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Depletion of Catalytic and Regulatory Subunits of Protein Kinase CK2 by Antisense Oligonucleotide Treatment of Neuroblastoma Cells

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SUMMARY

1. The use of antisense oligonucleotides to inhibit expression of the genes coding for the catalytic (α/α') and regulatory (β) subunits of protein kinase casein kinase 2 (CK2) has allowed study of the role of this enzyme in mouse neuroblastoma cells.

2. Selective depletion of catalytic (α/α') subunits results in the blocking of neuritogenesis. The depletion of catalytic subunits also affects the sorting of the regulatory (β) subunit of CK2, as the absence of catalytic subunits prevents the translocation of the regulatory subunit to the nuclei. These results emphasize the existence of a control mechanism linking the expression and sorting of CK2 catalytic and regulatory subunits.

3. Selective depletion of the regulatory (β) subunit of protein kinase CK2 by an specific antisense oligonucleotide causes partial inhibition of neurite extension.

INTRODUCTION

Protein kinase CK2 (also referred to as casein kinase 2) is an oligomeric enzyme with the structure $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ (Issinger, 1993). The predicted molecular weights of human CK2 subunits are 45,160 (α), 41,450 (α'), and 24,925 (β), and

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they are very similar in most other organisms. The α and α' subunits are catalytic, whereas the β subunit seems regulatory. In particular, the β subunit is thought to stabilize the holoenzyme, increase catalytic activity, and favor substrate recognition (Grankowski *et al.*, 1991; Lin *et al.*, 1991; Filhol *et al.*, 1991; Jakobi and Traugh, 1992; Issinger *et al.*, 1992; Boldyreff *et al.*, 1992a, b, 1993).

Protein kinase CK2 is a multifunctional enzyme present in all eukaryotic cells. We have focused our attention on the possible role of CK2 in the regulation of microtubule-associated proteins during neuritogenesis in mouse neuroblastoma cells. Phosphorylation of microtubule-associated protein MAP1B by CK2 seems to be associated with neurite growth in these neuroblastoma cells (Díaz-Nido *et al.*, 1988, 1992; Ulloa *et al.*, 1993).

The implication of CK2 in neuritogenesis has been confirmed using antisense oligonucleotides in neuroblastoma cells (Ulloa *et al.*, 1993). Neuronal cells appear to be particularly suitable for this type of experiments due to their low oligonucleotide degradation activity and their high oligonucleotide uptake compared with other cell types (Wahlestedt, 1994). Thus, several reports have described the use of antisense oligonucleotides to inhibit the function of specific neuronal proteins including tubulin (Teichman-Weinberg *et al.*, 1988), microtubule-associated proteins tau (Cáceres and Kosik, 1990), MAP1B (Brugg *et al.*, 1993), and MAP2 (Shanna *et al.*, 1994), and intermediate filament proteins (Troy *et al.*, 1992) and synaptic vesicle proteins (Ferreira *et al.*, 1994).

Antisense oligonucleotide technology has also been used to test whether the down-regulation of a particular protein can alter the function of another protein which interacts with the depleted protein (Ferreira *et al.*, 1994). Here we have studied how the depletion of either the catalytic or the regulatory subunits of an enzyme (CK2) modifies the expression, activity, and sorting of each other.

MATERIALS AND METHODS

Oligonucleotides. CK2 α/α' antisense oligodeoxyribonucleotide with the sequence GTA GTC ATC TTG ATT TCC GCA, its complementary sense oligonucleotide, CK2 β antisense oligonucleotide with the sequence TCC TCA GAG CTA CTC ATC TT, and its complementary sense oligonucleotide were purchased from Isogen Bioscience (The Netherlands). The localization of the DNA sequence complementary to the α/α' antisense oligonucleotide has been indicated previously (Ulloa *et al.*, 1993), whereas that for the β subunit is at residues -3 and $+17$ of the coding sequence (according to Kopatz *et al.*, 1990). The prediction of duplex formation, by using the previous oligonucleotides, was done by calculating the D score (D score = ΔG duplex formation/2), as indicated by Stull *et al.* (1992)

Antibodies. Antibodies raised against CK2 α/α' subunits (purchased from Upstate Biotechnology Inc., Lake Placid, NY) or the CK2 β subunit (to be described elsewhere) were tested as indicated previously (Ulloa *et al.*, 1993).

