A Novel, Extraneuronal Role for Cyclin-dependent Protein Kinase 5 (CDK5)

MODULATION OF cAMP-INDUCED APOPTOSIS IN RAT LEUKEMIA CELLS*

Received for publication, December 21, 2001, and in revised form, March 21, 2002
Published, JBC Papers in Press, March 21, 2002, DOI 10.1074/jbc.M112248200

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A number of cyclin-dependent protein kinase (CDK) inhibitors were tested for the ability to protect IPC-81 rat leukemic cells against cAMP-induced apoptosis. A near perfect proportionality was observed between inhibitor potency to protect against cAMP-induced apoptosis and to antagonize CDK5, and to a lesser extent, CDK2 and CDK1. Enforced expression of dominant negative CDK5 (but not CDK1-dn or CDK2-dn) protected against death, indicating that CDK5 activity was necessary for cAMP-induced apoptosis. The CDK inhibitors failed to protect the cells against daunorubicine-, staurosporine-, or okadaic acid-induced apoptosis. The inhibition of CDK5 prevented the cleavage of pro-caspase-3 in cAMP-treated cells. The cells could be saved closer to the moment of their onset of death by inhibitors of caspases than by inhibitors of CDK5. This suggested that the action of CDK5 was upstream of caspase activation. The cAMP treatment resulted in a moderate increase of the level of CDK5 mRNA and protein in IPC-81 wild-type cells. Such cAMP induction of CDK5 was not observed in cells expressing the inducible cAMP early repressor. The cAMP-induced increase of CDK5 contributed to apoptosis since cells overexpressing CDK5-wt were more sensitive for cAMP-induced death. These results demonstrate the first example of a proapoptotic CDK action upstream of caspase activation and of an extra-neuronal effect of CDK5.

Cell death with the phenotype of apoptosis (for a review, see Refs. 1 and 2) can be triggered by cellular damage (“death by accident”), by withdrawal of survival factors (“death by neglect”), or by activation of pathways committed to death induction (“death by design”). Death by design can rely on preformed molecules (3, 4), such as members of the caspase family of proteases (5, 6). This latter pathway can also be programmed in the sense that it requires ongoing gene transcription and protein translation (1, 2).

The rat promyelocytic IPC-81 cell line represents a unique cell system for the study of programmed cell death (7–9). Cells start to undergo apoptosis within 3 h after activation of the cAMP-dependent protein kinase type I by physiological stimulators of adenylate cyclase-like prostaglandin E1 or by cAMP analogs, and the cells become apoptotic within 7 h (8, 10, 11).

IPC-81 cells with eneasaurus of the cAMP-responsive element (CRE) transcriptional blocker (ICER) (12) do not undergo apoptosis in response to cAMP (13). This suggests that CRE-dependent gene transcription is essential for the cAMP-induced death. In a first approach to identify gene products involved in cAMP-induced cell death, a limited Atlas Array analysis was performed to compare cAMP-induced mRNA expression in IPC-81WT and IPC-81ICER cells. Among gene products already incrinated in apoptosis, only cyclin-dependent protein kinase 5 (CDK5) mRNA appeared to be selectively up-regulated in the wild-type cells (the present study). This observation spurred a closer study of the role of CDK5 in cAMP-induced cell death.

The common general function of the CDK family members is to ensure the normal progression through the cell cycle, and they are tightly regulated by the sequential expression of cyclins (14, 15). Abnormal cell cycle control has been proposed to be a major mechanism for apoptotic cell death (16, 17). The unscheduled activation of cell cycle-related CDKs such as CDK1 and CDK2 (18–23) might have an impact late in apoptosis since they are activated by caspases (18, 19).

Cdk5 has high sequence similarity to the cell cycle regulating CDK family members, but it is neither activated by cyclins nor involved in cell cycle regulation (24, 25); for a review, see Refs. 26 and 27. Until recently, the expression of CDK5 and its activators, p35/25 and p39, were believed to be restricted to the nervous system (28, 29), where it contributes to neurite extension (27, 30). Cdk5 has been implicated in cell death during brain development (31), in neurodegenerative diseases such as Alzheimer’s dementia, Parkinson disease, and amyotrophic lateral sclerosis (32–37), and in heat-shocked astrocytoma cells (38).

The evidence for a role of CDK5 outside the nervous system is scant. Both CDK5 and p35 have been detected in developing tissues during periods of programmed cell elimination (39, 40). Cdk5 has been shown by immunohistochemistry to be concentrated in dying cells (31, 40) and in terminally differentiated cells (39).

