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Effects of Retinoic Acid and cAMP on the Differentiation of *Naegleria gruberi* Amoebas into Flagellates

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During the differentiation of *Naegleria gruberi* amoebas into flagellates, the amoebas undergo sequential changes in cell shape and form new cellular organelles. To understand the nature of the signal which initiates this differentiation and the signal transduction pathway, we treated cells with four agents, PMA, retinoic acid (RA), okadaic acid, and cAMP. Retinoic acid and cAMP had specific effects on the differentiation of *N. gruberi* depending on the time of the drug treatment. Addition of (100 μM) retinoic acid at the initiation of differentiation inhibited differentiation by blocking the transcription of differentiation specific genes (e.g., β-tubulin). This inhibition of differentiation by retinoic acid was overcome by co-treatment with cAMP (or dbcAMP, 20 μM). Addition of retinoic acid at later stages (30 and 70 min) had no effect on the transcriptional regulation of the β-tubulin gene, however the differentiation was inhibited by different degrees. Co-treatment of cAMP at these stages did not overcome the inhibitory effect of retinoic acid. These results suggest that the role of retinoic acid as a transcriptional regulator might be conserved throughout the evolution of eukaryotes.

Naegleria gruberi is an amoebo-flagellate and is found ubiquitously on earth (Fulton, 1977). In laboratory conditions, *Naegleria* amoebas differentiate into flagellates rapidly and synchronously (Fulton and Dingle, 1967; Fulton, 1977). This differentiation accompanies distinct changes in cell shape and *de novo* formation of cellular organelles including basal bodies, flagellar axonemes, and cytoskeletal microtubules (Fulton, 1977; Walsh, 1984). After the initiation of differentiation, amorphous *Naegleria* amoebas begin to round up into spheres and basal bodies are formed on the cell periphery. At 70 min after the initiation, most of the differentiating cells are spheric in shape and 50% of these round cells have growing flagella (T_{50}, time after the initiation of differentiation when 50% of cells in the population have visible flagella). As differentiation proceeds further, these round cells begin to elongate and a complex array of cytoskeletal microtubules is rapidly formed from the base of the flagella toward the opposite end of these flagellated cells. At 120 min after the initiation, more than 95% of the cells have completed the formation of the flagella and the cytoskeletal microtubule system, and the majority of these flagellates are elongated in shape (Walsh, 1984).

In the process of this differentiation, *N. gruberi* reprograms its gene expression. During the differentiation, three mRNAs which encode proteins for flagellar axonemes and/or cytoskeletal microtubules, flagellar calmodulin, α- and β-tubulin mRNAs, are transiently and coordinately accumulated. Hence these mRNAs are called differentiation-specific (DS) mRNAs (Mar et al., 1986; Shea and Walsh, 1987). DS mRNAs are not found in amoebas and begin to accumulate in differentiating cells 10 to 20 min after the initiation of differentiation. Concentrations of the three mRNAs rapidly increase until 70 min when 50% of the cells have visible flagella, and then decrease. At the end of differentiation (120 min), the concentrations of these mRNAs are below 20% of their respective maximum values (Mar et al., 1986). This transient accumulation of the three mRNAs is due, in part, to transient transcriptional activation of the respective genes after the initiation of differentiation (Lee and Walsh, 1988). Transcription of these three genes is not detected in amoebas, but activated rapidly after the initiation of differentiation. At 20 min after the initiation of differentiation, the rate of transcription of the three genes reaches to 60-90% of the peak rate. The rate of transcription of these genes peaks at 40 min when concentration of the mRNAs increases most rapidly in the differentiating cells. At 70 min when the concentration of the mRNAs is at the

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maximum, the rate of transcription of these genes has already decreased to 50-70% of the peak rate. At the end of differentiation (120 min), these genes are transcribed slowly, below 10-20% of the peak rate (Lee and Walsh, 1988).

Continuous and programmed synthesis of new proteins is required to accomplish this complex regulation of gene expression and to complete differentiation (Fulton and Walsh, 1980; Walsh, 1984; Bok et al., 1995). Inhibition of protein synthesis has distinct effects on the transient accumulation of DS mRNAs depending on the time of inhibitor treatment (Bok et al., 1995). Inhibition of protein synthesis from the beginning of differentiation completely prevents the transient accumulation of β-tubulin mRNA. Addition of cycloheximide at 20 or 40 min after the initiation when the rate of transcription of the β-tubulin gene is rapidly increasing prevents further increases in the concentration of the mRNA However, addition of cycloheximide at 60 or 80 min when the transcriptional activity of β-tubulin gene is rapidly decreasing induces further increases in the concentration of the tubulin mRNA. Using a nuclear run-on transcription assay, we have shown that the inhibition of protein synthesis at the early stages (20 or 40 min) terminated the transcription of the β-tubulin gene, but the inhibition of protein synthesis at later stages (after 50 min) increased the rate of transcription of the β-tubulin gene (Bok et al., 1995). It was also shown that the addition of cycloheximide at later stages prevented mRNA from being rapidly degraded (Bok et al., 1995).

