8-Chloro-cAMP Inhibits Transforming Growth Factor α Transformation of Mammary Epithelial Cells by Restoration of the Normal mRNA Patterns for cAMP-dependent Protein Kinase Regulatory Subunit Isoforms Which Show Disruption upon Transformation*

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Differential regulation of the regulatory subunits of cAMP-dependent protein kinase isoforms correlates with the growth inhibitory effect of site-selective 8-Cl-cAMP demonstrated in cancer cell lines (Ally, S., Tortora, G., Clair, T., Grieco, D., Merio, G., Katsaros, D., Ogreid, D., Döskeland, S. O., Johansen, T., and Cho-Chung, Y. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 85, 6319–6322). Such selective modulation of protein kinase isozyme regulatory subunits was also found in the 8-Cl-cAMP-induced inhibition of both transformation and transforming growth factor α (TGFα) production in Ki-ras-transformed rat kidney fibroblasts (Tortora, G., Ciardiello, F., Ally, S., Clair, T., Salomon, D. S., and Cho-Chung, Y. S. (1989) FEBS Lett. 242, 363–367). In this work, we have demonstrated that 8-Cl-cAMP antagonizes the TGFα effect in TGFα-transformed mouse mammary epithelial cells (NOG-8TFC17) at the level of gene expression for cAMP receptor protein isoforms, RI and RII (the regulatory subunits of protein kinase isozymes). Northern blot analysis demonstrated that in the transformed NOG-8TFC17 cells, compared with the nontransformed counterpart NOG-8 cells, the mRNA levels for the RI cAMP receptor protein markedly increased, whereas the mRNA levels for the RII and RIIc cAMP receptor proteins decreased. 8-Cl-cAMP, which induced growth inhibition and phenotypic reversion in NOG-8TFC17 cells, caused an inverse change in the mRNA patterns of the cAMP receptor proteins; RI cAMP receptor mRNA sharply decreased to levels comparable with that of the nontransformed NOG-8 cells, whereas RIIc mRNA increased to a level even greater than that in the NOG-8 cells. In addition, one mRNA species of RII increased, whereas the other RIIc mRNA species decreased during the treatment. The mRNA level for the catalytic subunit of protein kinase, however, did not change during 8-Cl-cAMP treatment. In addition, 8-Cl-cAMP brought about a reduction in both TGFα mRNA and protein levels. These coordinated changes in the expression of the cAMP receptor proteins and TGFα were not observed during cis-hydroxyproline- or TGFα-induced growth inhibition of the NOG-8TFC17 cells. Thus, the antagonistic effect of 8-Cl-cAMP toward TGFα-induced transformation involves modulation of the expression of a specific set of cellular genes.

In mammalian cells, cAMP functions through two classes of cAMP-dependent protein kinases (1, 2), designated types I and II. Type I and II protein kinases are distinguished by their regulatory subunits (RI and RII, respectively) (3, 4). Four different regulatory subunits (RI, previously designated R1 (5), RII (6), RIIc (RIIc) (7), and RIIb (RIIb) (8)) have now been identified at the gene/mRNA level (see Ref. 9 for nomenclature). Two different catalytic subunits (Cα (10) and Cβ (11, 12)) have also been identified; however, preferential coexpression of either one of these catalytic subunits with either the type I or II protein kinase regulatory subunit has not been found (12).

Recently, we discovered that site-selective cAMP analogs, which show a preference for binding to type II rather than type I protein kinase of purified preparations in vitro (13, 14), provoke potent growth inhibition, differentiation, and reverse transformation in a broad spectrum of human and rodent cancer cell lines (15–17). We have also demonstrated that the antineoplastic effects of site-selective 8-Cl-cAMP are associated with an antagonistic activity on the effects of exogenous transforming growth factor α (TGFα) in normal rat kidney fibroblasts and on the production of TGFα in Ki-ras-transformed rat kidney cells (18).

