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Type II Protein Kinase A Up-regulation Is Sufficient to Induce Growth Inhibition in SK-N-SH Human Neuroblastoma Cells

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We have previously reported that overexpression of RIIβ subunit of protein kinase A, which markedly reduces RIα protein, induces growth inhibition in SK-N-SH human neuroblastoma cells. To determine whether the reduction of RIα or protein kinase A isozyme type I is essential in the growth inhibition of SK-N-SH cells, we overexpressed RIα in sense and antisense orientation. Type I protein kinase A activity was increased in the RIα-overexpressing cells and was decreased in the RIα antisense-expressing cells. However, the changes in type I protein kinase A activities did not affect cell growth. Overexpression of RIIβ or Cα increased type II protein kinase A and inhibited cell growth in both cell lines regardless of the type I protein kinase A level. These results indicate that type II protein kinase A is the main effector in the cAMP-mediated growth regulation of SK-N-SH human neuroblastoma cells.

Protein Kinase A (PKA) is a serine/threonine kinase composed of a regulatory (R) subunit dimer and two catalytic (C) subunits. There are two different regulatory subunits, RI and RII, which bind to common catalytic subunits to form type I and type II PKA holoenzyme, respectively (1). Two types of PKA are distinguished by several cellular and biochemical characteristics (2-8). Four isoforms of R subunits, RIα, RIβ, RIIα, and RIIβ(9-12), and three isoforms of C subunits, Cα, Cβ, and Cγ (13, 14) have been identified.

Differential expression of two types of PKA has been correlated with cell growth and differentiation (15). Cells in early developmental stage or undergoing active proliferation show a high level of type I PKA and/or high RI/RII ratio. However, growth-arrested cells or terminally differentiated cells exhibit a high type II PKA level and low RI/RII ratio.

We have previously reported that the overexpression of RIIβ induces growth inhibition in SK-N-SH human neuroblastoma cells (16). Since the growth inhibition was accompanied by the reduction of RIα protein, it was not clear whether the growth inhibition was administered by the increased RIIβ or by the decrease in RIα. In LS-174T human colon carcinoma cells and DT *ras*-transformed NIH/3T3 cells, it was found that the overexpression of mutant RIIβ which caused marked reduction in RIα and type I PKA activity could not induce growth inhibition (17, 18). These results suggested that functional type II PKA is essential in the growth inhibition of cancer cells. However, the possibility that the dominant negative effect of mutant RIIβ restrained the growth inhibition caused by the reduction of RIα/type I PKA activity could not be excluded.

In the present study, we examined the effect of varying the type I PKA activity levels on the growth of SK-N-SH human neuroblastoma cells. RIα sense-and antisense-expression constructs were introduced into SK-N-SH cells to change the type I PKA level. RIIβ and Cα expression constructs were also introduced to modulate both type I and type II PKA levels. Using these cells, we found that the cell growth is mainly regulated by type II PKA.

MATERIALS AND METHODS

Materials. Human RIα (9), RIIα (11), and RIIβ (12) cDNAs were provided by Dr. Tore Jahnsen, Rikshopitalet, Oslo, Norway. Human

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Cα (13) cDNA was provided by Dr. S. Hanks, Salk Institute, San Diego, CA. Most reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Eagle's minimum essential medium and heat-inactivated fetal bovine serum were purchased from GIBCO-BRL (Gaithersburg, MD). 8-N_3-[^{32}P]-cAMP (60 Ci/mmole; 1 Ci = 37 GBq) was obtained from ICN pharmaceuticals (Irvine, CA).

Cell culture. SK-N-SH human neuroblastoma cell line (provided by L. Neckers, National Cancer Institute) was maintained in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

Construction of expression vectors and introduction into SK-N-SH cells. OT1521 retroviral vector (19) was used to generate expression constructs. The constructs for the overexpression of RIα, RIIβ and Cα (17), and RIα antisense-expression construct (20) were prepared as previously described. Introduction of expression constructs into SK-N-SH cells and selections of high expressor clonal lines were performed as described previously (16).