The requirement of continuous protein synthesis in the regulation of the β-tubulin gene during differentiation is reflected by the effect of protein synthesis inhibition on the differentiation. For example, the formation of microtubule-based cellular organelles (basal body, flagella, and cytoskeletal microtubules) requires discrete periods of protein synthesis after the initiation of differentiation. To form flagella, protein synthesis has to continue for 53 min after the initiation of differentiation (Fulton and Walsh, 1980). To form cytoskeletal microtubule systems, protein synthesis has to continue for 10 min more after the completion of protein synthesis required for the formation of the flagella (Walsh, 1984). Formation of the flagellate shape requires about 75 min of continuous protein synthesis after the initiation of differentiation (Walsh 1984).

Starvation and a fluid environment are two well known factors which cause *Naegleria* amoebas to differentiate into flagellates (Fulton, 1977). However, it is not known how a *Naegleria gruberi* amoeba senses these changes in the growing conditions and by which pathway these signals are transduced to the cell nucleus and how the complex reprogramming of gene expression is accomplished. As a first step to answer these questions, cells were treated with various agents (phorbol 12-myristate 13-acetate [PMA], okadaic acid, retinoic acid [RA], dibutyryl cyclic AMP [dbcAMP], and cyclic AMP [cAMP]) which are known to stimulate signal transduction pathways or to provoke changes in gene expression. Among the four reagents, RA and cAMP showed specific effects on the differentiation of *N. gruberi*.

RA is one of the key molecules which regulate the growth and differentiation of a cell and the development of multicellular organisms (see for recent review: Means and Gudas, 1995; and references therein). RA (and other retinoids) binds to its receptors (RARs; retinoic acid receptor α, β, and γ or RXRs; retinoid X receptor α, β, and γ) in a target cell and this RA-receptor complex acts as a transcriptional regulator (Zelent et al., 1989; Durand et al., 1992; Heyman et al., 1992; Nagpal et al., 1992; Tran etal., 1992; Allenby et al., 1994; Pan et al., 1995; Minnucci et al., 1996; Caelles et al., 1997; see also Kastner et al., 1995; Perlmann and Evans, 1997 for review). Despite these extensive studies, the possible role of RA in regulating transcription of specific genes in single cell eukaryotes has been rarely studied. cAMP also has many functions. In multicellular organisms, cAMP regulates various cellular activities as a second messenger (for a review on cAMP in transcriptional regulation see Montminy, 1997). cAMP can also act outside of a cell by binding to a receptor on the surface of a target cell. In the development of *Dictyostellium discoidium,* cAMP acts as a chemoattractant and the binding of cAMP to its receptor on the cell surface initiates a complex signal transduction pathway and the development into a multicellular organism (Parent and Devreotes, 1996). Data in this report show that RA could act as a transcriptional regulator in *N. gruberi*, a single cell eukaryote, and this action of RA is antagonized by cAMP.

Materials and Methods

Cell growth and differentiation

N. gruberi strain NB-1 was used throughout this study. 2.5×10^5 *N. gruberi* cysts were inoculated on an NM agar plate with 0.1 ml of *Klebsiella pneumoniae* (Kp) cultured overnight and incubated at 34℃ (Bok et al., 1995). For differentiation, cells were harvested with 2 mM ice-cold Tris-HCl (pH 7.6 at 25℃). The cell suspension was centrifuged three times (at 2,500 rpm for 30 sec) to remove Kp. Differentiation was initiated by resuspending the final pellet in the Tris buffer prewarmed to 25℃. At every 10 min, a small portion of the cells were fixed with Lugol's iodine and examined under a phase contrast microscope to monitor the differentiation. Differentiation was evaluated by the percentage of cells with visible flagella (Fulton, 1977).

RNA preparation

During differentiation, 0.5 ml samples of differentiating cells ($\sim 5 \times 10^7$/ml) were taken every 10 min from each flask. After brief centrifugation, the cells were resuspended in 460 μl of 5 M guanidine thiocyanate, 10 mM

Na_2EDTA, 2% Sarkosyl, 25 mM Tris-HCl (pH 7.6). The cell lysate was extracted with two volumes of phenol: chloroform (2:1). The aqueous layer was further extracted with a 1:1 solution of phenol and chloroform until the interface was clean. The final aqueous layer was adjusted to 150 mM NaCl with 5 M NaCl, and RNA was precipitated with two volumes of 100% ethanol (Mar et al., 1986).

