and PacC (30) are located at nts −166 to −159, −10 to −1, and −188 to −172, respectively.

To identify sequences responsible for the transcriptional regulation of *Skp2*, we transiently transfected HeLa cells with various luciferase reporter constructs containing progressively truncated portions of the

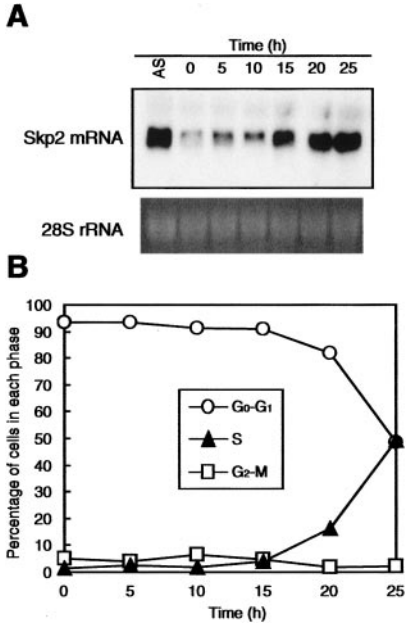

Fig. 1. Cell cycle dependence of the abundance of Skp2 mRNA in NIH 3T3 cells. *A*, total RNA was isolated either from cells in asynchronous culture (*AS*) or from cells that had been synchronized by serum deprivation and subsequently released from growth arrest by reexposure to serum for the indicated times. RNA samples (40 μg) were subjected to Northern analysis with an [α-^{32}P]dCTP-labeled mouse Skp2 cDNA probe (*top panel*). Equal application of samples was verified by staining of 28S rRNA with ethidium bromide (*bottom panel*). *B*, NIH 3T3 cells synchronized as in *A* were stained with propidium iodide and analyzed by flow cytometry. The percentages of cells in G_0-G_1, S, and G_2-M phases of the cell cycle are shown.

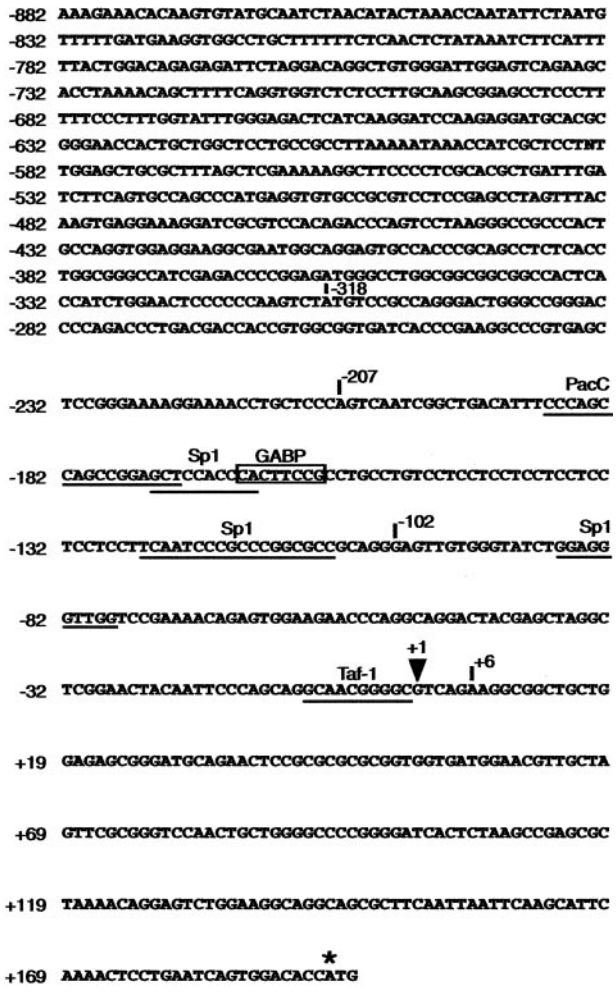

Fig. 2. nt sequence of the 5′ flanking region of mouse *Skp2*. The translational initiation codon (*ATG*) is indicated with an ∗, and the transcriptional start site (designated +1) is indicated with an *arrowhead*. Predicted binding sites for Taf-1, Sp1, and PacC are *underlined*, and a predicted binding motif for GABP is *boxed*. The 5′ ends of deletion constructs studied in Fig. 3 are indicated *above* the sequence.