TGFα is a potent mitogen for fibroblasts and epithelial cells (19) and has been implicated in the autocrine growth of rodent and human tumor cells. In fact, enhanced expression of TGFα has been detected in a number of rodent and human breast cancer cell lines and in a majority of primary rodent and human breast carcinomas (20–24). In addition, the ability of estrogens to stimulate the growth of estrogen-dependent breast cancer cells may be related in part to an increased

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1 The abbreviations used are: RI and RII, regulatory subunits of isozymes I and II of cAMP-dependent protein kinase, respectively; TGFα, transforming growth factor α; DMEM, Dulbecco's Modified Minimal Essential Medium; PBS, Phosphate-Buffered Saline; CHF, 4-cis-hydroxy-L-proline; RIA, radioimmunoassay; RRA, radioreceptor assay; EGF, epidermal growth factor; bp, base pair(s); kb, kilobase pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOFS, 3-(N-morpholino)propanesulfonic acid; 8-NeAMPS, 8-azidodeadenosine 3′,5′-monophosphate.
production of TGFα in response to estrogen stimulus (20, 21, 24). Also, experimentally, overexpression of the human TGFα gene in an expression vector plasmid successfully converts in vitro (25) an immortalized nontransformed mouse mammary epithelial cell clone (NOG-8) to a transformed phenotype (NOG-8TFC17) that produces tumors in vivo (25). NOG-8 TFC17 cells, therefore, represent a novel provided by R. K. Robins (Nucleic Acid Research Institute). Pepsticals, Inc. Dulbecco's modified minimal essential medium (DMEM), to investigate the regulatory mechanisms underlying cell proliferation and transformation and to eventually identify the antitransforming factor that has the ability to interfere with the production and/or action of TGFα.

In this work, we have examined the effect of 8-Cl-cAMP on TGFα-mediated transformation of NOG-8TFC17 cells in comparison with that of two other known growth inhibitors, cis-hydroxyproline (26, 27) and TGFβ (28, 29). The results are correlated with the expression of cAMP-dependent protein kinase isozymes and TGFα production in NOG-8TFC17 cells.

**EXPERIMENTAL PROCEDURES**

*Materials*—8-Cl cAMP/Na⁺ and Nβ-benzyl cAMP were kindly provided by R. K. Robins (Nucleic Acid Research Institute). Pepsticals, Inc. Dulbecco's modified minimal essential medium (DMEM), tetal bovine serum (FBS), trypsin/EDTA solution, penicillin/streptomycin solution, and L-glutamine were obtained from Gibco. 4-cis Hydroxy-L-proline (CHP) was obtained from Calbiochem. Porcine TGFβ was purchased from R and D systems. HEPES, pH 7.4, 4 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For monolayer growth, the cells (2 × 10⁴ cells/well) were plated in multiwell cluster dishes (Costar) in 2 ml of DMEM containing 10% FBS. At 24 h after seeding (day 0), the medium was changed, and fresh medium was added every 48 h thereafter. Starting at day 0, 8-Cl-cAMP, CHF, or TGFβ was added when the medium was replenished. At the indicated times, cells were harvested by trypsinization (Gibco), and cell counts were performed on a ZBI staining of the gels revealed that equivalent amounts of RNA were present in all of the samples. The gels were then transferred to Biorens nylon membranes (ICN Biomedicals) by the method of Thomas (33) and hybridized to the following “P-labeled nick-translated cDNA probes: a 406-bp EcoRI-Apal restriction fragment derived from a human TGFα cDNA clone; TGFα-C1 (34); a 600-bp PstI restriction fragment of the mouse cAMP-dependent protein kinase type I regulatory subunit, RL, cDNA clone (35); a 1600-bp BamHI restriction fragment of the human cAMP-dependent protein kinase type II regulatory subunit, RII, cDNA clone (36); and a 1600-bp EcoRI restriction fragment of the human cAMP-dependent protein kinase catalytic subunit, C, cDNA clone (38).