RNA slot blot hybridization

RNA samples (5 μg/slot) were transferred to nylon membranes (Amersham Hybond N^+) according to the manufacturer's manual using a slot blot apparatus (Hoefer Scientific Instrument). Membranes were prehybridized in hybridization buffer (50% formamide, 0.25 M $NaHPO_4$ [pH 7.2], 0.25 M NaCl, 1 mM Na_2EDTA, 100 μg/ml wheat germ tRNA, and 7% SDS) for 6 h at 42℃. For hybridization, the used buffer was drained and fresh hybridization buffer was added to the bag with denatured ^{32}P-labeled cDNA probe. After 16-19 h of hybridization at 42℃, the membrane was rinsed briefly with 0.1% SDS in 2×SSC and then washed two times with the same washing buffer for 15 min each. The membrane was further washed twice in 25 mM $NaHPO_4$ [pH 7.2], 1 mM Na_2EDTA, 1% SDS for 15 min each. All washes were carried out at 50℃ (Bok et al., 1995).

Probe preparation

A cDNA fragment of each mRNA (*Pst* I fragment of pcNg 8-5, 580 bp, for β-tubulin; PCR fragment of 380 bp, for actin; *Pst* I fragment of pcNg 3-28, 700 bps, for a non-specific mRNA [Lee and Walsh, 1988]) was labeled with ^{32}P-dCTP by using a DNA labeling kit (Boehringer Mannheim).

Results

Effects of retinoic acid on differentiation of N. gruberi

Cells were differentiated in the presence of PMA (100, 300, or 500 ng/ml), dbcAMP (or cAMP, 0.1 μM-5 mM), okadaic acid (10, 50, or 100 nM,), or RA (0.1-200 μM). Except RA, these reagents had no significant effect on the differentiation of *N. gruberi* (data not shown). RA did not inhibit differentiation at low concentrations (0.1 and 1 μM, data not shown). However, differentiation was inhibited in a dose dependent manner at higher concentrations of RA. In the presence of 10 μM of RA, cells differentiated normally except for a slight delay (2-5 min) in T_{50}. In the presence of 50 μM of RA, T_{50} was further delayed (T_{50} was 81 min) and about 90% of the cells formed flagella. In the presence of 100 μM RA, less than 20% of the cells formed flagella, however the change in cell shape was almost completely inhibited. Almost all of the cells were amoeboid in shape except for a few round flagellated cells (Fig. 1A).

Because the addition of RA (100 μM) at the beginning

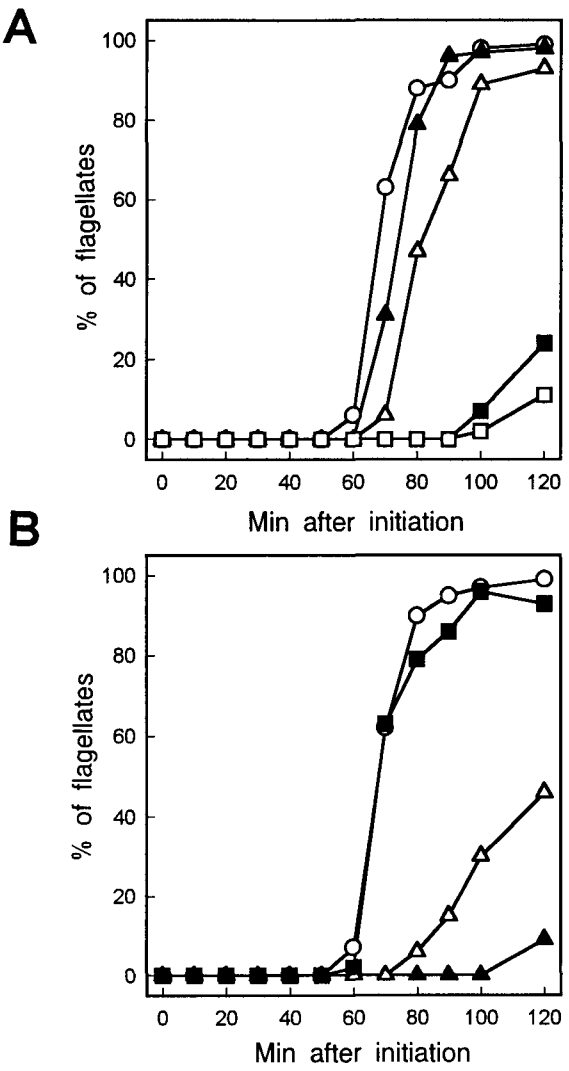

Fig. 1. The effects of retinoic acid on differentiation of *Naegleria gruberi*. A, *N. gruberi* amoebas were harvested, divided into five flasks, and differentiated. At the initiation of differentiation, various amounts of retinoic acid (RA) (10 mM in DMSO) were added into the flasks to final concentrations of 0, 10, 50, 70, or 100 μM. Differentiation of *N. gruberi* was monitored as described in Materials and Methods. Each curve represents the average of three independent experiments. ○-○, control (without RA); ▲-▲, 10 μM; △-△, 50 μM; ■-■, 70 μM; □-□, 100 μM. B, *N. gruberi* amoebas were harvested, divided into four flasks, and differentiated. RA was added to a final concentration of 100 μM at 0, 30, and 70 min after the initiation of differentiation. ○-○, control; ▲-▲, RA was added at 0 min; △-△, 30 min; ■-■, 70 min.