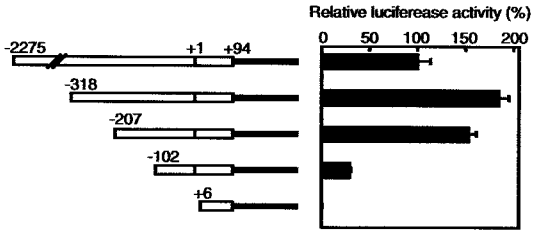

Fig. 3. Analysis of promoter activity of the 5′ flanking region of mouse *Skp2*. HeLa cells were cotransfected with 2 μg of a luciferase reporter plasmid containing one of the indicated fragments of the *Skp2* promoter (*left panel*) and with 0.1 μg of pRL-Tk. Firefly luciferase activity was normalized by that of *Renilla* luciferase and then expressed relative to the normalized activity observed with pGL2–2275 (*right panel*). Data are means of values from three independent experiments; *bars*, ±SE.

5′ flanking region of *Skp2* and then measured the luciferase activity of cell extracts (Fig. 3). Cells transfected with pGL2–2275, containing nts −2275 to +94 of *Skp2*, exhibited a high level of luciferase activity. Deletion of the 5′ flanking sequence from nts −2275 to −208 did not result in a reduction in luciferase activity, whereas deletion of this region to nt −103 resulted in an ~80% decrease in activity. These results suggest that a *cis* element located between nts −207 and −103 is required for transcription of *Skp2*.

Specific Binding of Nuclear Proteins to the *Skp2* Core Promoter Region. To characterize the proteins that interact with the *Skp2* regulatory element, we performed EMSA analysis with a probe comprising nts −207 to −103 of the *Skp2* promoter. We detected two protein-probe complexes (bands I and II) with nuclear extracts of HeLa cells (Fig. 4*A*), NIH 3T3 cells, or T98G cells (data not shown). Shorter exposure of the gels revealed that band I actually comprises two distinct bands of similar mobility (data not shown). Specificity of complex formation was suggested by the observation that a 200-fold molar excess of the corresponding unlabeled oligonucleotide markedly reduced the intensity of bands I and II (Fig. 4*A*). Furthermore, whereas an unlabeled oligonucleotide comprising nts −177 to −103 of *Skp2* also greatly reduced the intensity of bands I and II, one corresponding to nts −207 to −177 did not. We next synthesized unlabeled oligonucleotides corresponding to nts −175 to −153, −152 to −126, or −125 to −103. Whereas the −175/−153 oligonucleotide prevented the formation of bands I and II, the −152/−126 and −125/−103 oligonucleotides had no such effect (Fig. 4*B*). These results thus suggest that a DNA binding protein (or proteins) specifically interacts with the upstream region of *Skp2* comprising nts −175 to −153, which contains binding sites for Sp1 (−174 to −165) and GABP (−164 to −157).

We introduced clustered mutations (M1, M2, and M3) into the GABP consensus motif of oligonucleotide −175/−153 and plasmid pGL2 −207 (Fig. 5*A*). The unlabeled mutant oligonucleotides did not affect the formation of bands I and II by the ^{32}P-labeled −175/−153 probe (Fig. 5*B*). Furthermore, the luciferase activity of cells transfected with pGL2 −207 containing the mutations was reduced by 45–75% compared with that of cells transfected with wild-type pGL2 −207 (Fig. 5*C*). Then we measured the activity of the wild-type and mutant promoters in arrested *versus* growing cells (Fig. 5*D*). The response of luciferase activity to serum stimulation was partially

