The Localization and Activity of cAMP-dependent Protein Kinase
Affect Cell Cycle Progression in Thyroid Cells*

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The growth and differentiation of several cell types is controlled by cAMP (1, 2). In eukaryotes, cAMP binds the regulatory subunit of cAMP-dependent protein kinases (PKA). This releases the catalytic subunit (C-PKA), which phosphorylates a wide variety of substrate proteins. A fraction of the C-PKA migrates to the nucleus and phosphorylates nuclear proteins and transacting factors. Phosphorylated transcriptional factors activate the transcription of several genes (3–5).

cAMP also drives the cell cycle of thyroid cells. Thyrotropin (TSH), a pituitary hormone, binds to and stimulates a specific Gα-coupled receptor on the membrane of thyroid cells. Stimulation of adenylyl cyclase raises cAMP levels and induces the exit of thyroid cells from G0 into the cell cycle. TSH or cAMP depletion drives cells out of the cycle into quiescence (2, 6).

There are multiple isoforms of PKA, typically determined by their specific regulatory subunit. We have been studying the PKAII isoform, which is abundantly expressed in neural and endocrine tissues. The regulatory subunits of this isozyme, RIα and RIβ, have low affinity to cAMP (7–9). The RI subunit binds to a PKA anchoring protein (AKAP), which localizes the PKAII in the Golgi-centrosome area and in the cytoskeleton (10–12). Our evidence links intracellular targeting of PKAII to cAMP nuclear signaling and gene regulation. Overexpression of the bovine brain anchor protein AKAP75 restored cAMP-induced transcription in variant PC12 cells defective in cAMP nuclear signaling (13). Conversely, thyroid cell expression of a mutant derivative of AKAP45 that displaces membrane-bound PKAII depressed cAMP-induced thyroglobulin gene expression (8). Finally, AKAP75 expression increased the rate and the magnitude of c-fos expression induced by cAMP in human embryonic kidney cells and increased cAMP-response element-directed gene transcription in cerebellar granule cells (14, 15).

We describe here changes in the components of the cAMP signaling pathway during cycle progression in synchronized thyroid cells. We find that the G0 and G1–S transitions are accompanied by significant modifications in the localization and activity of PKA. Cells entering G1 display increased nuclear and cytoplasmic PKA activity. Later in G1, PKA activity is markedly down-regulated in the absence of significant variations of cAMP levels. Altering the activity of PKA affects the onset of S phase. Expression of a transdominant negative variant of a PKAII anchor protein, which translocates endogenous PKA from membranes to the cytosol, reduces PKA levels, down-regulates nuclear cAMP signaling, and significantly shortens G1.

MATERIALS AND METHODS

Cell Lines, DNA Plasmids, Deletion Mutagenesis, and Transfections—The TL cell line is derived from the FRTL-5 thyroid cell line, which has been extensively characterized with respect to thyroglobulin expression and TSH-dependent growth (6, 16). AKAP45 and AKAP75 plasmids contain the AKAP coding region under the control of the CMV promoter; the plasmids also carry the aminoglycoside transferase gene, which confers resistance to the neomycin analogue G-418 (8, 14, 17, 18). RSV-NEO is a construct expressing the aminoglycoside transferase
gene under the control of the long terminal repeat of Rous sarcoma virus. The AKAP45 mutant (AKAP45-RII), which is deleted for the RII binding site of AKAP45, was generated by the polymerase chain reaction using specific oligonucleotide primers. The primers were designed to create NotI and XhoI restriction sites at their 5′- and 3′-ends, respectively. Polymerase chain reaction products were digested with these enzymes, NotI and XhoI restriction enzymes and cloned in the pBluescript II vector. The correct AKAP45-RII coding region was confirmed by DNA sequencing. DNA transfections in mammalian cells were carried out by the calcium phosphate procedure. We have used also an expression vector carrying the aminoglycoside transferase gene under the CMV promoter to control for promoter-dependent effects. Transfected cell lines (AKAP45 and control) were pooled of at least 100 clones.