of differentiation inhibited differentiation, we examined the effect of RA added at two later stages of differentiation (30 and 70 min). At 30 min after the initiation, the rate of transcription of DS genes is about 90% of the peak rate (Lee and Walsh, 1988) and the concentration of DS mRNAs is 40% of the maximum and rapidly increasing (Mar et al., 1986). Addition of RA at 30 min significantly inhibited differentiation. Even though flagella were observed in approximately 40% of cells of the population, the flagellated cells remained amoeboid or spherical in shape. At 70 min, the rate of transcrizption of the DS genes has already passed the

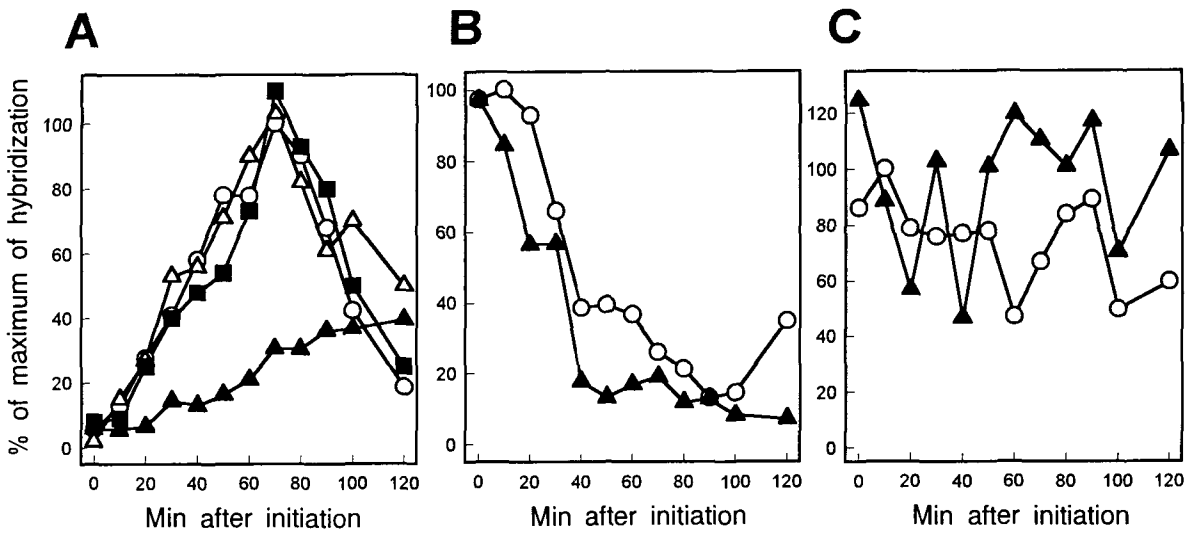

Fig. 2. The effect of RA treatment on the changes in the concentration of β-tubulin mRNA, actin mRNA, and a non-specific mRNA during differentiation. RA was added at the beginning (0 min), 30 min, or 70 min after the initiation of differentiation to a concentration of 100 μM. Every 10 min, 0.5 ml of the cell suspension was taken out. RNA was purified and transferred to nylon membranes as described in Materials and Methods. After RNA slot blot hybridization with respective DNA probes labeled with ^{32}P, the amount of each mRNA was estimated by scintillation counting. A, Effect of RA added at different stages of differentiation on the transient accumulation of β-tubulin mRNA. ○-○, control; ▲-▲, RA at 0 min; △-△, 30 min; ■-■, 70 min. B, RA had no effect on the rapid decrease in the concentration of actin mRNA. ○-○, control; ▲-▲, RA at 0 min. C, Effect of RA treatment of the concentration of a non-specific mRNA. ○-○, control; ▲-▲, RA at 0 min.

peak rate (50-70%, Lee and Walsh, 1988), the concentration of DS mRNAs is at the maximum (Mar et al., 1986), and protein synthesis required for the formation of the flagellar apparatus has been completed (Walsh, 1984). Addition of RA at 70 min did not inhibit the formation of flagella, but the formation of the flagellated shape was still inhibited. These flagellated cells were spherical in shape (Fig. 1B).

Effect of RA on accumulation of β-tubulin mRNA

The above results showed that RA inhibited differentiation of *N. gruberi* at different degrees depending on the time of the drug treatment. To understand the mechanism of inhibition, we examined the effect of RA treatment on the accumulation of β-tubulin mRNA. Addition of RA at the initiation of differentiation significantly inhibited the accumulation of β-tubulin mRNA (Fig. 2A). Concentration of β-tubulin mRNA increased very slowly and continuously until the end of differentiation (120 min). At 70 min, the concentration of the tubulin mRNA was about 30% of that of the control and increased continuously until the end of differentiation, reaching 40% of the control's maximum.