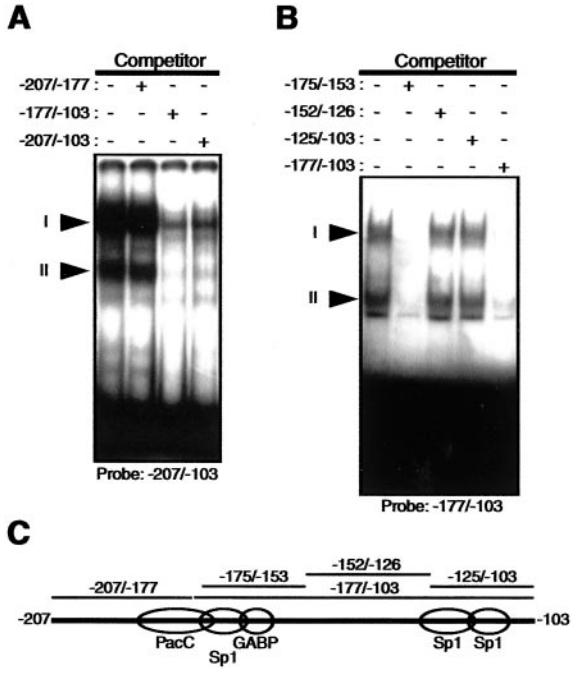

Fig. 4. Specific binding of nuclear proteins to the *cis* element of the *Skp2* promoter. *A* and *B*, EMSA analysis was performed with [α-^{32}P]dCTP-labeled −207/−103 (*A*) or −177/−103 (*B*) *Skp2* probes and a nuclear extract of HeLa cells. The effects of a 200-fold molar excess of the indicated unlabeled oligonucleotides on protein-probe complex formation were determined. *Arrowheads* indicate specific DNA-protein complexes (bands I and II). *C*, schematic representation of oligonucleotides used as probes or competitors in *A* and *B*, and of the binding motifs for transcription factors in the corresponding region of the *Skp2* promoter.

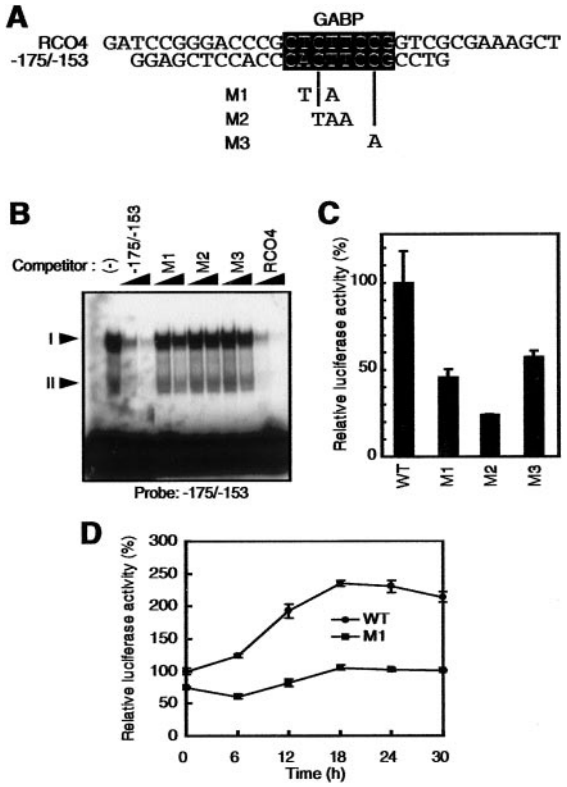

Fig. 5. Functional role of the GABP binding motif in the *Skp2* promoter. *A*, a series of mutations (*M1* to *M3*) was introduced into the putative GABP binding site (■) of the *Skp2* oligonucleotide −175/−153. The RCO4 oligonucleotide contains the GABP binding site of the rat cytochrome *c* oxidase subunit IV gene promoter (+9/+41). *B*, EMSA analysis was performed with the ^{32}P-labeled −175/−153 *Skp2* probe and nuclear extract of HeLa cells. The effects of 20- or 200-fold molar excesses of the indicated unlabeled oligonucleotides on protein-probe complex formation were determined. *C*, the M1, M2, and M3 mutations were also introduced into the putative GABP binding motif of the *Skp2* promoter construct pGL2 −207. HeLa cells were cotransfected both with wild-type (*WT*) pGL2 −207 or the mutant constructs and with pRL-Tk, and the luciferase activities of cell extracts were determined. *D*, NIH3T3 cells were transiently transfected with pGL2–207 (*WT*, ●) or mutant pGL2–207 (*M1*, ■). Cells were synchronized by serum deprivation and subsequently released from growth arrest by reexposure to serum, and harvested at the indicated times for luciferase assays. Results are expressed as the ratio of luciferase activity in each extract relative to that in the extract from WT transfected cells at time 0. Data are means of values from three independent experiments; *bars*, ±SE.