Northern hybridization. Total cellular RNA from the cells treated with 120 μM $ZnSO_4$ for three days to induce the expression of introduced genes were extracted, electrophoresed, and blotted onto nitrocellulose membrane as described previously (21). Membranes were prehybridized at 65°C for riboprobes and at 40°C for DNA probes in 5× SSC, 50% formamide, 5× Denhardt's solution, 0.1% SDS, and 200 μg/ml calf thymus DNA for 2 hrs. Hybridization was performed in the same buffer at the respective temperature with ^{32}P-labeled probe for 16 hrs. To obtain single strand RNA probes in antisense orientation, a 451 bp *HindIII* fragment (nucleotide positions 354 to 804, assuming the translation start site as +1) of human RIIβ cDNA (12), and an 845 bp *EcoRI* fragment (-103 to 742) of human RIα cDNA (9) which were cloned into pBluescript SK(+) (Stratagene, La Jolla, CA) were used as templates for *in vitro* transcription with T3 RNA polymerase. The RIα sense riboprobe was generated by *in vitro* transcription of the 845 bp *EcoRI* fragment of RIα cDNA using T7 RNA polymerase. Other probes, such as the 1.5 kb *EcoRI* fragment (92-1554) of RIIα cDNA (11), the 1.1 kb human Cα total cDNA (13), and the *BamHI-EcoRI* fragment of 18 S rRNA gene (22), were labeled by random-priming method. After hybridization, membranes were washed twice at room temperature with 2× SSC for 10 min, and then four times at 65°C with 0.1× SSC/0.1% SDS for 30 min for RIIβ and RIα riboprobes. For DNA probes, membranes were washed twice at room temperature with 2× SSC for 10 min, twice at room temperature with 0.1× SSC/0.1% SDS for 20 min, and then twice at 50°C with 0.1× SSC/0.1% SDS for 15 min. Washed membranes were exposed to X-ray films.

Monolayer and soft agar growth assay. For the monolayer growth assay, 2×10^4 cells were seeded on 35 mm culture dish at day 0. After 24 hrs, $ZnSO_4$ was added to the final concentration of 120 μM. Cell number was determined at day 6 using Coulter counter. For the soft agar growth assay, 5×10^4 cells in 1 ml of 0.3% Difco Noble agar in culture medium were layered over 1 ml of 0.8% agar-medium in 35 mm dish. After nine days, colonies were stained with nitro blue tetrazolium for overnight. Colonies larger than 0.3 mm were counted with MiniCount (IPI, Chantilly, VA).

8-N_3-[^{32}P]-cAMP labeling and Western blot analysis. Cells were treated with 120 μM $ZnSO_4$ for three days before analysis. Cell extracts were prepared as described before (23). The 8-N_3-[^{32}P]-cAMP labeling (23) and Western blot analysis (16) were performed as described previously.

DEAE-cellulose chromatography and protein kinase A assay. Exponentially growing cells were treated with 120 μM $ZnSO_4$ for three days. Preparation of cell extract, DEAE-cellulose chromatography, and protein kinase A activity assay were performed as described previously (24). For the assay of total protein kinase A activity, thirty micrograms of cell extract were used.

RESULTS AND DISCUSSION

In an attempt to define the primary cause of the growth inhibition caused by the overexpression of RIIβ (16), we introduced RIα-and RIα antisense-expression constructs into SK-N-SH cells to either increase or suppress the level of RIα or type I PKA without any changes in the type II PKA level. RIIβ and Cα-expression constructs were also introduced to bring about changes in both type I and type II PKA levels. After introduction of each construct into SK-N-SH cells, high expressor clonal cells for RIα, RIIβ, and Cα were selected with Northern blot analysis using RIα, RIIβ, and Cα cDNAs as probes, respectively. For RIα antisense-expressing cells, RIα sense riboprobe was used to select the cells highly expressing RIα antisense RNA. For the characterization of each cell line, one high expressor clonal line was used for RIIβ-overexpression (16), each pool of three high expressor clonal lines was used for RIα-and Cα-expressing cells, and a pool of two clonal lines was used for RIα antisense-expressing cells. The pool of OT1521-introduced cells was used as a vector-introduced control.