Addition of RA at 30 or 70 min after the initiation did not inhibit the accumulation of β-tubulin mRNA. When RA was added at 30 min, the concentration of the tubulin mRNA increased as in the control cells until 70 min and then began to decline. However, the rate of decrease in the concentration of mRNA was slightly slower than that of the control. Addition of RA at 70 min after the initiation had little effect on the accumulation of the tubulin mRNA (Fig. 2A).

To examine whether this effect of RA on the accumulation of β-tubulin mRNA was specific, we surveyed the concentrations of two other mRNAs during differentiation. One is actin mRNA which is amoeba-specific (i.e., an mRNA which disappears rapidly after the initiation of differentiation, Sussman et al., 1984), and the other is a non-specific mRNA (i.e., an mRNA which is present both in amoebas and in differentiating cells) represented by a cDNA clone pcNg 3-28 (Mar et al., 1986). To examine the changes in the concentration of actin mRNA more accurately during differentiation, we cloned a fragment of the genomic actin DNA by polymerase chain reaction (PCR) and subsequently a full-length genomic actin DNA by screening an *N. gruberi* NB-1 genomic library with the PCR amplified fragment as a probe. The concentration of actin mRNA decreased rapidly after the initiation of differentiation. At 40 min after the initiation, the concentration of the mRNA decreased to 40% of the maximum value (Fig. 2B). The amount of actin mRNA further decreased until 100 min after the initiation reaching less than 20% of the peak value and then began to increase. Addition of RA at the beginning of differentiation had no significant effect on the decrease in the concentration of actin mRNA except that the concentration of actin mRNA in RA treated cells was slightly lower than that of the control cells. RA treatment also had no specific effect on the amount of non-specific mRNA (Fig. 2C).

cAMP overcame the inhibitory effect of RA

Above results suggested that the addition of RA at the beginning of differentiation inhibited the cellular differentiation of *N. gruberi* by specifically inhibiting the accumulation of β-tubulin mRNA. There have been

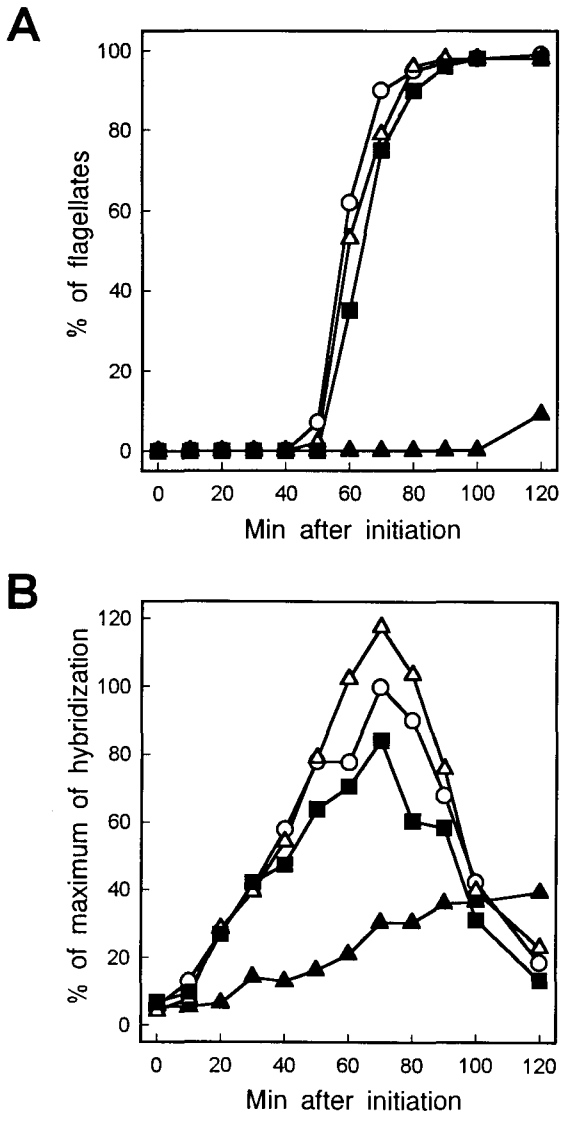

Fig. 3. cAMP antagonized the inhibitory effect of RA on the differentiation of *Naegleria gruberi*. RA and dbcAMP were added at the initiation of differentiation to a final concentration of 100 μM and 20 μM, respectively. A, Effect of RA and dbcAMP co-treatment on the differentiation of *N. gruberi*. B, Effect of RA and dbcAMP co-treatment on the concentration of β-tubulin mRNA. ○-○, control; ▲-▲, RA (100 μM) only; △-△, dbcAMP (20 μM) only; ■-■, RA (100 μM) and dbcAMP (20 μM) co-treated.

numerous reports on the effect of RA treatment on the transcriptional regulation of a gene. In some cases, this effect of RA was augmented or inhibited by PMA (Fukuo et al., 1986; Towers et al., 1995; Huang et al., 1997) or dbcAMP (Martin et al., 1990; Gianni et al., 1993; Desai et al., 1996). Based on these reports, we co-treated cells with RA and PMA or RA and dbcAMP at the beginning of differentiation. Addition of PMA (100 ng/ml) had no effect on the inhibitory effect of RA (data not shown). However, dbcAMP completely overcame the inhibitory effect of RA (Fig. 3). In the presence of 100 μM of RA and 10 or 20 μM of dbcAMP, the cells differentiated normally except for a slight

