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Selective modulation of protein kinase isozymes by the site-selective analog 8-chloroadenosine 3′,5′-cyclic monophosphate provides a biological means for control of human colon cancer cell growth

(cAMP receptor proteins/nuclear translocation/gene transcription)

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ABSTRACT **Differential expression of type I and type II cAMP-dependent protein kinase isozymes has been linked to growth regulation and differentiation. We examined the expression of protein kinase isozymes in the LS 174T human colon cancer cell line during 8-chloroadenosine 3′,5′-cyclic monophosphate (8-Cl-cAMP)-induced growth inhibition. Two species of R^{II} (the regulatory subunit of protein kinase type II) with apparent M_r 52,000 (R^{II}_{52}) and M_r 56,000 (R^{II}_{56}) and a single species of R^{I} (the regulatory subunit of protein kinase type I) with M_r 48,000 were identified in the cancer cells. R^{I} and both forms of R^{II} were covalently labeled with 8-azidoadenosine 3′,5′-cyclic [^{32}P]monophosphate, and two anti-R^{II} antibodies that exclusively recognize either R^{II}_{52} or R^{II}_{56} resolved two forms of the R^{II} receptors. 8-Cl-cAMP treatment induced a decrease of R^{I} and an increase of both R^{II}_{52} and R^{II}_{56} in the cytosols of cancer cells and rapid translocation (within 10 min) of R^{II}_{52} from the cytosol to nucleus. 8-Cl-cAMP caused transcriptional activation of the R^{II}_{52} receptor gene and inactivation of the R^{I} receptor gene. It also exhibited high-affinity site-1-selective binding to the purified preparations of both R^{II} receptor proteins. Thus, differential regulation of various forms of cAMP receptor proteins is involved in 8-Cl-cAMP-induced regulation of cancer cell growth, and nuclear translocation of R^{II}_{52} receptor protein appears to be an early event in such differential regulation.**

The actions of cAMP in mammalian cells are mediated through two classes of cAMP-dependent protein kinases (1–4), designated type I and type II. The type I and type II enzymes have a common catalytic subunit (3, 4) but differ in the physical properties, immunological characteristics, and primary sequences of their cAMP-binding regulatory subunits (3, 4). There appears to be one principal form of the regulatory subunit in the type I enzyme (R^{I}) and two (or more) types of the regulatory subunit in the type II enzyme (R^{II}), expressed in a tissue-specific manner (5–8). Multiple forms of R^{II} subunits have been found in normal and neoplastic tissues of various cell types (5–10). However, very little is known about the cellular mechanisms that control the expression of R^{I} or the multiple forms of R^{II}.

We discovered (11) that differential regulation of type I versus type II protein kinase is involved in the inhibition of human cancer cell growth by site-selective cAMP analogs. Depending on their substituents on the adenine ring, these analogs selectively bind to site 1 (site B) (C-2 and C-8 analogs) or site 2 (site A) (C-6 analog), the two known cAMP-binding sites of protein kinase (12, 13). Moreover, among the site 1-selective analogs, C-8-thio or -halogen analogs preferentially bind to site 1 of type II rather than type I protein kinase (14, 15). This distinctive binding specificity of site-selective cAMP analogs for two types of protein kinase, in fact, correlated with the potency of cAMP analogs in inhibiting growth (11).

To investigate the role of protein kinase isozymes in growth regulation, we have examined the concentration, subcellular localization, and transcriptional activity of genes for these isozymes during 8-chloroadenosine 3′,5′-cyclic monophosphate (8-Cl-cAMP)-induced growth inhibition of the LS 174T human colon cancer cell line [8-Cl-cAMP is the most potent analog (11)]. The results were analyzed in relation to the binding affinity of 8-Cl-cAMP for the purified preparations of protein kinase isozymes.