**RESULTS**

*Effect on Cell Growth*—The effect of 8-Cl-cAMP on the growth of the transformed NOG-8TFC17 cells and nontransformed counterpart NOG-8 cells is shown in Fig. 1. 8-Cl-cAMP inhibited the monolayer growth of the NOG-8TFC17 cells in a concentration-dependent manner. At 10 and 25 μM, the analog produced 50 and 70% growth inhibition, respectively, when compared with that in the untreated control cells (Fig. 1A). In contrast, up to a 50 μM concentration of 8-Cl-cAMP produced no growth inhibition in the nontransformed NOG-8 cells. A greater degree in the growth inhibitory effect of 8-Cl-cAMP was shown when the transformed NOG-8TFC17 cells were grown in soft agar. At 5 μM, the analog produced over 90% inhibition of colony formation compared with that in the untreated control cells (Fig. 1B). Fig. 1 also shows the effect of CHP on the growth of the NOG-8 and NOG-8TFC17 cells. The proline analog CHP has been shown to inhibit the growth of rat mammary tumors in vivo (26) and of virally transformed rodent cell lines in vitro (27). CHP exhibited growth inhibition in both the NOG-8 and NOG-8TFC17 cells, although it produced a more potent effect on the transformed cells than on the nontransformed cells (Fig. 1C). At 25 μg/ml, CHP inhibited over 90% of the colony formation of the NOG-8TFC17 cells in soft agar (Fig. 1D).

**Radioimmunoassay (RIA) and Radioreceptor Assay (RRA) for TGFα**—The levels of immunoreactive TGFα were determined using a liquid-phase competitive RIA with a polyclonal rabbit anti-rat TGFα antiserum that is specific for TGFα and fails to recognize either mouse or human epithelial growth factor (EGF) as previously described (21, 22). Labeled rat synthetic 125I-TGFα, rabbit anti-TGFα antiserum, and other reagents were purchased from Biotope Inc. EGF receptor-competing activity was analyzed as previously described (23) by using representative cultures of NOG-8TFC17 cells, a human mammary epithelial cell line that possesses approximately 10⁵ EGF receptor sites/cell. The amount of TGFα equivalent units in the samples was calculated in comparison with the competition curves produced by different concentrations of unlabeled human TGFα (Bachem) with 1 ng/ml mouse 125I-EGF (specific activity = 100 μCi/μg, Amersham Corp.).

**Isolation of Total RNA and Northern Blot Analysis**—The cells (10⁶ washed twice with phosphate-buffered saline) were lysed in 4.2 M guanidine isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl (N-lauroylsarcosine/Na+), and 0.1 M β-mercaptoethanol; the lysates were homogenized; and total cellular RNA was sedimented through a CsCl cushion (5.7 M CsCl, 0.1 M EDTA) as described by Chirgwin et al. (32). Total cellular RNA containing 20 mm MOPS, pH 7.0, 50% formamide, and 6% formaldehyde was denatured at 65°C for 10 min and electrophoresed through a denaturing 1.2% agarose, 2.2 M formaldehyde gel. Ethidium bromide staining of the gels revealed that equivalent amounts of RNA were present in all of the samples.

**Fig. 1. Effect of 8-Cl-cAMP and CHP on monolayer (A and C) and soft agar (B and D) growth of NOG-8 and NOG-8TFC17 cells. The data represent an average ± S.D. of four experiments. Colonies in the untreated controls were 1300 ± 55/dish.**
TGFβ at 1–5 ng/ml produced a weak growth inhibitory effect (~30% inhibition) on both NOG-8 (in monolayer culture) and NOG-8TFC17 (in monolayer culture or in soft agar) cells.

Time courses of the growth inhibition produced by 8-Cl-cAMP (25 μM) and CHP (30 μg/ml) in the NOG-8TFC17 cells in monolayer culture are shown in Fig. 2. The growth inhibition by these analogs required two to three population doublings. On day 3, both analogs produced a 30–50% growth inhibition; and by day 4, a 60–70% growth inhibition was achieved compared with that in the untreated control cells.

The growth inhibition in NOG-8TFC17 cells brought about by 8-Cl-cAMP or CHP accompanied distinct morphological changes. The growth-arrested cells exhibited a reverted morphology that resembled that of nontransformed NOG-8 cells (data not shown).