repressed in the mutant construct. Thus, the GABP consensus motif in the *Skp2* promoter appears to be required for optimal expression of *Skp2* promoter-reporter constructs.

Given that the nt sequence of the GABP element in *Skp2* is similar to that of the binding site for GABP (nts +22 to +29) in the promoter of the cytochrome oxidase subunit IV (RCO4) gene (Fig. 5*A*; Refs. 31, 32), we tested the effect of an unlabeled oligonucleotide containing this sequence in the EMSA with the −175/−153 *Skp2* probe. The RCO4 oligonucleotide markedly inhibited the formation of bands I and II (Fig. 5*B*). Thus, these data indicate that the DNA binding protein (or proteins) that recognizes the *cis* element of *Skp2* shares DNA sequence specificity with GABP.

Binding of GABP to the *Skp2* Core Promoter Region. GABP is a heterodimer of GABPα and either GABPβ or GABPγ subunits (19, 20). We examined the effects of antibodies specific for GABPα, or for GABPβ or GABPγ (GABPβ/γ) on complex formation by the −175/−153 *Skp2* probe in EMSA analysis. Antibodies to GABPα markedly reduced the intensity of bands I and II and generated "supershifted" complexes (Fig. 6*A*). Antibodies to GABPβ/γ also induced a supershift, although the effect was less pronounced than was that of the antibodies to GABPα, possibly as a result of a lower affinity of the antibodies or of an allosteric effect. Antibodies to Elk-1 (33), used as a control, did not influence complex formation, suggesting that the effects of the antibodies to GABP were specific, and that bands I and II contain GABP. Furthermore, we performed ChIP in HeLa cells using antibodies to GABPα or Elk-1, and detected association between GABPα and *Skp2* promoter (Fig. 6*B*).

We next examined the abilities of *in vitro*-translated GABPα, GABPβ, and GABPγ to bind to the *Skp2* core promoter region (Fig. 6*C*). Whereas GABPα alone formed a complex (band c) with the −175/−153 probe, neither GABPβ nor GABPγ alone bound the oligonucleotide. In contrast, a mixture of GABPα and either GABPβ or GABPγ yielded prominent complexes (bands a and b, respectively) of lower mobility than band c. The mobilities of bands a and b, and that of band c were identical or highly similar to those of bands I and II, respectively, formed by HeLa nuclear proteins. These data indicate that bands II and I formed by HeLa cell nuclear extract contain monomeric GABPα and a GABPα-GABPβ or GABPα-GABPγ heterodimer, respectively.

Cell Cycle-dependent Induction of GABP Binding to the *Skp2* Promoter. We next examined the effect of cell cycle progression on the binding of GABP to the core motif of the *Skp2* promoter. Thus, we performed EMSA analysis with the −175/−153 *Skp2* probe and with nuclear extracts prepared from T98G cells that were either synchronized at G_0 by serum deprivation or released from growth arrest by