Cell Culture. Mouse neuroblastoma N2A cells were cultured and induced

to differentiate as indicated (Díaz-Nido *et al.*, 1988; Ulloa *et al.*, 1993). Oligonucleotides were added to the culture medium at a concentration of 0.05 to 0.3 mg/ml and the oligonucleotide-containing medium was changed every 2 hr for a period of up to 6–8 hr. In some experiments, lipofectine (2 μg/μg oligonucleotide-containing) was added to the culture medium. Isolation of nuclear and cytoplasmic protein fractions was performed by gentle homogenization in 10 m*M* sodium phosphate buffer, pH 7.4, m*M* $MgCl_2$, 2 m*M* EGTA, 50 m*M* NaF, 0.5 m*M* dithiothreitol (DDT), 1 m*M* phenylmethylsulfonylfluoride (PMSF), 10 μg/ml pepstatin, 10 μg/ml aprotinin. After centrifugation at 1000*g* for 20 min, the pellet was resuspended in this buffer and centrifuged at 20,000*g* for 1 hr at 2°C on a 1.5 *M* sucrose cushion (prepared in the same phosphate buffer). The previous supernatants (cytoplasmic fractions) were collected and the resulting pellet containing purified nuclei was resuspended in 50 m*M* Tris–HCl, pH 7.0, 2 m*M* EGTA, 50 m*M* NaF, 20 m*M* DTT, and 2.5% sodium dodecyl sulfate (SDS) and boiled for 5 min. Lysed nuclei were centrifuged at 100,000*g* to remove nucleic acid and the supernatant was referred to as nuclear protein fraction.

Other Procedures. Neurite extension counting was used to test for the function of CK2 and was performed as described by Ulloa *et al.* (1993). Immunofluorescence and Western blot analyses have also been described (Ulloa *et al.*, 1993).

RESULTS AND DISCUSSION

Effect of Antisense Oligonucleotides Complementary to CK2 (α/α') mRNA. We have designed suitable antisense oligonucleotide to prevent the expression of CK2 α/α' subunits and inhibit neurite extension in mouse neuroblastoma cells (Ulloa *et al.*, 1993). Antisense oligonucleotides should be able to enter into cells and hybridize with their complementary mRNAs. The incorporation of oligonucleotides into the cell depends on their size but not on their sequence (Loke *et al.*, 1989), since only the phosphate moiety appears to be essential for oligonucleotide uptake through a cellular surface protein receptor (Loke *et al.*, 1989; Akhtar and Juliano, 1992). We chose an antisense oligonucleotide with 21 nucleotides, which is just below the limit of 22 nucleotides that are efficiently incorporated into cells (Ferreira *et al.*, 1994).

An antisense oligonucleotide should also hybridize with its mRNA sequence in an efficient way as a prerequisite to halt the expression of the protein. An index used to predict such efficiency is the duplex score or *D* score, which estimates the free energy of formation of the antisense oligonucleotide mRNA duplex (Stull *et al.*, 1992). Our calculation indicates that the chosen oligonucleotide GTAGTCATCTTGATTTCCCCA has a *D* score of about −14, a value that is correlated with a partial inhibition of protein synthesis in cell-free systems at low oligonucleotide concentrations (Stull *et al.*, 1992). However, a high oligonucleotide concentration is required to deplete cultured neuroblastoma cells of CK2 activity, as measured by the oligonucleotide amount added to the culture

Table I. Effect of the Addition of Antisense Oligonucleotides Complementary to CK2 Catalytic Subunit mRNA on Neuritogenesis[a]

Oligonucleotide concentration (mg/ml)	% neurite-bearing cells
—	92
0.05	84
0.10	68
0.30	8

[a] Oligonucleotides, at the concentrations indicated, were added to the culture medium 4 hr before the cells were induced to differentiate by serum deprivation, then replaced every 1.5 hr for a period of up to 8 hr.

medium that results in inhibition of neuritogenesis (Ulloa *et al.*, 1993). Table I shows that an oligonucleotide concentration of 0.3 mg/ml is required to prevent neurite extension in more than 92% of the cells. This high concentration may be needed because of the existence of a partial degradation of the oligonucleotides in the culture medium. Western blotting analysis shows that antisense oligonucleotide treatment indeed leads to depletion of CK2, which is the major CK2 catalytic subunit in N2A cells (Fig. 1).