**Effect of TGFα Production**—We examined whether the growth inhibitory effects of 8-Cl-cAMP, CHP, and TGFβ on the NOG-8TFC17 cells are associated with a specific interference by these agents on TGFα production. The conditioned media (see “Experimental Procedures”) were collected from the culture of the NOG-8TFC17 cells, and the levels of the immunoreactive and biologically active TGFα were measured by RIA and RRA, respectively. As shown in Table I, 8-Cl-cAMP caused a decrease of TGFα production in the transformed NOG-8TFC17 cells in a concentration-dependent manner. Twenty and sixty percent reductions in TGFα production were achieved by 25 and 50 μM 8-Cl-cAMP, respectively. 8-Cl-cAMP (5 μM) and Nα-benzyl-cAMP (15 μM) each alone had no inhibitory effect on TGFα production. However, these analogs in combination at these respective concentrations exerted as much inhibitory effect as that produced by these analogs alone at higher concentrations (~50 μM), demonstrating a synergistic effect (data not shown).

In contrast, both CHP and TGFβ at the concentrations that demonstrate growth inhibition exhibited little or no effect on the TGFα production in NOG-8TFC17 cells (Table I). The results in Table I indicate that the growth inhibitory effect of 8-Cl-cAMP is associated with inhibition of TGFα production, whereas that of CHP and TGFβ does not involve interference of TGFα production.

**mRNAs of cAMP Receptor Proteins and TGFα**—The effects of 8-Cl-cAMP, CHP, and TGFβ on the mRNA levels of the regulatory (RI, RIIα, and RIIβ) and catalytic (Cα) subunits of cAMP-dependent protein kinases and TGFα were determined in the NOG-8TFC17 cells. The RNA filters prepared were probed with [3P]-labeled cDNAs of RIIα, RIIβ, and TGFα; Northern blot analysis is shown in Fig. 3. The results are expressed in relation to the mRNA levels in the nontransformed NOG-8 cells (Fig. 3, lower). The human RIIα cDNA probe detected 6.0- and 2.2-kb mRNA species in the NOG-8 cells (Fig. 3, upper lane NOG-8). Transformation with TGFα caused a 70% decrease in both the 6.0- and 2.2-kb RIIα mRNA species (lane C). 8-Cl-cAMP treatment of the transformed cells brought about a further decrease in the 6.0-kb mRNA, but an increase in the 2.2-kb mRNA species (lane 8-Cl-

**TABLE I**

**Effect of 8-Cl-cAMP, CHP, and TGFβ on TGFα production in NOG-8TFC17 cells**

| Treatment     | TGFα in CM | RIα | RIIα | RIIβ | RRA  |
|---------------|------------|-----|------|------|------|
| None          | 660 ± 60   | 680 ± 66 | 100   | 100 | 100   |
| 8-Cl-cAMP     | 550 ± 47 (83) | 530 ± 52 (78) | 250 ± 20 (38) | 250 ± 20 (37) |
| 25 μM         | 640 ± 55 (97) | 670 ± 63 (99) | 570 ± 55 (86) | 590 ± 57 (87) |
| TGFβ (1 ng/ml)| 460 ± 62 (103) | 650 ± 83 (96) | 4.2 ± 0.3 | 3.0 ± 0.9 | 12.3 ± 1.3 |

**Fig. 2.** Temporal course of effect of 8-Cl-cAMP and CHP on monolayer growth of NOG-8TFC17 cells. The data represent an average ± S.D. of four experiments.