The present study demonstrates an essential role for CDK5 in the apoptotic process induced by the cAMP analog 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate (8-CPT-cAMP) in promyelocytic cells. By using cell-permeable

* This work was supported by grants from the Norwegian Cancer Society (to T. S., S. D., and L. H.), from the Novo Nordic Insulin Foundation (to S. D.), and from Grethe Harbitz legate (to L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: CRE, cAMP-dependent protein kinase responsive element; CREB, cAMP-response element-binding protein; CREM, CRE modulator; ICER, inducible cAMP early repressor; CDK, cyclin-dependent protein kinase; GSK-3 β, glycogen-synthase kinase3; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate; zVAD-fmk, z-val-ala-DL-asp-fluormethylketone; dn, dominant-negative; RC, roscovitine; CHAPS, (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate; GFP, green fluorescent protein; EGFP, enhanced GFP; dn, dominant-negative; wt, wild-type.
CDK5 antagonists (41), a requirement for CDK activity is demonstrated in a narrow time window preceding caspase activation. Finally, overexpression of CDK5-wt is shown to enhance death, whereas enforced expression of dominantly negative CDK5 will protect against apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Constructs—The protein kinase inhibitors butyrolactone-1 and olomoucine were from Calbiochem, alsterpaullone and purvalanol A were from Alexis Corp. (Lausen, Switzerland), 6-γ,y-di-methylallylamine) purine (isopenetyladenine) was from Sigma, roscovitine (RCV) was from Biomol (Plymouth, MA), and KN93 was from Seikagaku America (Rockville, MD). The cAMP-dependent protein kinase activator 8-CPT-cAMP was from BioLog (Bremen, Germany). The caspase inhibitor z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was from Bachem Feinchemikal G.U. (Bubendorf, Switzerland). [α-32P]dCTP was from Amersham Biosciences. 96J-G (pJim) retrovirus vector was a kind gift from Jim Loren and Garry Nolan at Stanford Medical University (42). The pIRE52-EGFP expression vector was from CLONTECH (No. 6029-1). The pcDNA3.1-CDK5-wt (43) was from John Eriksson at Turku Biocenter, Turku, Finland, and pCMV-CDK5-T33 (30) was a kind gift from Zarah Zakeri at the City University of New York, New York. The pcMv-CDK1-dn and pcMv-CDK2-dn (14) was a gift from David Ucker at the University of Illinois, Chicago, IL. Mouse brain extract was from Santa Cruz Biotechnology (Santa Cruz, CA, SC-2255).

Cell Culturing and Scoring of Apoptosis—The cells used were either the wild-type IPC-81 rat pteryomelytic leukemia cell line or a subclone, IPC-S11CER (kindly provided by Dr. M. Lanotte, Hôp, St. Louis, Paris, France), with enforced, stable expression of ICER, which is an inhibitor of gene transcription via the CREB and CREM family of cAMP-regulated transcription factors (13). The cells were cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 7% horse serum (Invitrogen), streptomycin (5 μg/ml), and penicillin (5 units/ml) (7).

The cells had a doubling time of 11–12 h and were kept in continuous logarithmic growth. The cell density was adjusted to 200,000 cells/ml when experiments were started. Apoptosis was determined as described and validated previously (7, 44) by microscopy of cells fixed in phosphate-buffered saline containing 2% formaldehyde and 10 μg/ml of the DNA-specific dye bisbenzimide, Hoechst 33342, (Calbiochem). The evaluation was done blindly by two independent and experienced evaluators. Random microscopic fields were selected for study with cells with more than 50% of the area within the chosen sector being included for analysis.

Determination of mRNA Expression—IPC-81 cells were treated for various periods of time with 8-CPT-cAMP or vehicle (control), and their RNA was isolated using the acidic guanidinium thiocyanate-phenol-chloroform extraction method (45). The RNA was treated with RNase-free DNase to remove contaminating genomic DNA, and its integrity was assessed by electrophoresis (12.5% acrylamide SDS-denaturing gels). The RNA was treated with RNase-free DNase to remove contaminating genomic DNA, and its integrity was assessed by electrophoresis (12.5% acrylamide SDS-denaturing gels). The RNA was treated with RNase-free DNase to remove contaminating genomic DNA, and its integrity was assessed by electrophoresis (12.5% acrylamide SDS-denaturing gels). The RNA was treated with RNase-free DNase to remove contaminating genomic DNA, and its integrity was assessed by electrophoresis (12.5% acrylamide SDS-denaturing gels). The RNA was treated with RNase-free DNase to remove contaminating genomic DNA, and its integrity was assessed by electrophoresis (12.5% acrylamide SDS-denaturing gels).

RESULTS

Protein Kinase Inhibitors Showed a Strict Proportionality in Their Ability to Inhibit CDK5 and to Protect against 8-CPT-cAMP-induced Apoptosis—Transcriptional activity is essential during the first 1–2 h of the preapoptotic period in IPC-81 cells stimulated to die by cAMP-elevating agents or by cAMP analogs such as 8-CPT-cAMP (8, 13). In preliminary experiments, a commercially available rodent cDNA Atlas Array, containing 597 non-overlapping cDNA probes from different mouse genes, was used to search for mRNAs showing increased expression during this period. CDk5 mRNA showed the highest increase in expression, along with Fos-related antigen 2 and thrombomodulin. Known proapoptotic genes such as Bax and Bag-1 (48, 49) did not show any increased expression (data not shown).