It is also important to determine the toxicity of oligonucleotide addition. For that purpose, the antisense oligonucleotide was removed from the cultured medium after the usual treatment and the number of viable cells was measured. Ninety-one percent (±5%) of the cells were viable, a value similar to that (93 ± 5%) found for a parallel culture of neuroblastoma cells grown in the absence of oligonucleotide. These results indicate the lack of side toxic effects due to the oligonucleotides added to cultured neuroblastoma cells.

Depletion of CK2 α/α′ Subunits Prevents Transport of the CK2 β Subunit to the Cell Nucleus. In undifferentiated neuroblastoma cells the CK2 holoenzyme is localized mainly in the nucleus, whereas a translocation of the enzyme to the cytoplasm takes place in differentiated cells (Serrano *et al.*, 1989; Díaz-Nido *et*

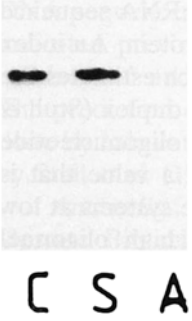

Fig. 1. Depletion of the casein kinase α subunit in neuroblastoma cells upon antisense oligonucleotide treatment. Immunoblotting analyses show the reaction of an antibody to CK2 α/α′ with N2A protein extracts from cells under control conditions (C) or treated with the sense (S) or antisense (A) oligonucleotides to CK2 α/α′.

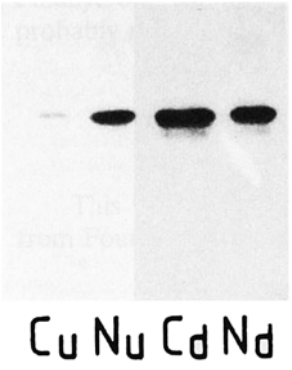

Fig. 2. Distribution of CK2 α subunits. Nuclear (N) and cytoplasmic (C) protein fractions from undifferentiated (u) or differentiated (d) mouse neuroblastoma N2A cells were fractionated by gel electrophoresis, blotted onto nitrocellulose and assayed with an antibody raised against CK2 α/α' subunit. CK2 α is the major catalytic subunit in N2A cells.

al., 1992). This result has been confirmed by Western blotting. Figure 2 shows the amount of CK2 α subunit present in nuclear and cytoplasmic fractions from differentiated or undifferentiated cells.

Since it has been discussed which subunit could be the one responsible for the transport of the holoenzyme to the nucleus, we looked for the localization of the CK2 β subunit when the catalytic subunits were depleted by antisense oligonucleotide addition (see Fig. 2). Figure 3 shows that the CK2 β subunit is present in the cytoplasmic protein fraction obtained from CK2 α-depleted cells. The localization of CK2 β in the cytoplasm of CK2 α-depleted cells has also been confirmed by immunofluoresce microscopy (see Fig. 4). These results suggest that the transport of the CK2 β regulatory subunit to the nuclei is prevented when it is not bound to the catalytic subunits. These results are consistent with the observation in CK2 catalytic subunits of a putative signal for nulcear transport containing the sequence lysine–lysine–lysine (Lozeman *et al.*, 1990; Boldyreff *et al.*, 1992a,b).

Effect of Antisense Oligonucleotides Complementary to CK2 β mRNA. By following an approach similar to that indicated for the preparation of the oligonucleotides complementary to CK2 α/α' mRNA, we used the oligonucleotide TCC TCA GAC CTA CTC ATC TT to study the effect of CK2 β depletion in neuroblastoma cells. Essentially no effect was found on cell morphology in the presence of the oligonucleotide at 0.3 mg/ml. This might be due to the fact that the amount of the incorporated oligonucleotide into the cell is below the concentration required for observing any effect or that a large amount of the oligonucleotide is degraded in the culture medium. Thus, the previous experiment was done in the presence of lipofectine to improve oligonucleotide uptake. Under these conditions expression of the CK2 β subunit is prevented

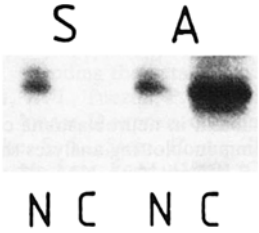

Fig. 3. Distribution of the CK2 β subunit in the absence of the CK2 α subunit. N2A neuroblastoma cells were treated with CK2 α sense (S) or antisense (A) oligonucleotides, and the presence of the β subunit in the nuclear (N) or cytoplasmic (C) fraction was determined by Western blotting using an antibody raised against CK2 β subunit.