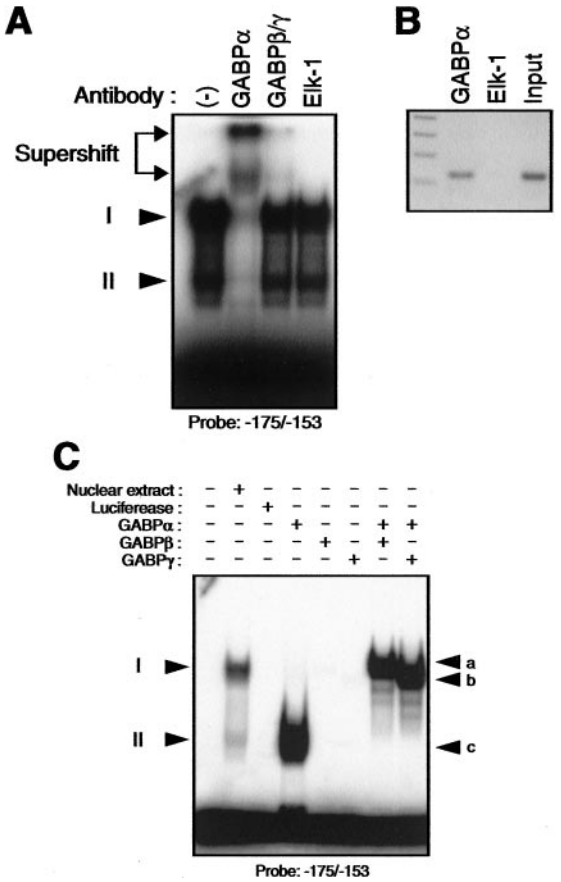

Fig. 6. Binding of GABP to the *Skp2* promoter. *A*, EMSA analysis was performed with the ^{32}P-labeled −175/−153 *Skp2* probe and HeLa cell nuclear extract in the absence or presence of antibodies to GABPα, GABPβ/γ, or Elk-1 (control), as indicated. The positions of the supershifted bands are indicated. *B*, ChIP from HeLa cells was performed with antibodies to GABPα or Elk-1 (control). Input corresponds to PCR reactions containing 0.5% of total amount of chromatin used in immunoprecipitation reactions. *C*, EMSA analysis was performed with the ^{32}P-labeled −175/−153 *Skp2* probe and either HeLa cell nuclear extract or the indicated combinations of *in vitro*-translated GABPα, GABPβ, GABPγ, and luciferase (control). The predominant bands formed by GABPα-GABPβ, GABPα-GABPγ, and GABPα alone are designated *a*, *b*, and *c*, respectively.