MATERIALS AND METHODS

Materials. cAMP and N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP) were obtained from Boehringer Mannheim. 8-Cl-cAMP was kindly provided by R. K. Robins (The Nucleic Acid Research Institute, Costa Mesa, CA). Pepstatin, antipain, chymostatin, leupeptin, and soybean trypsin inhibitor were from Sigma. 8-Azidoadenosine 3′,5′-cyclic [^{32}P]monophosphate (60.0 Ci/mmol; 1 Ci = 37 GBq) and ^{125}I-labeled protein A (30 mCi/mg) were obtained from ICN. Eagle's minimal essential medium, fetal bovine serum, the trypsin/EDTA solution, the penicillin–streptomycin solution, L-glutamine, Hepes buffer (1 M stock solution; pH 7.3), and minimal essential medium nonessential amino acids were obtained from GIBCO.

Cell Culture. The LS 174T human colon carcinoma cell line (provided by J. Greiner, National Cancer Institute) was grown in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum, Eagle's minimal essential medium nonessential amino acids, 20 mM Hepes, 2 mM glutamine, and penicillin–streptomycin; the medium was changed every 48 hr. Growth inhibition of LS 174T cells was induced by addition of 1–10 μM 8-Cl-cAMP (using 100× concentrated stock solutions) starting at 24 hr after seeding and every 48 hr thereafter.

Preparation of Cell Cytosol and Nuclear Fraction. All procedures were performed at 0–4°C. The cell pellets (2×10^7 cells), after two washes with isotonic phosphate-buffered

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Abbreviations: R^{I} and R^{II}, regulatory subunits of cAMP-dependent protein kinase types I and II, respectively; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; 8-Cl-cAMP, 8-chloroadenosine 3′,5′-cyclic monophosphate.

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saline, were suspended in 0.5 ml of buffer A [0.25 M sucrose/2 mM $MgCl_2$/1 mM $CaCl_2$/10 mM KCl/20 mM Tris·HCl, pH 7.5/0.1 mM pepstatin/0.1 mM antipain/0.1 mM chymostatin/0.2 mM leupeptin/aprotinin (0.4 mg/ml)/soybean trypsin inhibitor (0.5 mg/ml)] and homogenized with a Dounce homogenizer (60 strokes). The cytosols and nuclear fractions were prepared as described (16). The purified nuclear pellets were suspended in 200 μl of buffer A and used as nuclear suspension. Nuclei were judged to be free of cytoplasmic contamination and intact cells by the absence of glucose-6-phosphate dehydrogenase activity and by phase-contrast microscopy.

Photoaffinity Labeling of cAMP Receptor Proteins. The photoactivated incorporation of 8-azidoadenosine 3',5'-cyclic [^{32}P]monophosphate was performed as described (11, 17). The samples containing 25–75 μg of protein were subjected to electrophoresis in 0.05% $NaDodSO_4$/12% polyacrylamide gels (18), and the separated proteins were transferred to nitrocellulose sheets (19). The sheets were air-dried and exposed to Kodak XAR film overnight at −20°C.

Immunoblotting of R^{II} cAMP Receptor Protein. Cellular proteins present in the cell cytosol and nuclear fraction (see above) were separated and transferred to nitrocellulose sheets (19). The sheets were first incubated with 3% (wt/vol) bovine serum albumin (20) and then were sequentially incubated with medium containing the affinity-purified monospecific anti-R^{II} antibodies [the R^{II}_{56} antibody, our designation (bovine heart R^{II} antibody, provided by R. K. Sharma, University of Tennessee, Memphis), and the R^{II}_{52} antibody, our designation (described in ref. 21 as rat skeletal muscle R^{II} antibody)] overnight at 4°C and with ^{125}I-labeled protein A (5×10^5 cpm/ml) for 1 hr in an ice–water bath. The nitrocellulose sheets were air-dried and exposed to Kodak XAR film for 12–36 hr at −20°C.