RNA Analysis—Total RNA was purified by homogenization in guanidium isothiocyanate and phenol-chloroform-isomyl alcohol extraction (19). Probes were 0.35-kilobase PCR fragments corresponding to RIA, RIIα, RIIβ, and GAPDH labeled as follows. 10 ng of the amplified DNA fragment (purified by electrophoresis) were labeled via 10 PCR cycles (1 min at 95°C, 1 min at 60°C, and 3 min at 72°C) in a volume of 30 µl of PCR buffer (see below) containing 50 µCi (3000 Ci/mmol) of [32P]dGTP; 2 nmol of dATP, dTTP, and dCTP; 10 pmol of each of the appropriate oligonucleotide primers; and 0.75 units of Taq DNA polymerase.

Single strand cDNA synthesis was performed as previously described (8).

Semiquantitative reverse transcriptase-PCR was performed amplifying RIA, RIIα, RIIβ, and GAPDH cDNAs (GAPDH primers were added after the first five cycles). 20 µl of the PCR products were resolved on four different 1.5% agarose/TBE gels, blotted onto different nylon membranes (Amerham Pharmacia Biotech), and hybridized with specific probes (see above).

The DNA fragments corresponding to the RIA, RIIα, and RIIβ were amplified with primers: RIA-F (5′-GGCGGTATGGGGAAGCAGC-3′; 5′-end at position +1) and RIA-R (5′-GATTGTTGTGGGCTGAGGG-3′; 5′-end at position 361) (20); RIIα-F (5′-TGGAGGACTCTCTTACAGC-CTG-3′; 5′-end at position 8) and RIIα-R (5′-AAGCTTTCCTAGGAGG-AGT-3′; 5′-end at position 342) (21); RIIβ-F (5′-ACCCCGGAGAAG-GTTGAAACA-3′; 5′-end at position 161) and RIIβ-R (5′-GTTCTCTGTGT-CGTCAGCT-3′; 5′-end at position 514) (22).

Total RNA (15 µg) preparation and hybridization were as described previously (8, 14, 19). cDNA probes for 18 S RNA or GAPDH were used as internal control.

DNA Synthesis—DNA synthesis in TL cells was monitored by [3H]thymidine incorporation as described previously (16). Experiments were conducted in triplicate, and the data represent a mean of four independent experiments, all which gave similar results. Cell cycle progression (G0, G1, and S phases) was monitored by fluorescence-activated cell sorter analysis. Where indicated, the cells were stimulated with 8-bromo-cyclic AMP (8-Br-cAMP), 8-chlorophenylthio-cyclic AMP (CPT-cAMP), or forskolin.

Quiescent (G0) TL cells did not express early genes (c-myc and c-fos). These induced upon exposure to TSH (23). Cyclin D and cyclin A were induced 24 h after continuous treatment with TSH. PCNA analyzed by immunofluorescence appeared 36 h after continuous TSH treatment.

Antibodies and Immunoblot Analyses—Polyclonal antibodies against RIIα, RIIβ, RIA, and PKA catalytic subunits were a gift of C. Rubin (Albert Einstein College of Medicine, Bronx, NY). In addition, specific anti-RIIα or anti-RIIβ antibodies were generated by immunizing rabbits with a synthetic RIIα peptide (peptide 31–57 from the AUG of the rat sequence) or RIIβ (peptide containing residues 53–73 from the start codon of the rat protein), respectively, cross-linked to soybean trypsin inhibitor. Total IgGs were purified, and the specificity of each preparation was tested by immunoprecipitation, immunofluorescence, and immunoblot by preadsorbing the antibodies to the specific peptides or control peptides. Anti-c-mannosidase antibodies were a gift of K. Moreman (University of Georgia, Athens, GA). Western blot analyses were carried out as described previously (8).

Nuclear and Cytoplasmic PKA Assay—Cells were lysed in AT buffer containing 0.1% Triton X-100 for 5 min on ice. The lysate was layered on 1 volume of a sucrose cushion (AT buffer containing 1 x sucrose) and centrifuged at 100,000 g for 5 min. The pellet and the upper phase representing purified nuclei and cytoplasm, respectively. The nuclear fraction contained ~90% of the transcription factors TTF1, cAMP-response element-binding protein, and PAX5 (24). In addition, each preparation was stained with propidium iodide to check purity. Assays (final volume 25 µl) were performed as described previously (8). Holoenzyme activity was calculated by subtracting values obtained in the absence of cAMP and in the presence of protein kinase inhibitor (+ cAMP) from the values obtained in the presence of cAMP. Free C-PKA activity was evaluated by subtracting counts/min obtained in the absence of cAMP from the values obtained in the presence of protein kinase inhibitor. Data were expressed as pmol of [32P]phosphate transferred to peptide substrate during a 10-min incubation in the presence (PKA holoenzyme) or absence (PKA catalytic subunit) of cAMP. At the concentrations used, protein kinase inhibitor did not inhibit the binding of phosphorylated kemptide to phosphocellulose filters (8).