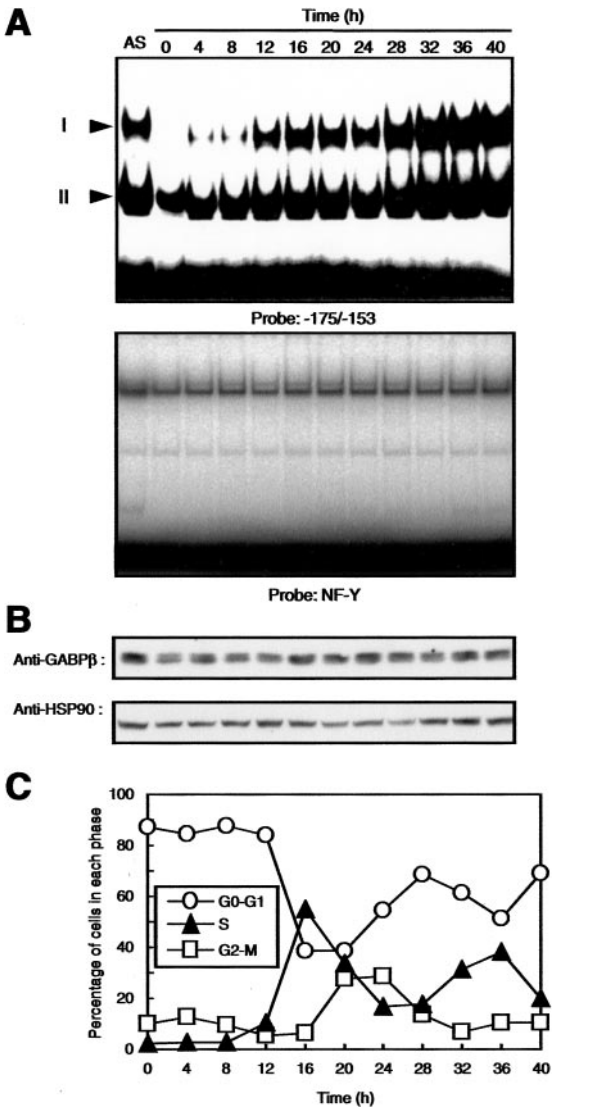

Fig. 7. Cell cycle-dependent binding of GABP to the *Skp2* promoter. *A*, EMSA analysis was performed with the ^{32}P-labeled −175/−153 *Skp2* probe (*top panel*) or NF-Y probe (*bottom panel*) and nuclear extracts of T98G cells prepared at the indicated times after release from serum deprivation. *B*, nuclear extracts prepared in *A* were subjected to immunoblotting analysis with anti-GABPβ and anti-HSP90 (control). *C*, T98G cells synchronized as in *A* were stained with propidium iodide and analyzed by flow cytometry.

subsequent reexposure for various times to serum (Fig. 7*A*). Serum addition resulted in a marked increase in the intensity of band I and a smaller (~3-fold) increase in that of band II during the subsequent 40 h, whereas NF-Y binding did not virtually vary in the cell cycle. The abundance of GABPβ did not change, suggesting that its DNA binding is regulated (Fig. 7*B*). Cell synchronicity was monitored in parallel by flow cytometry (Fig. 7*C*). Binding of GABP to the *Skp2* promoter continued in the cycling cells. These data suggested that the binding of the GABP heterodimer to the *Skp2* promoter is induced during cell cycle progression after serum deprivation and may underlie the transactivation of *Skp2* apparent at this time.

Effects of Overexpression of GABPα and GABPβ on *Skp2* Promoter Activity. Given that the core motif of the *Skp2* promoter is recognized by GABP, we examined the effects of overexpression of GABPα and GABPβ on *Skp2* promoter activity. Transfection of HeLa cells with an expression vector for GABPα (0.01–0.4 μg) resulted in a dose-dependent inhibition of *Skp2* promoter activity (Fig. 8*A*). In contrast, transfection of HeLa cells with various amounts of an expression vector for GABPβ resulted in either slight inhibition of (0.01 or 0.1 μg) or a 2.6-fold increase in (0.4 μg) *Skp2* promoter activity. Cotransfection of cells with the vectors for GABPα and GABPβ resulted in a marked inhibition of promoter activity. These data suggest that overexpression of a GABP subunit may result in the sequestration of a factor (or factors) required for transactivation of *Skp2*. They are also consistent with the possibility that both GABPα and GABPβ subunits contribute to the transcriptional activation of *Skp2*, as well as with the notion that the amount of GABPβ is more limiting than that of GABPα in HeLa cells. Similar "squelching" effects of overexpression of Ets protein have also been observed with the human thrombopoietin gene promoter and insulin-mediated prolactin gene expression (34, 35). We also examined the effect of transient transfection of HeLa cells with the expression vectors for GABPα or GABPβ on the abundance of Skp2 mRNA by Northern blot analysis. Overexpression of GABPβ resulted in a dose-dependent increase in the abundance of Skp2 mRNA (Fig. 8*B*), whereas that of GABPα had no such effect (data not shown).

Reduced *Skp2* Promoter Activity Induced by siRNA Targeting of GABP. We next investigated the functions of endogenous GABP with the use of RNA interference in HeLa cells. Transfection of HeLa cells with siRNA targeting either GABPα or GABPβ partially reduced the abundance of GABPα or GABPβ mRNA, respectively (Fig. 9*A*). We examined the effect of GABP depletion on the Skp2 transcription level by Northern blot analysis. In cells depleted of either GABPα or GABPβ by specific siRNA, Skp2 mRNA was reduced