Nuclear Run-off Transcription Assay. Nuclear run-off transcription assays were performed as described by McKnight and Palmiter (22). LS 174T cells were treated for various times with 8-Cl-cAMP (10 μM) and compared with untreated logarithmic-phase cells. DNA probes (2 μg per slot) were blotted onto nitrocellulose by using a slot blotter (Schleicher & Schuell). The R^I_α probe was a 0.6-kilobase (kb) *Pst* I fragment (mouse), entirely from inside the open reading frame (23), and the C_α probe was a 0.6-kb *Eco*RI fragment (mouse) containing a 3' coding region and ≈160 base pairs of 3' nontranslating sequence (24). Plasmids of these probes were kindly provided by S. McKnight (University of Washington, Seattle). The R^{II}_α (R^{II}_{56}) probe was a 1.65-kb *Eco*RI fragment (human) containing the whole reading frame (except for ≈80 nucleotides) and the 3' nontranslated region, and the R^{II}_β (R^{II}_{52}) probe was a 1.6-kb *Bam*HI fragment (human) containing half of the inside open reading frame and half of the 3' nontranslating region. The actin probe was human β actin (Oncor p7000 β actin) and the pUC probe was pUC19 (Pharmacia). After overnight prehybridization, the slots were hybridized with ^{32}P-labeled RNA (generated from 5×10^7 nuclei per culture condition) for 48 hr at 42°C, washed, air-dried, and exposed to Kodak XAR film for 4 days at −20°C.

RESULTS

Photoaffinity Labeling of cAMP Receptor Proteins During 8-Cl-cAMP Treatment. Since the type I and II protein kinases differ only in their regulatory subunits (the cAMP-binding receptor proteins) (3, 4), by using the photoaffinity ligand 8-azidoadenosine 3',5'-cyclic [^{32}P]monophosphate (17), we measured the R^I and R^{II} cAMP receptor proteins in the cytoplasmic and nuclear fractions of the untreated control cells and the cells treated with 8-Cl-cAMP.

As shown in Fig. 1, the untreated LS 174T cells contained

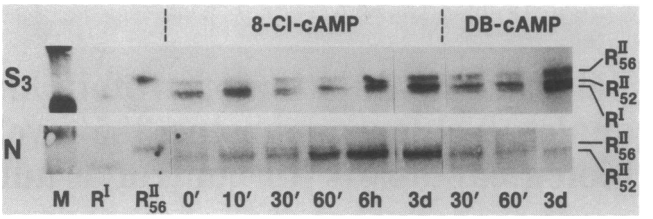

FIG. 1. Photoaffinity labeling of cAMP receptor proteins in cytosolic (S_3) and nuclear (N) fractions of LS 174T colon cancer cells during 8-Cl-cAMP and Bt_2cAMP treatment. Lanes: M, marker proteins of known molecular weight; 0', untreated control cells; 10'–3d, cells treated for indicated times with 8-Cl-cAMP (10 μM) or Bt_2cAMP (DB-cAMP) (1 mM). Each lane contained 25 μg protein for $NaDodSO_4$/PAGE. d, Days; ', min; h, hr.

a major cAMP receptor protein (M_r, 48,000), designated R^I (1), in the cytosols but not in the nuclei (lane 0'). Low intensity bands of M_r 52,000 and M_r 56,000 receptor proteins (R^{II}_{52} and R^{II}_{56}) (5–10) were also detected in the cytosol and nuclei of untreated cells (lane 0'). At 10 min after 8-Cl-cAMP (10 μM) treatment, the R^{II}_{52} receptor protein appreciably increased in the nuclei, reaching its maximum level by 6 hr and remaining elevated during treatment. Concomitant with the increase of the R^{II}_{52} receptor in the nuclei, there was a decrease of R^{II}_{52} in the cytosols (30 and 60 min). At later times, R^{II}_{52} in the cytosols increased (at 6 hr and 3 days). R^{II}_{56} also increased in the cytosols during treatment (6 hr and 3 days), but the nuclear R^{II}_{56} remained unchanged (Fig. 1). The R^I receptor protein in cytosol, after its transient increase (at 10 min of treatment), sharply decreased within 1 hr to a level below that in untreated cells and remained low during treatment (Fig. 1).

Bt_2cAMP (1 mM), which does not induce growth inhibition (11), brought about an increase in all three species of cAMP receptor protein in the cytosols and little or no increase in the nuclei (Fig. 1). The autoradiograph in Fig. 1 was scanned by densitometry; the results (Fig. 2) indicate that the R^{II}_{52} and R^{II}_{56} in the cytosol increased 3- to 5-fold and 2- to 3-fold, respectively, between 6 and 72 hr of 8-Cl-cAMP treatment, while R^I in the cytosol, after an initial transient increase (2-fold) at 10 min of treatment, decreased within 1 hr to 50%