**Fig. 3.** Northern blot analysis of RIIα, RIIβ, and RIα cAMP receptor and TGFα mRNAs in NOG-8 (untreated) and NOG-8TFC17 cells untreated and treated with 8-Cl-cAMP (25 μM), CHP (30 μg/ml), or TGFβ (1 ng/ml) for 3 days. Control and treated cells were harvested, and total RNA was extracted by the guanidine isothiocyanate method as described under "Experimental Procedures." Twenty micrograms of total RNA/lane were run on 1.2% agarose-formaldehyde gels and blotted onto nylon membranes. Untreated control cells (lane C) were also grown for 3 days. The data in the table represent quantification by densitometric scanning of the autoradiograms. The data are expressed relative to the levels in nontransformed NOG-8 cells, which are set equal to 1 arbitrary unit. The TGFα data are expressed relative to the levels in the untreated control NOG-8TFC17 cells, which are set equal to 10 arbitrary units. N.D., not detectable. The data represent an average ± S.D. of four experiments.
cAMP); in total quantity, RIIa mRNA decreased to 30% of that in the untreated transformed cells (Fig. 3). CHP and TGFβ treatment brought about little or no effect on the levels of the 6.0- and 2.2-kb RII, mRNA species (Fig. 3). The human RIIα cDNA detected a 1.9-kb mRNA species. The RIIα mRNA level decreased by 50% in the transformed NOG-8TFC17 cells (lane C) compared with that in the nontransformed cells (lane NOG-8). 8-Cl-CAMP treatment of NOG-8TFC17 cells brought about a 16-fold increase in the RII, mRNA level over that in the untreated control cells (Fig. 3). CHP caused little or no effect on the RII, mRNA level, but TGFβ caused a substantial increase (6-fold) in the RII, mRNA level (Fig. 3). The mouse RIIα cDNA probe detected a 3.2- and 1.7-kb mRNA species. Both the 3.2- and 1.7-kb mRNA species markedly increased (~10-fold) in the transformed NOG-8TFC17 cells (lane C) compared with the nontransformed cells (lane NOG-8). 8-Cl-CAMP caused a marked decrease in both the 3.2- and 1.7-kb mRNA levels comparable to that in the nontransformed cells (Fig. 3). CHP also decreased the RII, mRNA level by 60%, but TGFβ caused little or no effect on RII, mRNA (Fig. 3). The mouse C, cDNA probe detected a 2.4-kb mRNA species in the transformed and nontransformed cells in a similar quantity, and treatment with 8-Cl-CAMP, CHP, or TGFβ caused no change in the mRNA levels (data not shown).

The human TGFα cDNA probe detected a 2.3-kb mRNA species in the transformed NOG-8TFC17 cells (Fig. 3, upper, lane C), but not in the nontransformed cells (lane NOG-8). 8-Cl-CAMP treatment brought about a 90% reduction in the TGFα mRNA level of NOG-8TFC17 cells (Fig. 3), whereas CHP decreased the TGFα mRNA level only by 20%, and TGFβ caused a slight increase in the TGFα mRNA level (Fig. 3).

In previous work, we demonstrated that site 1- and 2-selective cAMP analogs, which in combination produce synergistic enhancement of binding to type II rather than type I cAMP-dependent protein kinase (13, 14), also produces a synergistic growth inhibitory effect (15-17, 39). We examined whether such synergism exists between 8-Cl-CAMP (site 1-selective) and N'-benzyl-cAMP (site 2-selective) in changing the mRNA levels for CAMP receptor proteins and TGFα in NOG-8TFC17 cells. When cells were treated with 5 μM 8-Cl-CAMP, which alone gave a 20% growth inhibition (Fig. 1), the mRNA levels of cAMP receptor and TGFα were not significantly different from those in the untreated control cells (Table II). However, 5 μM 8-Cl-CAMP in combination with 15 μM N'-benzyl-cAMP brought about changes in these mRNA levels to an extent similar to that produced by higher concentrations of 8-Cl-CAMP (25 μM) alone (Table II). In addition, 15 μM N'-benzyl-cAMP alone neither exerted growth inhibition or changed these mRNA levels (Table II). However, 50 μM N'-benzyl-cAMP, which demonstrated a moderate growth inhibitory effect, brought about changes in these mRNA levels to a lesser extent than that produced by 25 μM 8-Cl-CAMP (Table II).

**DISCUSSION**

Our study presents the first evidence of the cAMP antagonism for TGFα at the level of cellular gene expression. The data presented in this study show that there is an antagonistic interaction between TGFα and 8-Cl-CAMP in the regulation of mammary epithelial cell proliferation. TGFα induces transcription in an immortalized population of mouse mammary epithelial cells (25), whereas 8-Cl-CAMP, a potent growth inhibitor for a spectrum of human cancer cell lines (18-17), can reverse such transformation. This antagonism between TGFα and 8-Cl-CAMP is expressed in the modulation of the genes for different species of the cAMP receptor proteins. The transformation of mammary epithelial cells with TGFα resulted in a marked increase in the RII, cAMP receptor mRNA level along with a decrease in the RII, and RIIα cAMP receptor mRNA levels. In the reverse transformation by 8-Cl-CAMP, the RII, mRNA level decreased, whereas one species of RII, mRNA and RII, mRNA levels sharply increased, converting the RII/RI CAMP receptor mRNA ratio to a value similar to that in nontransformed mammary epithelial cells.