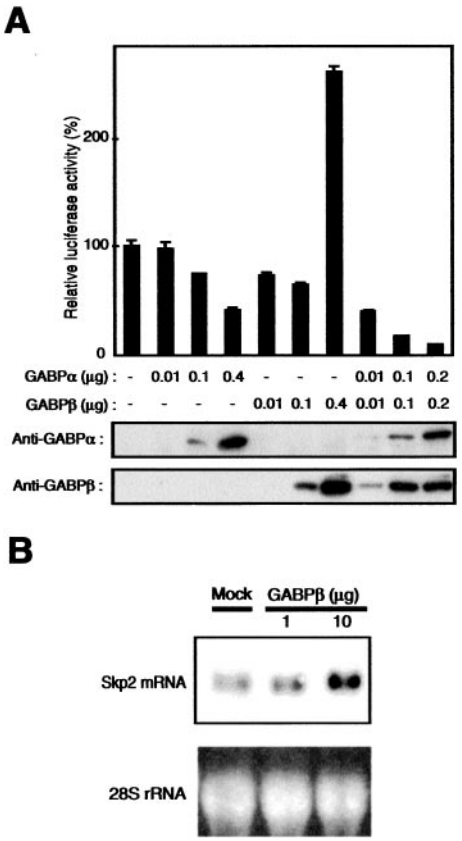

Fig. 8. Effects of GABPα and GABPβ overexpression on *Skp2* promoter activity. *A*, HeLa cells were transfected with 1 μg of pGL2 −207, 0.1 μg of pRL-tk, and the indicated amounts of pCAGGS-GABPα and pCAGGS-GABPβ. The amount of transfected DNA was adjusted to 2 μg by the addition of empty pcDNA3 vector. The luciferase activities of cell extracts were then determined. Data are means of values from three independent experiments (*top panel*); *bars*, ±SE. Expression levels of GABPα and GABPβ were monitored by immunoblotting (*bottom two panels*). *B*, HeLa cells were transfected with 0, 1, or 10 μg of pCAGGS-GABPβ, with the amount of transfected DNA being adjusted to 10 μg by the addition of pcDNA3 (*mock*). After 48 h, total RNA was isolated and subjected to Northern blot analysis with a Skp2 cDNA probe.

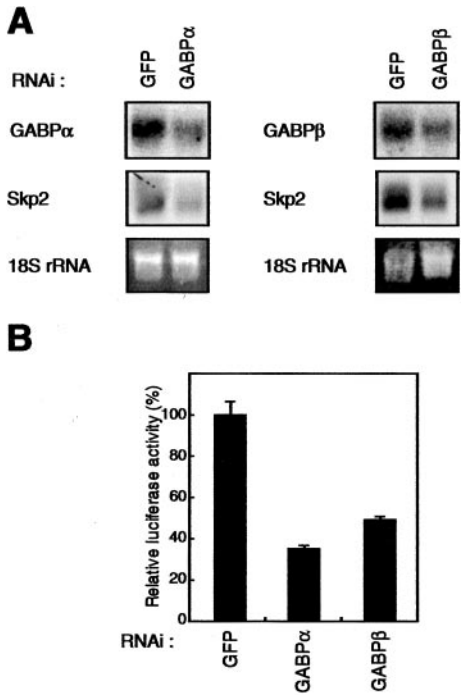

Fig. 9. Effect of GABP knockdown on Skp2 promoter activity. *A,* HeLa cells were transfected with 200 nM each duplex RNAs. Total RNA was isolated and subjected to Northern blot analysis with human GABPα, GABPβ, and Skp2 cDNA probe. Equal application of samples was verified by staining of 18S rRNA with ethidium bromide (*bottom panel*). *B,* HeLa cells were transfected with each duplex RNAs, and transfected with 2 μg of pGL2–207 and 0.1 μg of pRL-Tk 36 h before harvest. The luciferase activities of cell extracts were then determined. Data are means of values from three independent experiments; *bars,* $\pm$SE.

significantly compared with that in cells transfected with control siRNA (Fig. 9*A*).

We also examined the effect of GABPα or GABPβ knockdown on Skp2 promoter activity by luciferase reporter assay. Transfection of HeLa cells with siRNA targeting either GABPα or GABPβ resulted in inhibition of Skp2 promoter activity (Fig. 9*B*). The percentage in G_0/G_1 population slightly but significantly increased in cells transfected with GABPα- (58.3 $\pm$ 0.4) or GABPβ-siRNA (54.7 $\pm$ 0.9) compared with that with GFP-siRNA (52.6 $\pm$ 0.6). Collectively, these data suggest that GABP plays a pivotal role in the regulation of the Skp2 promoter.