RESULTS

Amplification of Nuclear cAMP Signaling in G1—TL thyroid cells forced into quiescence by TSH withdrawal were exposed to 10 milliunits/ml of TSH and 1 µg/ml of insulin to induce cell cycle entry. The onset of DNA synthesis occurred 36–48 h later (Fig. 1, upper panels). TSH stimulates cAMP synthesis; cAMP or agents that induce cAMP accumulation substitute for TSH and move TL cells into the cell cycle (2). The timing of DNA synthesis was monitored by thymidine incorporation (left panels, broken line) and fluorescence-activated cell sorting analysis (right panels).

To determine how cAMP induces cell cycle entry, we analyzed several parameters in the cAMP transduction pathway. cAMP levels increased during the first 30 min after hormone addition, returned to base line 1–3 h later, and did not vary significantly after (data not shown; see Ref. 2). Nuclear C-PKA activity, a marker of nuclear response to cAMP, increased in early G1 cells and decreased 36 h after TSH and insulin stimulation (Fig. 1, upper panel, continuous line). Similarly, cytoplasmic C-PKA activity slowly decreased in late G1 (Fig. 1, middle panel, continuous line). The reduction of PKA activity paralleled the onset of S phase (36 h) (Fig. 1, upper and middle panels). To determine whether PKA down-regulation was necessary for S phase onset, we stimulated cells in late G1 (30 h, arrow) with 8-Br-cAMP and 3-isobutyl-1-methylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, to maintain high and constant cAMP levels. Persistent activation of PKA inhibited and delayed DNA synthesis (Fig. 1, lower panels). Note that PKA activity declines in stimulated cells and becomes refractory to cAMP (25). Down-regulation of nuclear and cytoplasmic PKA in late G1 was not affected by other inductive stimuli.

To determine if the sensitivity of TL cells to cAMP nuclear signaling changed during G1, we measured C-PKA levels (Fig. 1A, insets) and activity (Fig. 1A) in nuclei of hormone-treated cells exposed to short (40-min) pulses of exogenous cAMP. Nuclear C-PKA accumulation was relatively insensitive to cAMP in G0 cells (Fig. 24). In contrast, cells in early G1 (12–24 h after TSH and insulin treatment) responded efficiently to cAMP. At 36–49 h, cAMP-induced accumulation of C-PKA in the nucleus was less sensitive. To monitor the transcription of cAMP-induced genes, we measured the accumulation of c-fos mRNA in G0, mid-G1, and late G1 cells treated for 30 and 60 min with CPT-cAMP. c-fos mRNA accumulated in early G1 cells 30 min earlier than in G0 or in late G1 cells, while GAPDH mRNA accumulation did not vary (Fig. 2B). C-PKA levels rose rapidly in nuclei of G1 cells compared with G0 and G1-S cells despite constant CAMP levels (Fig. 2C). Additionally, phosphorylated cAMP-response element-binding protein accumulated in G1 and markedly decreased in G0 and G1-S transition (data not shown). These data indicate that the ability of nuclei to take up and/or to retain C-PKA varies during the cycle. It is low in G0, increases in G1, and falls at the G1-S transition. Different cAMP analogues reproduced the results described above (data not shown).

The enhanced sensitivity to cAMP of G1 cells relative to quiescent cells might explain why cyclin A gene transcription is
efficiently induced by cAMP in G₁ but not in G₀ cells (26).

PKAII Is Diffuse in the Cytoplasm in Quiescent Cells and Concentrates in the Golgi-centrosome Area as Cells Enter the Cycle—Efficient accumulation of nuclear C-PKA in G₁ cells without corresponding increases in cytoplasmic cAMP levels suggested to us that the location of PKAII, known to affect nuclear cAMP signaling, might vary during cell cycle (8, 14).