Thus, transformation by TGFα brought about a disruption in the normal mRNA patterns of cAMP receptor species, and 8-Cl-CAMP restored the normal cAMP receptor mRNA patterns in the mammary epithelial cells. The changing patterns of cAMP receptor mRNAs brought about by 8-Cl-CAMP are the specific effects of the analog rather than a nonspecific event related to growth inhibition in general since two other known growth inhibitors, namely CHP (a proline analog) and TGFβ, inhibited the growth of the transformed mammary epithelial cells without reverting the abnormal cAMP receptor mRNA patterns to the normal patterns.

That these effects of 8-Cl-CAMP are due to the interaction of this cAMP analog with type II cAMP dependent protein kinase is supported by the synergistic effect observed with 8-Cl-CAMP and N'-benzyl-cAMP in combination (Table II). Such synergism between site 1- and 2-selective cAMP analogs has been previously demonstrated with purified preparations of protein kinase in vitro (13, 14) and in the growth inhibition and differentiation induced in cancer cells by these cAMP analogs (15-17, 39). Moreover, in the growth inhibition induced by 8-Cl-CAMP of LS-174T human cancer cells, there was rapid translocation of RIIα cAMP receptor protein from the cytoplasm to the nucleus that preceded increased transcription of the RIIα gene with decreased transcription of the RIIα gene (40). Based on these previous findings, we interpret our present results of the changing levels of mRNAs for cAMP receptor protein to be due to changes in transcription rather than to effects on mRNA stability.

Concomitantly, with these changes in mRNAs for the cAMP receptor proteins, 8-Cl cAMP also brought about a reduction in the TGFα mRNA level. In these cells, TGFα production is driven by the SV40 promoter. Whether the inhibitory effects of 8-Cl-CAMP were directed against the SV40 promoter is not known at present. A CAMP- and phorbol ester-responsive element (activator protein 2)-binding sequence has been identified in the transcription enhancer of

**TABLE II**

| Treatment | RIIα | RIIβ | RIIα | TGFα |
|-----------|-----|-----|-----|-----|
| None      | 0.2 ± 0.02 | 0.5 ± 0.05 | 10.0 ± 1.0 | 10.0 ± 1.0 |
| 8-Cl-CAMP | 0.07 ± 0.01 | 0.8 ± 0.08 | 1.5 ± 0.2 | 1.0 ± 0.1 |
| 5 μM      | 0.2 ± 0.02 | 0.7 ± 0.08 | 8.5 ± 0.8 | 9.0 ± 0.9 |
| 25 μM N'-benzyl-cAMP | 0.2 ± 0.02 | 0.5 ± 0.05 | 10.0 ± 1.0 | 10.0 ± 1.0 |
| 8-Cl-CAMP (5 μM) + N'-benzyl-cAMP (15 μM) | 0.07 ± 0.01 | 0.7 ± 0.08 | 2.5 ± 0.3 | 1.5 ± 0.2 |
| 8-Cl-CAMP (50 μM) | 0.10 ± 0.01 | 5.0 ± 0.7 | 4.5 ± 0.5 | 5.0 ± 0.5 |

*The data are expressed relative to the levels in nontransformed NOG-8 cells, which are set equal to 1 arbitrary unit (see Fig. 3) and represent an average ± S.D. of three experiments.
the SV40 promoter (41). However, no negative control by cAMP of such a viral promoter is known.

The results presented here suggest that 8-Cl-cAMP, binding to its specific site at cAMP receptor proteins (40) by substituting for endogenous cAMP, modulates its own receptor expression as well as those of proliferative signal(s), thereby regulating cell proliferation and transformation. Furthermore, the precise antagonism between TGFα and 8-Cl-cAMP demonstrated at the level of cellular gene expression in the growth control of the experimental mammary epithelial cells suggests the possibility that such antagonism may be a fundamental mechanism for the growth regulation of human cancers.

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