DISCUSSION

Skp2 is the F-box protein component of an SCF-type ubiquitin ligase complex. The substrates of SCF^{Skp2} are thought to include $p27^{Kip1}$ (9, 11, 36), free cyclin E (11), E2F-1 (37), ORC1 (38), CDK9 (39), B-Myb (40), and p130 (41). Although $p27^{Kip1}$, free cyclin E, CDK9, and p130 all accumulate in the cells of Skp2-deficient mice (11, 39), the principal target of Skp2 may be $p27^{Kip1}$, given that most of the abnormalities of Skp2-deficient animals are abolished in a $p27^{Kip1}$-null background.[5] In normal lymphocytes, $p27^{Kip1}$ had almost completely disappeared 12 h (mid-G_1 phase) after mitogenic stimulation, whereas the expression of Skp2 was first evident at 18–24 h (S phase) after stimulation and was maximal at 30–36 h (G_2 phase; Ref. 42). These data suggest that the early decrease in the abundance of $p27^{Kip1}$ is mediated by a Skp2-independent mechanism. Consistent with this notion, the degradation of $p27^{Kip1}$ occurs normally at mid-G_1 phase but is impaired during S and G_2 phases in the cells of Skp2-deficient mice (42). Furthermore, injection of antibodies to Skp2 inhibits the progression of cells into S phase (8), whereas overexpression of Skp2 promotes such progression (43). Thus, the main role of Skp2 appears to be to promote cell cycle progression from G_1 to S phase by mediating the ubiquitylation-dependent proteolysis of $p27^{Kip1}$. Thus, the regulation of Skp2 expression may be an important determinant of progression through the G_1-S transition.

Skp2 was originally identified as a 45-kDa protein associated with the cyclin A-CDK2 complex, and the abundance of its mRNA was shown to increase as HeLa cells enter S phase (8). We observed a similar temporal profile of Skp2 mRNA abundance in NIH 3T3 cells in the present study. However, the amount of Skp2 mRNA in T98G cells was shown previously to increase by a factor of only ~2 to 3 on progression from G_0 to S phase, whereas the concentration of the encoded protein increased to a markedly greater extent (18). These observations suggest that the abundance of Skp2 is regulated by two independent mechanisms mediated at the transcriptional and post-translational levels, and that the predominant mechanism varies with cell type.

Extracellular mitogenic stimuli induce the exit of quiescent cells from G_0 phase into G_1 phase and subsequent progression to S phase. However, the molecular links between signaling pathways triggered by mitogenic stimulation and the cell cycle machinery during cycle progression have not been clear. Our data now demonstrate that transcriptional regulation of *Skp2* by GABP might constitute an important target of mitogenic signal transduction. The regulation of cyclin D appears to occur at the G_0-G_1 transition, whereas that of Skp2 is implicated in the G_1-S transition.

On re-entry of cells into the cell cycle and throughout G_1 phase, mitogenic signals are integrated through the GTPase Ras. Activation of the Ras-Raf-MAPK signaling pathway has been shown to increase GABP-dependent promoter-enhancer activity (44, 45). This conclusion is also supported by the phosphorylation of GABP both by extracellular signal-regulated kinase 2 *in vitro* as well as in response to c-Raf activation or to stimulation of cells with 12-*O*-tetradecanoylphorbol 13-acetate and serum *in vivo* (44). UV radiation and methylmethane sulfonate, both of which induce activation of the MAPKs c-Jun NH_2-terminal kinase and p38 (46), also increase GABP-mediated transcriptional activity both alone and synergistically with 12-*O*-tetradecanoylphorbol 13-acetate (47). Thus, these observations suggest that the activity of GABP is modulated not only by the classical Ras-Raf-MAPK pathway but also by other signaling pathways. The *Skp2* promoter activity in Ras-transformed cell lines was significantly elevated (data not shown), consistent with the notion that GABP is the critical regulator of *Skp2* in response to mitogenic signaling.

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