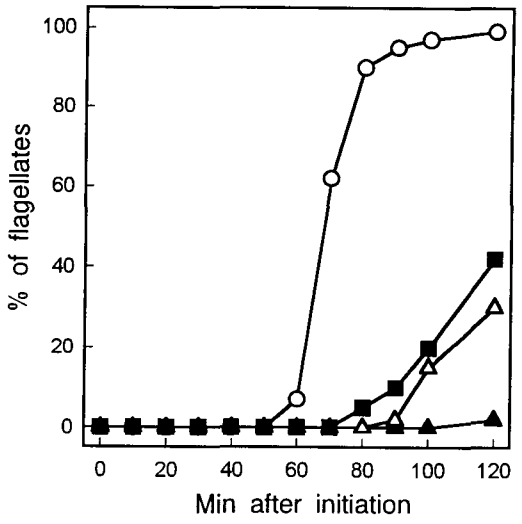

Fig. 4. Antagonistic effect of cAMP on RA was evident only when the two agents were treated at the beginning of differentiation. ○-○, control differentiation; ▲-▲, RA was added at 0 min and dbcAMP was added at 30 min; △-△, dbcAMP was added at 0 min and RA was added at 30 min; ■-■, dbcAMP was added at 0 min, and 30 min later, RA and dbcAMP were added.

delay in T_{50} (73 min) (Fig. 3A). Because the accumulation of β-tubulin mRNA was specifically inhibited by RA treatment, we examined the changes in the concentration of β-tubulin mRNA in RA and dbcAMP co-treated cells. dbcAMP alone had no effect on the accumulation of β-tubulin mRNA except for a slight increase in the concentration. However, in cells co-treated with RA and dbcAMP, dbcAMP overcame the inhibitory effect of RA on the accumulation of β-tubulin mRNA almost completely (Fig. 3B). The tubulin mRNA began to accumulate in the differentiating cells after the initiation of differentiation, reached a peak level at 70 min to about 80% of that of the control cells, and then decreased. This co-treatment of RA and dbcAMP had no effect on the change in the amount of actin mRNA during the differentiation (data not shown).

Addition of RA at 30 or 70 min after the initiation of differentiation had different effects on differentiation (see above). We examined if these effects of RA were also antagonized by dbcAMP (Fig. 4). When cells were differentiated with RA and dbcAMP was added into the differentiating cells 30 min later, less than 10% of the cells formed flagella. When cells were differentiated with dbcAMP and RA was added 30 min later, about 30% of the cells formed flagella. When cells were differentiated in the presence of dbcAMP (20 μM), and 30 min after the initiation, dbcAMP and RA were added to final concentrations of 40 and 100 μM respectively. As a result, 40% of the cells in the population formed flagella. These results showed that the effect of dbcAMP on offsetting the inhibitory effect of RA was only evident when the two reagents were added simultaneously at the beginning of differentiation.

When we started this study, we used dbcAMP instead

of cAMP because it was reported that cAMP had no effect on *N. gruberi* differentiation (Fulton, 1977). Our results also showed that dbcAMP alone had no visible effect on *N. gruberi* differentiation and the effect of dbcAMP was only visible when it was co-treated with RA. Based on these results, we tested whether cAMP can offset the inhibitory effect of RA as dbcAMP did. Addition of cAMP alone (20 μM) had no effect on the differentiation as reported. However, when the cells were differentiated in the presence of RA (100 μM) and cAMP (20 μM), they differentiated normally. These results show that cAMP can also offset the inhibitory effect of RA.

Discussion

RA treatment (100 μM) had drastic and distinct effects on the cellular differentiation of *N. gruberi*. Addition of RA at the beginning of differentiation inhibited differentiation. Only 10 to 20% of the population formed flagella and almost all of the cells were amoeboid in shape, even the flagellated ones. This inhibition of differentiation is due, at least in part, to the specific inhibition of the rapid and transient accumulation the β-tubulin and possibly other DS mRNAs. Inhibition of gene expression by RA treatment has been reported in many systems. For example, RA treatment repressed the transcriptional activation of the collagenase gene by AP-1. RA treatment inhibited the binding of the AP-1 protein to its binding site (Schroen and Brinckerhoff, 1996). Recently, Caelles et al. (1997) showed that RA (and other steroid/thyroid hormones) treatment inhibited the amino-terminal phosphorylation of c-Jun by Jun amino-terminal kinase (JNK) hence preventing AP-1 activation. RA treatment initiated the differentiation of embryonic stem cells and repressed Oct3/4 expression. Pikarsky et al. (1994) showed by a gel retardation assay that a cis-acting sequence in the promoter region bound with different proteins depending on whether RA treated or untreated nuclear extracts were used. Minucci et al. (1996) showed that RA treatment caused the displacement of bound proteins from cis-acting regulatory sequences. These results suggest that the RA-receptor complex inhibits the function of specific TFs which are required for the expression of target genes. Based on these reports, the observed inhibition of the β-tubulin gene by RA might be the result of the inhibition of a specific transcription factor which was synthesized after the initiation of differentiation (Bok et al., 1995).