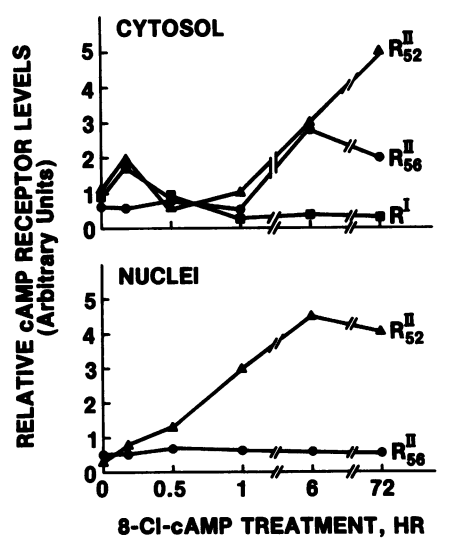

FIG. 2. Quantification of cAMP receptor proteins in the cytosolic and nuclear fractions of LS 174T cells during 8-Cl-cAMP treatment. The autoradiograph in Fig. 1 was scanned on a densitometer, and the levels of cAMP receptor proteins are expressed relative to the level of R^I receptor in the cytosol of untreated cells, which is set equal to 1 arbitrary unit.

of the level in untreated cells. R^{II}_{52} receptor in the nuclei increased 5- and 12-fold by 30 and 60 min of treatment, respectively; this increase coincided well with the decrease of R^{II}_{52} in the cytosol. The level of nuclear R^{II}_{56} remained unchanged during treatment, and R^{I} was not detected in the nuclei of either treated or untreated cells.

Immunoblotting Analysis of R^{II}_{52} and R^{II}_{56} cAMP Receptor Proteins. The different behaviors observed with the photoaffinity labeling experiments of the two forms of R^{II} cAMP receptor proteins of colon cancer cells during 8-Cl-cAMP treatment were further examined by immunoblotting analysis with two anti-R^{II} antibodies. Both antibodies are polyclonal and affinity-purified. One antibody, prepared from rat skeletal muscle R^{II} (21), specifically cross-reacted with R^{II}_{52} antigen but not with R^{II}_{56} antigen of colon cancer cells, and another antibody, prepared from bovine heart R^{II} (provided by R. K. Sharma), demonstrated monospecificity for R^{II}_{56} antigen but not for R^{II}_{52} antigen of colon cancer cells. These antibodies, therefore, are designated anti-R^{II}_{52} and anti-R^{II}_{56} antibodies, respectively. As shown in Fig. 3, the anti-R^{II}_{52} antibody detected a single band of M_r 52,000 R^{II} in the cytosol of untreated cells (lane 0′). The antibody barely detected the R^{II}_{52} antigen in the nuclei (lane 0′), but 10 min after 8-Cl-cAMP treatment, the R^{II}_{52} antigen appreciably increased in the nuclei (lane 10′). Further increase in the R^{II}_{52} antigen in the nuclei was accompanied by its decrease in the cytosols (lanes 30′ and 60′), indicating nuclear translocation of R^{II}_{52} receptor protein. After 6 hr of treatment, the R^{II}_{52} antigen increased in the cytosols to a level above that in the untreated cells (lanes 6h and 3d). Bt_2cAMP treatment brought about a less-pronounced increase of the R^{II}_{52} antigen than 8-Cl-cAMP treatment (Fig. 3).

The anti-R^{II}_{56} antibody detected a single band of M_r 56,000 R^{II} in both cytosol and nuclei of untreated cells. During the first hour of treatment with either 8-Cl-cAMP or Bt_2cAMP, there was little or no change in the amount of R^{II}_{56} antigen in both cytosol and nuclei (Fig. 3). At 6 hr after treatment with 8-Cl-cAMP, the R^{II}_{56} antigen in the cytosol increased (Fig. 3). These data confirmed qualitatively the results of photoaffinity labeling (Fig. 1). However, the magnitude of the changes in the amounts of R^{II}_{52} and R^{II}_{56} detected by the photoaffinity labeling was considerably greater than that observed with immunoblotting with the R^{II}_{52} and R^{II}_{56} antibodies. This may be due to the known limited capacity for R^{II} antibodies to cross-react with the heterologous antigens (25).