PKAII localization as a function of time after hormone stimulation was determined by immunofluorescence analysis. In dividing thyroid cells, the RIIβ and RIIα PKA regulatory subunits localized in a discrete area corresponding to the Golgi-centrosome area, whereas RΙα was diffuse in the cytosol (Fig. 3; Ref. 12). This area stained with anti-RIIβ/α, α-mannosidase, and β-tubulin antibodies and appeared as an intense bright spot in proximity to the nucleus (8, 11, 27). Fig. 4 shows RIIβ staining at 0, 24, 36, and 48 h after stimulation with TSH and insulin. Anti α starchase antibodies were used to monitor the position of the Golgi apparatus (28). In quiescent cells, the RIIβ signal was diffuse in the cytoplasm, quite distinct from the perinuclear Golgi signal (Fig. 4, c and b). As cells exited G₀ and progressed into G₁, the RIIβ signal concentrated in the Golgi area (12 h, Fig. 4, c and d). Upon entry into S phase, both the RIIβ and Golgi signals became progressively perinuclear (36–48 h; Fig. 4, e, f, g, and h). The staining pattern of RΙα was similar to that of RIIβ during G₁ (data not shown).

To demonstrate the localization of RIIβ in the Golgi-centrosome area, we stained cells in G₁ with antibodies to p58 formiminotransferase cyclodeaminase, a protein that binds microtubule and is localized in the Golgi apparatus (Fig. 4). Panels i, j, and k show the staining with anti-RIIβ, formimino-transferase cyclodeaminase, and the overlay of both signals, respectively.

During transition from quiescence into G₁, RII accumulated in the Golgi-centrosome area. At the G₁ to S transition, both RII and the Golgi apparatus appeared in a perinuclear ring. We suggest that this movement of PKAII plays a significant role in the transmission of cAMP signals to the nucleus and in the control of cell cycle progression.

Down-regulation of PKA Regulatory Subunits during G₁—Our data indicate that the intracellular location of RII changes after exit from G₀. To monitor these movements more precisely, we prepared extracts from quiescent and cycling cells and measured R subunit concentrations in particulate and soluble fractions (P and S, respectively, in Figs. 5 and 10). Cells were synchronized by starving from hormones and serum for 3 days and then induced into cell cycle by the addition of TSH, insulin, and serum. The peak of DNA synthesis under these conditions invariably occurred 48 h after induction (Fig. 1).
levels at 48 h, a point corresponding to the peak of S phase.

Cycle-dependent fluctuations in concentration of R subunits are not specific to TL cells. Cell cycle entry also induced changes in R subunit concentration in the PCCl3 thyroid cell line (29) with kinetics comparable with those of TL (data not shown).

The decrease in R subunit concentration in G1 cannot be accounted for by down-regulation of the corresponding mRNAs. In fact, R subunit mRNA concentrations were markedly elevated by TSH or cAMP treatment in early G1 (RIα) and mid-late G1 (RIIα and RIIβ) (Fig. 6 and Ref. 30). To determine further the mechanism of the R subunit down-regulation, we measured the RII subunit synthesis during cell cycle progression. As shown in Fig. 7 (upper panel), the translation rate of the RIIβ subunit was similar in G1 (12–24 h), G0 (0 h) and G1-S transition (36 h). The band of ~40 kDa present in the immunoprecipitates of RII in G0 phase and in G1-S transition (36 h) cells was C-PKA, as indicated by the in vitro kinase A assay (Fig. 7, lower panel). In contrast, anti-RII antibody did not coprecipitate the C-PKA subunit in early and middle G1. These data indicate that the association between RII and C-PKA fluctuates during cell cycle.

Taken together, these analyses indicate that 1) the decrease in R subunit concentration in G1 is post-transcriptionally regulated; 2) R subunit levels reach a minimum at a time in G1 that corresponds to the maximal activity of nuclear and cytoplasmic PKA (12–24 h; Fig. 1); 3) PKA activation stimulates R subunit mRNA accumulation (R subunit transcription is known to be induced by cAMP (30)); 4) protein and mRNA levels of RIIα and, to a lesser extent RIIβ, peaked at 36–48 h, a time corresponding to the onset of S phase and the minimum activity of the PKA pathway (Fig. 1); and 5) C-PKA is tightly bound to RII subunits in G0 and G1-S cells, thus reducing basal PKA activity.