Addition of RA at 30 min after the initiation had no effect on the transient accumulation of β-tubulin mRNA. These data suggest that RA treatment at this stage had no effect on the transcriptional regulation of the tubulin gene. However, the fact that only about 40% of the cells formed flagella and most of these flagellates were amoeboid in shape imply that RA treatment at this stage inhibited expression of other genes which are necessary for progression into the next stage of differentiation, e.g., formation of the spheric shape. When RA was added at 70 min, most of cells formed flagella but the formation the flagellate, shape was still inhibited. These results also suggest that the RA treatment inhibited the expression of genes required for the formation of the flagellate shape.

Based on the effects of protein (or RNA) synthesis inhibition, it has been suggested that different groups of genes have to be expressed on schedule during differentiation (Fulton and Walsh, 1980; Walsh, 1984). Although this hypothesis is very attractive, it is also possible that all the events required for the differentiation are accomplished at a certain stage of differentiation, e.g., 70 min, but the amount of protein synthesized until this stage is not enough to complete the differentiation. Our data showed that distinct cellular events were affected depending on the time of RA addition. These results further support the hypothesis that different groups of genes have to be expressed on schedule to complete the differentiation.

The effects of cAMP on the differentiation were intriguing. cAMP (or dbcAMP) alone had no visible effect on the differentiation and on the accumulation β-tubulin mRNA. However, cAMP antagonized the inhibitory effect of RA when it was co-treated with RA at the beginning of differentiation. The fact that addition of cAMP with RA at the beginning of differentiation which allowed the normal accumulation of β-tubulin mRNA suggested that cAMP repressed the effects of RA on transcription of the DS genes. Because both cAMP and dbcAMP are equally effective, it is suggested that this effect of cAMP is mediated by a receptor on the cell surface. The antagonistic effect of cAMP on RA has been reported (Desai et al., 1996).

RA (and other retinoids) is one of the key regulators of gene expression during cell differentiation and development of vertebrates. In this report, it has been shown that this compound could function in *N. gruberi* as a specific transcriptional regulator and that the effect of RA is related to the cAMP signal transduction pathway. These results have shown that the role of RA as a transcriptional regulator is evolutionarily conserved and suggest that the regulation of gene expression by RA during differentiation of *N. gruberi* could provide a simple model system to study the transcriptional regulation mechanism by RA.

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References

Allenby G, Jonocha R, Kazmer S, Speck J, Grippo JF, and Levin AA (1994) Binding of 9-cis retinoic acid and all-trans retinoic acid to retinoic acid receptor α, β, and γ. *J Biol*