To confirm the overexpression or suppression of PKA subunits in prepared cells, and to examine the effects of increased or decreased PKA subunit level on the expression of other subunits, we performed Northern (Fig. 1A) and Western (Fig. 1B) blot analysis. In Northern blot analysis, RIα mRNA was detected in all cell lines. The highest level of RIα mRNA (10-fold increase compared to parental cells) was observed in the RIα-overexpressing cells and a 3-fold increase of RIα mRNA was detected in the Cα-overexpressing cells. In the RIα antisense-expressing cells, the level of RIα mRNA was the same as parental cells (Fig. 1A). However, in Western blot analysis, RIα protein was increased only in Cα-overexpressing cells (3-fold compared to parental cells), and was not changed in RIα-overexpressing cells. In RIα antisense-expressing cells and RIIβ-overexpressing cells, RIα protein was reduced by 40 and 70%, respectively (Fig. 1B). The discrepancy between the mRNA and the protein level of RIα has been attributed to the lower affinity of RI to C subunit than that of RII (25). Because RII has higher affinity to C subunit than RI, overexpressed RII forms holoenzyme with C subunit leaving excess RI as free subunit. In contrast, most of the overexpressed RI cannot form holoenzyme and remains as free subunit. In both cases, the free RI protein is easily degraded. The decrease of RIα protein in RIα antisense-expressing cells without change in mRNA level suggests that the RIα antisense RNA might act at the translation level. RIIβ mRNA and protein were detected in only RIIβ-overexpressing cells (Fig. 1A,B). RIIα mRNA and protein levels were the same in all infectants (data not shown). Cα mRNA and protein levels were increased only in Cα-overexpressing cells (10-fold in mRNA and 1.5-fold in protein)

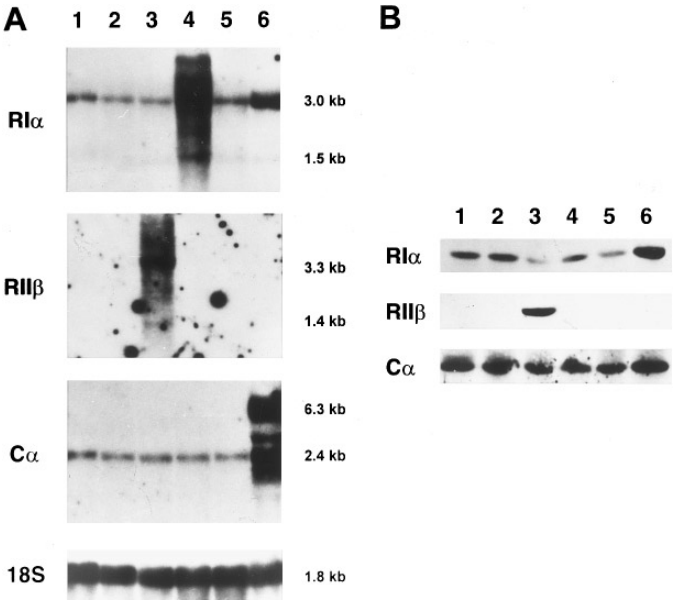

FIG. 1. mRNA and protein levels of protein kinase A subunits. Panel A, total cellular RNA from each cell line was analyzed with Northern hybridization as described in "Materials and Methods." Blots were probed with RIα, RIIβ, Cα, and 18 S rRNA genes as shown. The size of each band is indicated at the right side of the autoradiogram. Panel B, cell extracts were analyzed by Western blot analysis as described in "Materials and Methods." Each protein was detected with polyclonal antiserum generated against recombinant protein produced in bacteria. The membranes were stained with Ponceau S and an equal amount of protein on each lane was confirmed. Lane 1, SK-N-SH; lane 2, OT1521-introduced cell; lane 3, RIIβ-overexpressing cell; lane 4, RIα-overexpressing cell; lane 5, RIα antisense-expressing cell; lane 6, Cα-overexpressing cell.

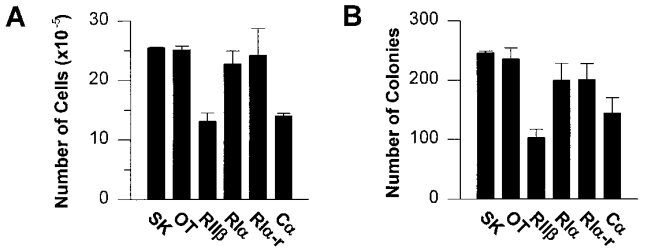

FIG. 2. Effects of overexpression or antisense expression of protein kinase A subunits on cell growth. Panel A, effects on the monolayer growth. Cells were grown in the presence of $ZnSO_4$ as described in "Materials and Methods." Cell number was determined 5 days after induction. Panel B, effects on soft agar colony formation. Cells were grown for 9 days in 0.3% agar medium in the presence of $ZnSO_4$. Colonies larger than 0.3 mm were counted. Designations are SK, parental cell; OT, vector-introduced cell; RIIβ, RIIβ-overexpressing cell; RIα, RIα-overexpressing cell; RIα-r, RIα antisense-expressing cell; Cα, Cα-overexpressing cell. Data show means of two separate experiments of triplicate determination with standard deviation.