**cAMP Reduces the Decline of p27 in G1 Cells**—We have shown that thyroid cells in G1 are extremely sensitive to nuclear cAMP signals (Fig. 2) and that this phenotype is associated with a significant down-regulation of RIα and RIIα subunits (Fig. 4) and a reduction in RII-bound C-PKA (Fig. 7). We also demonstrated that forced activation of cAMP-PKA signal-
ing in late G1 cells delayed the onset of S phase (Fig. 1). Since the Cdk inhibitor p27 regulates the timing of the G1-S phase transition, we asked if p27 levels might be influenced by cAMP. Fig. 8A indicates that the concentration of p27 in G0 cells fell as cells entered and progressed through G1. A pulse of cAMP in middle G1 (10–24 h) significantly increased p27 levels, whereas a pulse in G0 had no effect (Fig. 8B). p27 mRNA levels were not influenced by cAMP (data not shown). Our preliminary data suggest that the degradation of p27 protein is inhibited by cAMP.

Cytosolic Translocation of RII Subunits Impairs cAMP Signaling to the Nucleus and Shortens G1—

We have described a number of cellular events related to the cAMP signal transduction pathway that accompany entry into cell cycle. The amplification of cAMP nuclear signaling and the up-regulation of p27 by cAMP in G1 may be related to the targeting of PKAII in the Golgi-centrosome region (Fig. 4) or to the decrease in total R subunit concentration. To discriminate between these possibilities, we manipulated intracellular PKAII localization.

We overexpressed a defective RII anchor protein (AKAP45) that lacks the N-terminal membrane anchor domain. AKAP45 binds RII and translocates PKAII from membranes to the cytosol (10, 17, 18). Pools of clones expressing AKAP45 were isolated and characterized. Expression of AKAP45 has been shown to significantly increase cytosolic RII levels, accompanied by down-regulation of cAMP-response element-binding protein phosphorylation, nuclear C-PKA accumulation, and transcription of cAMP-regulated genes (8). We determined the effect of AKAP45 on the kinetics of c-fos mRNA induction by cAMP 24 h after entry into cell cycle. Fig. 9 shows that c-fos mRNA was induced significantly later in AKAP45-expressing (A45) cells than in control cells (TL), indicating that AKAP45 delocalized RIIβ was confirmed by immunostaining and immunoblot analysis of RII subunits in the cytosol and membranes (S and P in Figs. 5 and 6).
and 10). Unlike control cells, RIβ staining remained diffuse in the cytoplasm before and after entry into the cell cycle (Fig. 10B). In addition, AKAP45 raised the levels of cytosolic RIα and RIβ, although the total amount of RIβ subunit was reduced (Fig. 10A; Ref. 8).

The effects of PKAII delocalization on cycle progression were monitored by comparing the time course of DNA synthesis and the levels of p27 in A45 and control cells. Strikingly, A45 cells entered S phase 12 h earlier than controls (Fig. 10C). That this was the consequence of PKAII delocalization was confirmed by transfecting TL cells with an AKAP45 mutant (A45-RII*), deleted for the RII binding site (see “Materials and Methods”). The profile of DNA synthesis in cells expressing A45-RII* was comparable with control cells. As expected, A45 cells contained significantly less nuclear p27 at 24 h than control cells, as demonstrated by histochemical staining with p27 antibody (data not shown). The effect of AKAP45 overexpression on p27 levels was also shown by immunoblotting cell extracts with anti-p27 antibodies. AKAP45 induced a rapid cAMP-resistant down-regulation of p27 (Fig. 11). Thus, failure to localize PKAII in the membrane leads to an early decrease in p27 and a shorter G1 phase.