Chem 269: 16689-16695.
Bok JW, Jin YJ, and Lee J (1995) Independent mechanisms are utilized for the coordinate and transient accumulation of two differentiation-specific mRNAs during differentiation of *Naegleria gruberi* amoebae into flagellates. *Exp Cell Res* 219: 47-53.
Caelles C, Gonzalez-Sancho JM, and Munoz A (1997) Nuclear hormone receptor antagonism with AP-1 by inhibition of JNK pathway. *Genes & Dev* 11: 3351-3364.
Cheon H (1996) Study of α-tubulin gene promoter of *Naegleria gruberi.* MS Thesis, Yonsei University, Korea, pp 1-61.
Desai D, Michalak M, Singh NK, and Niles RM (1996) Inhibition of retinoic acid receptor function and retinoic acid-regulated gene expression in mouse melanoma cells by calreticulin. A potential pathway for cyclic AMP regulation of retinoid action. *J Biol Chem* 271: 15153-15159.
Durand B, Saunders M, Leroy P, Leid M, and Chambon P (1992) All-trans and 9-cis retinoic acid induction of CRABP II transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. *Cell* 71: 73-85.
Fukuo KM, Takigagawa M, Tajima K, Enomoto M, Kumahara Y, and Suziki F (1986) Comparision of inhibition by tumor promoter (12-O-tetradecanoyl-phorbol-13-acetate) of expression of the differentiated phenotype of chondrocytes in rabbit costal chondrocytes in culture with inhibition by retinoic acid. *J Biochem* 99: 385-396.
Fulton C (1977) Cell differentiation and flagellar elongation in *Naegleria gruberi. Annu Rev Microbiol* 31: 597-629.
Fulton C and Dingle AD (1967) Appearance of the flagellate phenotype in populations of *Naegleria* amebae. *Dev Biol* 15: 169-191.
Fulton C and Walsh C (1980) Cell differentiation and flagellar elongation in *Naegleria gruberi. J Cell Biol* 85: 346-359.
Fulton C, Lai EY, and Remillard SP (1995) A flagellar calmodulin gene of *Naegleria*, coexpressed during differentiation with flagellar tubulin genes, shares DNA, RNA, and encoded protein sequence elements. *J Cell Biol* 270: 5839-5848.
Gianni M, Terao M, Sozzani S, and Garaffini E (1993) Retinoic acid and cyclic AMP synergistically induce the expression of liver/bone/kidney-type alkaline phosphatase gene in L929 fibroblastic cells. *Biochem J* 296: 67-77
Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans M, and Thaller C (1992) 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406.
Huang C, Ma WY, Dawson Mi, Rincon M, Flawell RA, and Dong Z (1997) Blocking activator protein-1 activity, but not activating retinoic acid response element, is required for the antitumor promotion effect of retinoic acid. *Proc Natl Acad Sci USA* 94: 5826-5830.
Kastner P, Mark M, and Chambon P (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83: 859-869.
Lee JH and Walsh CJ (1988) Transcriptional regulation of coordinate changes in flagellar mRNA during differentiation of *Naegleria gruberi* amebae into flagellates. *Mol Cell Biol* 8: 2280-2287.
Mar J, Lee JH, Shea D, and Walsh CJ (1996) New poly(A)+ RNAs appear coordinately during the differentiation of *Naegleria gruberi* amobae into flagellates. *J Cell Biol* 102: 353-361.
Martin C, Ziegler LM, and Napoli JL (1990) Retinoic acid, dibutylyl-cAMP, and differentiation affect the expression of retinoic acid receptors in F9 cells. *Proc Natl Acad Sci USA* 87: 4804-4808.
Means AL and Gudas LJ (1995) The roles of retinoids in vertebrate development. *Annu Rev Biochem* 64: 201-233.
Minucci S, Botquin V, Yeom YI, Dey A, Sylvester I, Zand DJ, Ohbo K, Ozato K, and Scholer R (1996) Retinoic acid-mediated down-regulation of *Oct3/4* coincides with the loss of promoter occupancy *in vivo. EMBO J* 15: 888-899.
Montminy M (1997) Transcriptional regulation by cyclic AMP. *Annu Rev Biochem* 66: 807-822.
Nagpal S, Saunders M, Kastner P, Durand B, Nakshatri H, and Chambon P (1992) Promoter context- and responsive element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell* 70: 1007-1019.
Pan L, Eckhoff C, and Brinckerhoff CE (1995) Suppression of collagenase gene expression by all-trans and 9-cis retinoic acid is ligand dependent and requires both RARs and RXRs. *J. Cell Biochem* 57: 575-589.
Parent CA and Devreotes PN (1996) Molecular genetics of signal transduction in *Dictyostelium. Annu Rev Biochem* 65: 411-440.
Permann T and Evans RM (1997) Nuclear receptors in Sicily: all in the famiglia. *Cell* 90: 391-397.
Pickarsky E, Sharir H, Ben-Shushan E, and Bergman Y (1994) Retinoic acid represses *Oct-3/4* gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. *Mol Cell Biol* 14: 1026-1038.
Schroen DJ and BRinckerhoff CE (1996) Inhibition of rabbit collagenase (matrix metalloproteinase-1; MMP-1) transcription by retinoid receptors: evidence for binding of RARs/RXRs to the -77 AP-1 site through interactions with c-Jun. *J Cell Physiol* 169: 320-332.
Schüle R, Rangarajan P, Yang N, Kliewer S, Ransone LJ, Bolado J, Verma IM, and Evans RM (1991) Retinoic acid is a negative regulator of AP-1-responsive genes. *Proc Natl Acad Sci USA* 88: 6092-6096.
Shea DK and Walsh CJ (1987) mRNAs for α- and β-tubulin and flagellar calmodulin are among those coordinately regulated when *Naegleria gruberi* amebae differentiate into flagellates. *J Cell Biol* 105: 1303-1309.
Sussman DJ, Lai EY, and Fulton C (1984) Rapid disappearance of translatable actin mRNA during cell differentiation in *Naegleria. J Biol Chem* 259: 7355-7360.
Towers G, Harris J, Lang G, Collins MK, and Latchman DS (1995) Retinoic acid inhibits both the basal activity and phorbol ester-mediated activation of the HIV long terminal repeat promoter. *AIDS* 9: 129-136.
Tran P, Zhang X, Salbert G, Hermann T, Lehmann JM, and Pfahl M (1992) COUP orphan receptors are negative regulators of retinoic acid response pathway. *Mol Cell Biol* 12: 4666-4676.
Walsh C (1984) Synthesis and assembly of the cytoskeleton of *Naegleria gruberi* flagellates. *J Cell Biol* 98:449-456.
Zelent A, Krust A, Petkovich M, Kastner P, and Chambon P (1989) Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. *Nature* 339: 714-717.

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