whereas they stayed constant in control and other infectants (Fig. 1A,B). The level of RIα protein seems not to be related to the growth of SK-N-SH cells. We checked the growth of SK-N-SH cells to examine the effect of changes in PKA subunit level (Fig. 2A,B). Decrease of RIα in RIα antisense-expressing cells did not inhibit the cell proliferation in monolayer and soft agar culture condition. Cell growth was even decreased in Cα-overexpressing cells where RIα protein level was increased. However, the RIIβ overexpression, which has been known to decrease the cellular level of RIα protein (16), inhibited cell growth by 50% in monolayer culture and by 60% in soft agar culture as compared to either the parental cells or vector-introduced cells. These results indicate that the level of RIα is not related to the growth of SK-N-SH cells.

By photoaffinity labeling with 8-N_3-[^{32}P]-cAMP, we analyzed the ratio of RI to RII. The ratio of RI to RII was 2:1 in parental, vector-introduced, and in RIα-overexpressing cells, and was 6:1 in Cα-overexpressing cells. In RIα antisense-expressing and RIIβ-overexpressing cells, the ratios of RI:RII were 1:1 and 1:2, respectively (data not shown). Thus, the growth inhibition in RIIβ-and Cα-overexpressing cells could not be explained by the ratio of RI to RII, either.

Since we found that the growth rate of SK-N-SH is independent of either the intracellular level of RIα protein or RI/RII ratio, we decided to examine the relationship between the PKA isozyme distribution and the growth of SK-N-SH cells. We fractionated type I and type II PKA using DEAE-cellulose column chromatography and determined the activity of each isozyme (Fig. 3). Type I PKA activity peaked at 90 mM NaCl, and type II PKA activity was eluted at 230-260 mM NaCl. The parental and vector-introduced cells showed 1:1 ratio of type I to type II PKA in peak height. The type I PKA activities were increased by 1.5-and 2.7-fold in RIα-and Cα-overexpressing cells, respectively, and

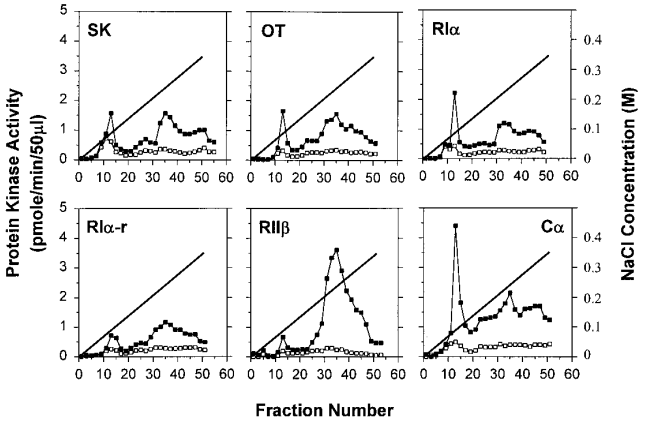

FIG. 3. Activities of protein kinase A isozymes. Cell extracts were subjected to the DEAE-cellulose chromatography and protein kinase A assay as described in "Materials and Methods." Kinase activities were determined in the absence (□) or presence (■) of 5 μM cAMP. Designations are the same as in Fig. 2. Solid line indicates NaCl concentration gradient for elution. Data show a representative from three separate experiments of similar results.

TABLE 1

Total Protein Kinase A Activity

Cell line	Kinase activity (fmole/min/μg)	
	Without cAMP	5 μM cAMP
SK[a]	23.75 ± 2.36[b]	71.03 ± 6.96
OT	27.30 ± 4.96	73.65 ± 2.45
RIIβ	29.09 ± 1.59	162.30 ± 4.94
RIα	21.57 ± 3.24	68.94 ± 10.08
RIα-r	17.68 ± 1.58	64.36 ± 5.91
Cα	69.44 ± 10.23	475.30 ± 28.46

[a] Designations for cell lines are the same as Fig. 2.