**DISCUSSION**

Activation of the cAMP-PKA signal transduction pathway can have either mitogenic or antimitogenic effects in mammary cells (31). These opposite responses might indicate cell-specific targets of the pathway. Alternatively, the intensity or the time of delivery of cAMP-PKA signal relative to the phase of cell cycle might critically influence the response of the cell to

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**TABLE I**

| Cells in G0-G1 (%) | 0 h | 12 h | 24 h | 36 h | 48 h |
|--------------------|-----|-----|-----|-----|-----|
| DNA synthesis     | 95  | 90  | 80  | 50  | 35  |
| Nuclear C-PKA+AMP | 0.8 ± 0.1 | 1.6 ± 0.15 | 2.0 ± 0.15 | 0.8 ± 0.11 | 0.9 ± 0.1 |
| RII localization  | Diffuse | Golgi* | Golgi* | Golgi*-perinuclear | Perinuclear |
| p27 content       | 100 | 80 ± 5 | 45 ± 4 | 25 ± 5 | 15 ± 4 |
| p27 + cAMP        | 100 | 80 ± 5 | 45 ± 4 | 25 ± 5 | 15 ± 4 |
| Nuclear C-PKA+AMP | 2.1 ± 0.5 | 7.2 ± 0.8 | 3.2 ± 1.1 | 3.5 ± 0.6 | ND |
| p27 in A45        | 100 | 50 | 40 | 70 | ND |
| DNA synthesis in A45 | 1 | 1 | 1 | 2 | 3.5 |

*The cells were synchronized by TSH starvation and stimulated with TSH (10 milliunits/ml) for various periods of time indicated in hours.

aSee Fig. 1.

bExpressed as pmol/μg of proteins of nuclear C-PKA induced by 15-min treatment with or without 0.1 mM 8-Br-cAMP.

cSee Fig. 4.

dDNA synthesis in cells treated with cAMP and IBMX at 24 h after initial TSH stimulation.

eExpressed as indicated in Footnote c in A45 cells (see also Figs. 2 and 10).
cAMP. In this paper, we study the effects of cAMP-PKA on cell cycle progression in thyroid cells, which are exquisitely dependent on cAMP for growth. We show that cAMP is required to enter G1 from G0. The cAMP-PKA pathway is initially up-regulated early in G1 and then down-regulated later in this phase. Down-regulation is critical for progression from G1 to S. We propose that regulation of the cAMP-PKA pathway after entry into cell cycle does not primarily reflect changes in cAMP levels. Instead it results from changes in the subcellular location of PKAII isoenzymes, in the concentrations of the RII subunits, and in the capacity of these subunits to bind C-PKA. Our data linking cAMP signal transduction to cell cycle progression are summarized in Table I.

**Entry in the Cycle of Thyroid Cells (G0-G1 Transition)**—Stimulation of quiescent thyroid cells with cAMP or with agents, such as TSH or forskolin, that elevate cAMP levels induces cell cycle entry and DNA replication (2). cAMP up-regulates the expression of immediate early genes (2, 23) and prepares the cell for DNA synthesis (26, 32, 33).

To stimulate gene transcription, C-PKA released from PKA holoenzyme must enter the nucleus. Quiescent cells respond weakly to cAMP, showing little accumulation of nuclear C-PKA (Fig. 2). 12 h after hormone stimulation, however, early G1 cells become highly responsive to cAMP. Increased cAMP signal transduction is associated with accumulation of PKAII in the Golgi-centrosome area (Fig. 4) and a decrease in R subunit...
A potential target of nuclear C-PKA is p27, an inhibitor of Cdk4. Cdk4 activation is essential for G1-S transition. p27 decays during the first 24 h after exposure to TSH and insulin. Treatment with cAMP significantly increased the levels of p27; similar findings have been reported in other cell lines that are growth-inhibited by cAMP (36). Another Cdk inhibitor, p21, was not affected by PKA stimulation (data not shown). In contrast, p27 concentrations in A45 cells were lower than in control cells. Thus, the levels of p27 and the timing and extent of DNA synthesis are correlated. The addition of cAMP later in G1-S (36 h) had no effect on DNA synthesis or on the down-regulation of p27 (data not shown).

How the PKA pathway interferes with p27 decay is not clear. p27 is degraded by the ubiquitin pathway, and it is possible that one or more components of this pathway are modified by PKA-dependent phosphorylation. Our preliminary evidence suggests that cAMP reduces p27 degradation. Note that R subunits are also degraded by the ubiquitin pathway and that the reductions in R subunit and p27 follow similar kinetics (Figs. 5, 6, and 8). Thus, it is possible that CAMP-PKA might regulate ubiquitin-mediated proteolysis of both p27 and R subunits. In yeast, some components of the proteasome are known to be controlled by the CAMP-PKA pathway (37).