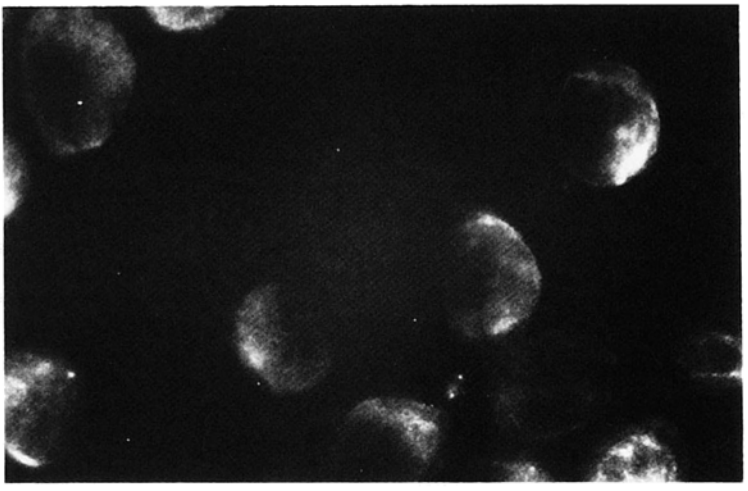

Fig. 4. Distribution of the CK2 β subunit in the absence of the CK2 α subunit, determined by immunofluorescence analyses. N2A neuroblastoma cells treated with CK2 α antisense oligonucleotides were analyzed by immunofluorescence using an antibody raised against the CK2 β subunit.

(Fig. 5). The addition of antisense oligonucleotide for the ß subunit, together with lipofectine, prevents neuritogenesis in 37% of neuroblastoma cells. On the other hand, the addition of lipofectine alone does not prevent neuritogenesis. This result suggests that the ß subunit, as suggested for yeast CK2 (Roussou and Draetta, 1994), could be required for a full enzymatic activity of CK2.

Interestingly, CK2 catalytic subunits are present in the nuclei of CK2 β-depleted cells (data not shown). This result agrees well with the previous suggestion that CK2 catalytic subunits are responsible for the nuclear transport of the holoenzyme.

In summary, CK2 holoenzyme is transported to the nuclei after being synthesized in the cytoplasm in undifferentiated neuroblastoma cells. Our results suggest that the catalytic subunits are responsible for this transport and can cotransport the regulatory subunits. In differentiated neuroblastoma cells, translocation of the holoenzyme to the nuclei decreases. The mechanism for this change in localization of the CK2 holoenzyme is unknown at present, although possible posttranslational modifications of the enzyme cannot be ruled out.

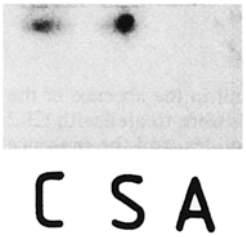

Fig. 5. Depletion of the casein kinase β subunit in neuroblastoma cells upon antisense oligonucleotide treatment. Immunoblotting analyses show the reaction of an antibody to the CK2 β subunit with N2A protein extracts from cells without any addition (C) or supplemented with the sense (S) or the antisense (A) oligonucleotides to the CK2 β subunit.

Finally, our experiments suggest that the presence of the regulatory subunit, probably needed for full enzymatic activity, facilitates neurite extension.

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REFERENCES

Akhtar, S., and Juliano, R. L. (1992). Cellular uptake and intracellular fate of antisense oligonucleotides. *Trends Cell Biol.* **2:**139–144.

Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O. G. (1992a). Casein kinase-2 structure-function relationship: Criterion of a set of mutants of the β subunit that variably surrogate the wild type β subunit function. *Biochem. Biophys. Res. Commun.* **188:**228–234.

Boldyreff, B., Wehr, K., Hecht, R., and Issinger, D. G. (1992b). Identification of four genomic loci highly related to casein kinase 2 alpha cDNA and characterization of a casein kinase 2 alpha pseudogene within the mouse genome. *Biochem. Biophys. Res. Commun.* **186:**723–730.

Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O. G. (1993). Reconstitution of normal and hyperactivated forms of casein kinase-2 by variably mutated β-subunits. *Biochemistry* **32:**12672–12677.

Brugg, B., Reddy, D., and Matus, A. (1993). Attenuation of microtubule-associated protein 1 B expression by antisense oligonucleotides inhibits initiation of neurite outgrowth. *Neuroscience* **52:**489–496.