[b] Data show means ± standard deviation for two separate experiments.

were reduced in RIIβ-overexpressing and RIα antisense-expressing cells by 50-60% (Fig. 3). Thus, the inhibition in cell growth could not be explained by the changes in type I PKA level. However, the RIIβ-and Cα-overexpressing cells, in which the growth rates were reduced compared to parental cells (Fig. 2A,B), showed increased type II PKA activity by 2.5-and 1.5-fold, respectively (Fig. 3). Furthermore, the increase of type II PKA activity inversely correlated with the ability of growth in soft agar (Fig. 2B), which is an important criterion for cell transformation. These data indicate that the type II, but not type I PKA, is the main regulator of cell growth.

To test the possibility that the increase of PKA catalytic activity affects the growth of SK-N-SH cells, we checked the total PKA activity in each cell line (Table 1). In the absence of cAMP, parental, vector-introduced, RIα-, RIα antisense-, and RIIβ-expressing cells showed similar level of total PKA activity. However, Cα-overexpressing cells showed about 3-fold increased PKA activity in the absence of cAMP. In the presence of 5 μM cAMP, only RIIβ-and Cα-overexpressing cells showed twice and seven times higher level of total PKA activity than that of parental or vector-introduced cells. However, the change in total PKA activity does not parallel the level of growth inhibition in RIIβ-and Cα-overexpressing cells. The increase of total PKA activity is much higher in Cα-overexpressing cells than RIIβ-expressing cells. In contrast, the degree of growth inhibition is higher in RIIβ-overexpressing cells (Fig. 2). Therefore, total PKA activity cannot account for the inhibition of SK-N-SH cell growth.

There have been reports that down-regulation of RIα using RIα antisense oligodeoxynucleotide causes growth inhibition and differentiation in a variety of cancer cells (26, 27). The RIα antisense oligodeoxynucleotide also suppressed tumor growth in nude mice (28). In these reports, RIα antisense treatment caused an increase in RIIβ protein as well as the reduction of RIα mRNA (27) and protein (26-28). However, in our RIα antisense-expressing cells, the expression of RIIβ was not induced as examined with Northern and Western blot analysis. This enabled us to analyze the effect of RIα down-regulation on the cell growth without the effect of RIIβ. The down-regulation of RIα/type I PKA was evident in RIα antisense-expressing cells as assessed by Western blot analysis and DEAE-cellulose chromatography combined with PKA assay, but the cell growth was not changed. Therefore, RIα/type I PKA is not the main effector of cell growth. But the increase in type II PKA by overexpression of RIIβ or Cα could cause growth inhibition. Thus, the growth of SK-N-SH cells are mainly regulated by the type II PKA. Accordingly, for the growth inhibition induced by RIα suppression, the increase of RIIβ may be necessary. Two possibilities may exist on why RIIβ was not induced in our RIα antisense-expressing cells. One possibility is that the expression level of RIα antisense RNA was not sufficient to induce RIIβ protein. The other possibility is that the antisense RNA against the whole mRNA may act differently from the antisense oligodeoxynucleotide directed to a specific region of RIα mRNA (26-28).

Previous reports presented the possibility that the two types of PKA may transduce different intracellular signals. Type II PKA is responsible for the CRE (cAMP-responsive element)-dependent transcription in PC-12 cells (29), and RII binds to CRE in a cAMP-dependent manner *in vitro* (30). Type II PKA shows different subcellular distribution from that of type I PKA. Type II PKA is attached to specific subcellular structures through the binding with AKAP (A-kinase anchoring protein; 31), whereas type I PKA is mainly cytosolic (32). This may confer different substrate specificity to type I and type II PKA. Colocalization of type I PKA with TCR-CD3 complex during T cell activation has also been reported, suggesting involvement of type I PKA in T cell activation (33). Thus, two types of PKA may act as different signal transducer by different cellular localization, differential binding with cellular proteins, and unique functions of their regulatory subunits.

Based on the previous reports that the overexpression of mutant RIIβ that down-regulates RIα protein and type I PKA cannot inhibit the growth of transformed cells (17, 18), the importance of functional type II PKA in the growth inhibition has been suggested. However, in these studies, the possibility that the mutant RIIβ protein act as a dominant-negative mutant cannot be excluded. In the present study, using the RIα antisense-expressing cells, we showed that type II PKA plays a major role in the growth regulation of SK-N-SH cells, and the level of RIα or type I PKA is not the main determinant in the growth regulation. In summary, we conclude that the up-regulation of type II PKA activity accounts for the growth inhibition through the PKA signalling pathway in SK-N-SH human neuroblastoma cells.

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