Effect of 8-Cl-cAMP on the Transcriptional Activities of cAMP Receptor Genes. We next examined the transcription of cAMP receptor genes after 8-Cl-cAMP treatment of colon cancer cells. Nuclear run-off transcriptional assays are shown in Fig. 4. Transcription of the R^{I} cAMP receptor gene remained unaffected during the initial 30 min of 8-Cl-cAMP treatment, then fell to 50% of that in untreated cells by 1 hr. In contrast, transcription of the R^{II}_{52} receptor gene increased 2-fold by 30 min and remained elevated during the course of the experiment. Transcription of the R^{II}_{56} receptor gene and the catalytic subunit gene (of protein kinase) remained unaffected throughout the course of the experiment, but the actin gene transcription was slightly enhanced at 30 min. A pUC control hybridized simultaneously gave little or no detectable signal.

DISCUSSION

We have demonstrated here that the growth inhibition induced by 8-Cl-cAMP of a human colon cancer cell line correlates with a selective modulation of various forms of cAMP receptor proteins, the regulatory subunits of cAMP-dependent protein kinases. Photoaffinity labeling and immunoblotting experiments identified three forms of cAMP receptor proteins, R^{I}, R^{II}_{52}, and R^{II}_{56}, in subcellular fractions of colon cancer cells and demonstrated changing levels of these receptors during 8-Cl-cAMP treatment. Nuclear run-off assays demonstrated that these changing levels of R^{I} and R^{II}_{52} receptors are, at least in part, due to changes in gene transcription. The transcriptional control of these receptors, however, did not appear to be the initial event in 8-Cl-cAMP-induced growth inhibition. Within 10 min after 8-Cl-cAMP treatment, there was a 2-fold increase in both R^{I} and R^{II}_{52} receptor levels even though the transcriptional changes of these genes could not yet be detected. Thus, 8-Cl-cAMP initially enhanced, at least transiently, the stability of R^{I} and R^{II}_{52} receptors. Change in the stability of R^{I} after treatment of S49 lymphoma cells with cAMP analogs (26) or an increase in the biosynthesis of R^{II}_{52} during differentiation of leukemic cells (27) has been shown.

In the present studies we report that, after initial stabilization, only R^{II}_{52} accumulates in the nucleus. The untreated control cells contained barely detectable levels of R^{II}_{52} in the nucleus; within 10 min after 8-Cl-cAMP treatment, the R^{II}_{52} increased in the nucleus, and its further increase there paralleled a decrease in the cytoplasmic R^{II}_{52}. Thus, 8-Cl-cAMP selectively induced nuclear translocation of the R^{II}_{52} receptor in intact cells *in vivo*.

To correlate the binding affinity of 8-Cl-cAMP for cAMP receptor proteins with the changes elicited by 8-Cl-cAMP of cAMP receptor levels in colon cancer cells, we examined the binding and activation by 8-Cl-cAMP of the purified preparations of protein kinases (Table 1). 8-Cl-cAMP demonstrated a high-affinity binding for site 1 of the R^{II} receptor

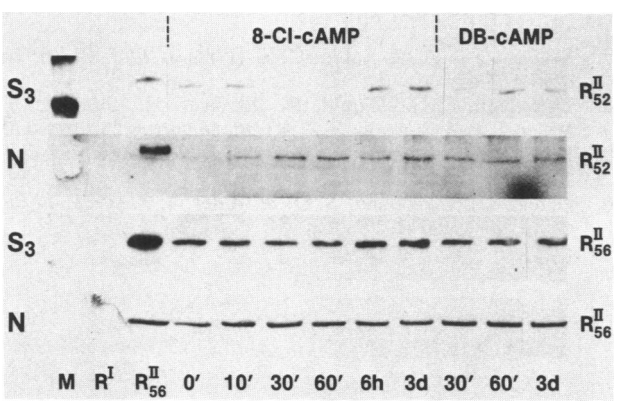

FIG. 3. Immunoblotting of R^{II}_{52} and R^{II}_{56} cAMP receptor proteins in cytosolic (S_3) and nuclear (N) fractions of LS 174T colon cancer cells during 8-Cl-cAMP and Bt_2cAMP (DB-cAMP) treatment. Immunoblotting of cAMP receptor proteins was performed using R^{II}_{52} and R^{II}_{56} antibodies. Lane designations are as described in Fig. 1.