Cáceres, A., and Kosik, K. (1990). Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebelar neurons. *Nature* **343:**461–463.

Díaz-Nido, J., Serrano, L., Méndez, E., and Avila, J. (1988). A casein kinase II related activity is involved in phosphorylation of microtubule associated protein MAP1B during neuroblastoma cell differentiation. *J. Cell Biol.* **106:**2057–2065.

Díaz-Nido, J., Armas-Portela, R. and Avila, J. (1992). Increase in cytoplasmic casein kinase II-type activity accompanies neurite outgrowth after DNA synthesis inhibition in NIA-103 neuroblastoma cells. *J. Neurochem.* **58:** 1820–1828.

Ferreira, A., Kosik, K., Greengard, P., and Han, H. O. (1994). Aberrant neurites and synaptic vesicle protein deficiency in synapsin II-depleted neurons. *Science* **264:**977–979.

Filhol, O., Cochet, C., Wedegaertner, P., Gill, G. N., and Chambaz, E. M. (1991). Coexpression of both α and β subunits is required for assembly of regulated casein kinase II. *Biochemistry* **30:** 11133–11140.

Grankowski, N., Boldyreff, B., and Issinger, O. G. (1991). Isolation and characterization of recombinant human CK2 subunits α and β from bacteria. *Eur. J. Biochem.* **198:**25–30.

Issinger, O. G. (1993). Casein kinases: Pleiotropic mediators of cellular regulation. *Pharmacol. Ther.* **59:**1–30.

Issinger, O. G., Brockel, C., Boldyreff, B., and Pelton, J. T. (1992). Characterization of the α and β subunits of casein kinase 2 by far-UV CD spectroscopy. *Biochemistry* **31:**6098–6103.

Jakobi, R., and Traugh, J. A. (1992). Characterization of the phosphotransferase domain of casein kinase II by site-directed mutagenesis and expression in *Escherichia coli. J. Biol. Chem.* **267:**23894–23902.

Kopatz, I., Naiman, T., Eli, D., and Canaani, D. (1990). The nucleotide sequence of the mouse cDNA encoding the beta subunit of casein kinase II. *Nucleic Acids Res.* **18:**3639–3642.

Lin, W.-J., Tuazon, P. T., and Traugh, J. A. (1991). Characterization of the catalytic subunit of casein kinase II expressed in *Escherichia coli* and regulation of activity. *J. Biol. Chem.* **166:**5664–5669.

Loke, S. L., Stein, C. A., Zhang, X., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S., and Neckers, L. M. (1989). Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA* **86:**3474–3478.

Lozeman, F. J., Litchfield, D. W., Piening, C., Tako, K., Walsh, K., and Krebs, E. G. (1990).

Isolation and characterization of human cDNA clones encoding the α and the α' subunits of casein kinase II. *Biochemistry* **29:**8436–8447.

Roussou, I., and Draetta, G. (1994). The S. pombe casein kinase II α and β subunits: Evolutionary conservation and positive role of the β subunit. *Mol. Cell Biol.* **14:**576–586.

Serrano, L., Hernández, M. A., Díaz-Nido, J., and Avila, J. (1989). Association of casein kinase II with microtubules. *Exp. Cell Res.* **181:**263–272.

Shanna, N., Kress, Y., and Shafitzagardo, B. (1994). Antisense MAP2 oligonucleotides induce changes in microtubule assembly and neuritic elongation in preexisting neurites of rate cortical neurons. *Cell. Mol. Cytoskel.* **27:**234–238.

Stull, R. A., Taylor, L. A., and Szoka, F. C. (1992). Predicting antisense oligonucleotide inhibitory efficacy: A computational approach using histograms and thermodynamic indices *Nucleic Acids Res.* **20:**3501–3508.

Teichmann-Weinberg, A., Littauer, U., and Ginzburg, I. (1988). The inhibition of neurite outgrowth in PC12 cells by tubulin antisense oligonucleotides. *Gene* **72:**297–307.

Troy, C. M., Greene, L. A., and Shelanki, M. L. (1992). Neurite outgrowth in peripherin-depleted PC12 cells. *J. Cell Biol.* **117:**1085–1092.

Ulloa, L., Díaz-Nido, J., and Avila, J. (1993). Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells. *EMBO J.* **12:**1633–1640.

Wahlestedt, C. (1994). Antisense oligonucleotide strategies in neuropharmacology. *Trends Pharmacol. Sci.* **15:**42–46.