Inhibition of S phase onset can be induced by cAMP in a wide variety of cell types, depending on the time of cAMP stimulation. CAMP advances S phase if added early in G1 (NIH 3T3 fibroblasts), whereas it inhibits entry if added later (31).

We showed previously that CAMP levels oscillate during cell cycle in Xenopus egg extracts and that such oscillations are required for mitosis-interphase transition (38) and for the onset of S phase. We described above how CAMP signaling may be amplified or down-regulated in G1 thyroid cells without marked fluctuations in CAMP concentrations (see also 2). These latter events, which are essential for G1 progression, are downstream to adenyllylcyclase and amplify small changes in CAMP concentrations. In Xenopus oocytes, CAMP levels fluctuate in response to internal signals, whereas CAMP concentrations in thyroid cells respond to external stimulation by TSH. By adjusting PKAI activity, thyroid cells can modulate their response at different times during cycle despite a constant blood TSH concentration. Conversely, down-regulation of the PKA pathway in late G1 may buffer the cells from surges in TSH levels, which would otherwise block G1-S transition.

In conclusion, we propose that the CAMP-PKA signal transduction pathway determines the length of G1 phase by affecting the concentrations of p27 and possibly other regulators of cell cycle progression. We have demonstrated this control system in thyroid cells, which are dependent on cAMP for growth, but we suggest that it plays a role in cycle progression in all cells. Our hypothesis is supported by studies of cycle progression in Saccharomyces cerevisiae, where CAMP-PKA signals have been demonstrated to regulate cell size sensors and the entry into S phase (39).