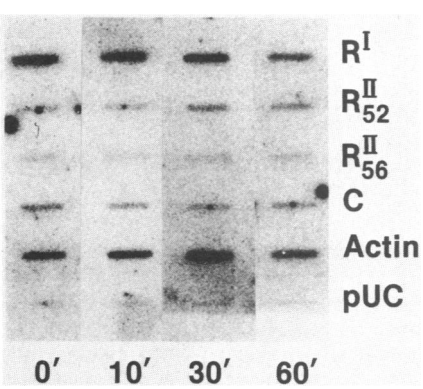

FIG. 4. Nuclear run-off transcriptional assay. Lanes: 0′, untreated control cells; 10′, 30′, and 60′, cells treated with 8-Cl-cAMP (10 μM) for 10 min, 30 min, and 60 min, respectively. The cDNA probes include R^{I}_{α} (R^{I}), R^{II}_{β} (R^{II}_{52}), R^{II}_{α} (R^{II}_{56}), C_{α} (C), Oncor p7000 human β actin (actin), and pUC19 (pUC).

Table 1. Affinity and activation potency of cAMP analogs for protein kinase

	K_i (cAMP)/K_i (analog)				K_a (cAMP)/K_a (analog)		
	R^I		R^{II}				
Analog	Site 1	Site 2	Site 1	Site 2	I	II	I/II
8-Cl-cAMP	2.0	2.71	4.61	0.051	2.3	0.70	3.3
BtcAMP	0.093	3.60	0.041	0.74	0.37	0.39	0.95

Site 1 (Site B) and Site 2 (Site A) are two distinct cAMP binding sites (12, 13) on receptor proteins (R^I and R^{II}); the affinities of analogs for site 1 and site 2 were determined by the measurement of kinetic constants (inhibition constant, K_i), and the values are expressed as the relative affinity K_i (cAMP)/K_i (analog)—i.e., the ratio between the apparent inhibition constant for cAMP and the analog, as described (15, 28). The R^{II}_{52} prepared from rat ovary produced similar results. Protein kinases types I (I) and II (II) were prepared from rabbit skeletal muscle and bovine heart, respectively, as described (29). The relative activation K_a (cAMP)/K_a (analog), where K_a is the concentration of cAMP or analog sufficient for half-maximum activation of the protein kinase (15, 30), is reported for protein kinases types I and II. The ratio of the relative activations of protein kinases types I and II is also presented (I/II). BtcAMP, N^6-butyryladenosine 3',5'-cyclic monophosphate.

(4.6-fold more than cAMP) while exhibiting a low-affinity binding for site 2 of R^{II} (5% that of cAMP). Therefore, it has a 90-fold greater affinity for site 1 than site 2 of R^{II} receptor protein compared with cAMP.

8-Cl-cAMP exhibited a lower affinity and a change in its site selectivity for R^I receptor. It showed a higher-affinity binding for site 2 than site 1 (2.7- versus 2.0-fold that of cAMP). Thus, 8-Cl-cAMP belongs to a specific class of cAMP analogs, such as 8-piperidinoadenosine 3',5'-cyclic monophosphate (15), that demonstrates a differential site selectivity toward R^I versus R^{II} receptors.

In the activation of protein kinase, 8-Cl-cAMP showed a 3.3-fold greater potency for type I protein kinase than for type II protein kinase (Table 1). N^6-Butyryladenosine 3',5'-cyclic monophosphate, which induces little or no growth inhibition (11), demonstrated a higher binding affinity for the R^I than the R^{II} receptor, and it activated with equal potency both type I and II protein kinase (Table 1).

These results indicate that the growth-inhibitory effect of cAMP analogs does not correlate with their potency for protein kinase activation *per se* but rather depends on their binding affinity for the receptor proteins, the regulatory subunits of protein kinases. 8-Cl-cAMP, the potent growth inhibitor of human colon cancer cells (11), exhibited a high-binding affinity for the R^{II} receptor and enhanced the stability, nuclear translocation, and synthesis of R^{II}_{52} receptor protein.

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