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REFERENCES
1. McKnight, G. S. (1991) Curr. Opin. Cell Biol. 3, 213–217
2. Tramontano, D., Moses, A. C., and Ingbar, S. H. (1988) Endocrinology 122, 133–136
3. Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 675–680
4. Gonzalez, G. A., Yamamoto, K. K., Fisher, W. H., Karr, D., Menzel, P., Biggs, W., Vale, W. W., and Montminy, M. R. (1989) Nature 343, 749–752
5. Montminy, M. (1997) Annu. Rev. Biochem. 66, 897–922
6. Ambesi-Impiombato, F. S., Parks, L. A. M., and Coon, H. G. (1980) Proc. Natl. Acad. Sci. USA 77, 5836–5840
7. De Brasi, D., De la Morena, R., and Giuliano, P. (1987) Proc. Natl. Acad. Sci. USA 84, 2789–2793
8. V. Sexl, personal communication.
9. A. Feliciello, unpublished observations.
Acad. Sci. U. S. A. 77, 3455–3459
7. Erlichman, J., Sarkar, D., Fleischer, N., and Rubin, C. S. (1980) J. Biol. Chem. 255, 8179–8184
8. Feliciello, A., Giuliano, P., Porcellini, A., Mele, E., Angotti, E., Grieco, D., Amabile, G., Cassano, S., Li, Y., Musti, A. M., Rubin, C. S., Gottesman, M. E., and Avvedimento, V. E. (1996) J. Biol. Chem. 271, 25350–25359
9. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005
10. Li, Y., Ndubuka, C., and Rubin, C. S. (1996) J. Biol. Chem. 271, 16862–16869
11. Rios, B. M., Celati, C., Lohmann, S. M., Bornens, M., and Keryer, G. (1992) EMBO J. 11, 1723–1731
12. Rubin, C. S. (1994) Biochim. Biophys. Acta 1224, 467–479
13. Cassano, S., Gallo, A., Bucciarelli, V., Porcellini, A., Cerillo, R., Gottesman, M. E., and Avvedimento, V. E. (1996) J. Biol. Chem. 271, 29870–29875
14. Feliciello, A., Li, Y., Avvedimento, V. E., Gottesman, M. E., Rubin, C. S. (1997) Curr. Biol. 7, 1011–1014
15. Paolillo, M., Feliciello, A., Porcellini, A., Garbi, C., Bifulco, M., Schinelli, S., Ventra, C., Stabile, E., Ricciardi, G., Bifulco, M., Schettini, G., and Avvedimento, V. E. (1999) J. Biol. Chem. 274, 6546–6552
16. Porcellini, A., Ruggiano, G., Pannain, S., Ciullo, I., Amabile, G., Fenzi, G. F., and Avvedimento, V. E. (1997) Oncogene 15, 781–789
17. Glantz, S. B., Li, Y., and Rubin, C. S. (1993) J. Biol. Chem. 268, 12796–12804
18. Hirsch, A. H., Glantz, S. B., Li, Y., You, Y., and Rubin, C. S. (1992) J. Biol. Chem. 267, 2131–2134
19. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
20. Kuno, T., Ono, Y., Hirai, M., Hasimoto, S., Shuntoku, K., and Tanaka, C. (1987) Biochem. Biophys. Res. Commun. 146, 878–883
21. Scott, J. D., Glaccum, M. B., Zoller, M. J., Ulmer, D. M., Helfman, D. M., McKnight, S. G., and Krebs, E. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 535, 5199–5204
22. Jahn, J., D. E., Hidk, L., Kidd, J. V., Beattie, W. G., Lohmann, S. M., Walter, U., Durica, J., Schulz, T. Z., Schultz, E., Gowers, C., Lawrence, C. B., Godman, D., Ratons, S. L., and Richards J. (1986) J. Biol. Chem. 261, 12532–12541
23. Colletta, G., Cirafici, A. M., Vecchio, G. (1986) Science 233, 488–490
24. Gallo, A., Benussiglio, E., Bonapace, I. M., Feliciello, A., Cassano, S., Garbi, C., Musti, A. M., Gottesman, M. E., and Avvedimento, V. E. (1992) Genes Dev. 6, 1621–1633
25. Armstrong, R., Wen, W., Meinkoth, J., Taylor, S. S., and Montminy, M. (1995) Mol. Cell. Biol. 15, 1826–1832
26. Desdouets, C., Matesic, G., Molina, C. A., Foulkes, N. S., Sassone-Corsi, P., Brechot, C., and Sobzak-Thepot, J. (1995) Mol. Cell. Biol. 15, 3301–3309
27. Nigg, E. A., Hiltz, H. M., Eppenberger, H. M., and Dutly, P. (1985) EMBO J. 4, 2801–2806
28. Zheng, J., Koda, T., Fujiwara, T., Kishi, M., Ikehara, Y., and Kakimura, M. A. (1998) J. Cell Sci. 111, 1061–1069
29. Battaglia, C., Berlingerio, M. T., Martelli, M. L., Trapasso, F., Delli Bovi, P., and Fusco, A. (1993) Cell Growth Differ. 4, 185–192
30. Oyen, O., Eskild, W., Beebe, S. J., Hansson, V., and Jahn, T. (1988) Mol. Endocrinol. 2, 1070–1076
31. Smets, I. A., and Van Roey, H. (1987) J. Cell. Physiol. 133, 395–399
32. Deportore, F., Van Keymeulen, A., Lukas, J., Castagliola, S., Bartkova, J., Dumont, J. E., Bartek, J., Roger, P. P., and Elmer, S. (1998) J. Cell Biol. 140, 1427–1439
33. Premier, S., Pohl, V., Poteet-Smith, C., Roger, P. P., Corbin, J., Doskland, S. O., Dumont, J. E., and Maenhaut, C. (1997) Mol. Cell. Biol. 17, 6717–6726
34. Chaim, D. J., Casadio, A., Schacher, S., Hedge, A. H., Vaubrun, M., Yamamoto, N., Goldberg, A. L., Bartsch, D., Andel, E., and Schwartz, J. H. (1999) Neuron 22, 147–156
35. Feliciello, A., Rubin, C. S., Avvedimento, V. E., and Gottesman, M. E. (1998) J. Biol. Chem. 273, 23361–23366
36. Kato, J., Matsuoka, M., Palyak, C., Massague, J., and Sherr, C. J. (1994) Cell 79, 487–496
37. Yamashita, Y. M., Nakaseko, Y., Samejima, I., Kumada, K., Yamada, H., Michaelson, D., and Yanagida, M. (1996) Nature 384, 276–279
38. Grieco, D., Porcellini, A., Avvedimento, V. E., and Gottesman, M. E. (1996) Science 271, 1718–1722
39. Baron, M. D., Menti, P., and Alberghina, L. (1994) Nature 371, 339–342
40. Hennings, D., Scales, S. J., Moreau, A., Murrely, L., Lee, T. D., and Kreis, T. E. (1998) J. Biol. Chem. 273, 19602–19611