To elucidate whether CDK5 activity was required for cAMP-induced death, IPC-81 cells were treated with 8-CPT-cAMP and various concentrations of CDK5 inhibitors for 6 h and scored for apoptosis. The structurally different CDK5 inhibitors roscovitine and butyrolactone-1 (50–52) were both able to almost completely block apoptosis, with IC50 values (IC50apopt) of 7 and 5 μM, respectively (Fig. 1A). The inhibitors had no apparent effect on cell proliferation (Fig. 1, A and D). After 24 h of incubation, the EC50 value for death induction was 50 and 70 μM, respectively (Fig. 1B). To examine whether the inhibitors protected the cells completely against death or only against the morphological features of apoptosis (Fig. 1F), cells were co-treated with 8-CPT-cAMP and roscovitine for 6 h and then transferred to fresh

CDk5 and cAMP-induced Apoptosis

Western Blot Analysis—Cells were washed in phosphate-buffered saline and then lysed in 10 mM K2HPO4, 10 mM KH2PO4, 1 mM EDTA (pH 6.8) containing 10 mM CHAPS, 50 μM NaF, 0.3 mM Na3VO4 (magnonovanadate) supplemented with Complete mini protease inhibi-
medium. Whereas cells treated with 8-CPT-cAMP alone were completely apoptotic (Fig. 1G), cells co-treated with 8-CPT-cAMP and roscovitine were viable and able to vigorously reproduce 24 h later (Fig. 1H and data not shown).

A battery of CDK inhibitors was tested to establish a firmer correlation between the inhibition of CDK activity and apoptosis. These inhibitors covered a wide range of IC50 values for CDK5 inhibition, and some of them could be used to discriminate between CDK family members and GSK-3β (see Ref. 53 and other references listed in Table I). All the CDK5 inhibitors tested were able to protect against cAMP-induced apoptosis with IC50apo values ranging from 0.2 μM (alsterpaullone) to 600 μM (isopentenyladenine). The IC50apo values were plotted against the IC50 values (see Table I for details) for CDK1, CDK2, and CDK5 (Fig. 2A) as well as for CDK4 and GSK-3β (Fig. 2B). A strict proportionality was observed over a range of 4 orders of magnitude (Fig. 2A) between IC50apo and IC50 for CDK5 (the calculated slope of the best fit straight line being 0.99) and slightly less for CDK2 (slope of 0.87) and CDK1 (slope of 1.14). In contrast, there was no strong correlation between IC50apo and IC50 for either CDK4 or GSK-3β (Fig. 2B). The CDK inhibitors did not protect the cells from death induced by okadaic acid, staurosporine, or daunorubicine (Table II and data not shown). In conclusion, soluble inhibitors of CDK5, CDK2, and CDK1 were able to specifically block the cAMP-dependent cell death.

**Fig. 1.** Inhibitors of CDK5 protected completely against 8-CPT-cAMP-induced leukemic cell death. IPC-81 rat leukemia cells were treated with various concentrations of roscovitine (●) or butyrolactone-1 (▲) in the absence (filled symbols) or presence (open symbols) of 200 μM 8-CPT-cAMP for 6 h. Apoptosis was scored as described under “Experimental Procedures.” The concentration of CDK inhibitor required for half-maximal protection (IC50apo) against apoptosis was 7 μM for roscovitine and 8.5 μM for butyrolactone-1 (A). B shows the apoptogenic effect of roscovitine or butyrolactone-1 alone upon prolonged incubation (24 h). Note that the concentration required to induce apoptosis (EC50) was much higher, 50 μM for butyrolactone-1 and 70 μM for roscovitine, than those required to protect against apoptosis. Data represent the mean ± S.E. of six (A) or three (B) experiments. C–H show IPC-81 cells stained with the DNA binding fluorescent dye (Hoechst 33342). C–F show IPC-81 cells after 6 h of incubation with vehicle (C), 20 μM roscovitine (D), 200 μM 8-CPT-cAMP (E), or 8-CPT-cAMP and roscovitine (F). G and H show cells preincubated for 6 h with 8-CPT-cAMP (G) or 8-CPT-cAMP and roscovitine (H) followed by wash and 18 h incubation in plain medium.

**Inhibition of the Cell Cycle-related CDK1 and CDK2 Failed to Protect against cAMP-induced Death**—Retroviral transduction of IPC-81 cells with dominantly negative CDK1 (CDK1-dn) or CDK2 (CDK2-dn) was performed to further discriminate the role(s) of the different CDKs in the apoptotic process. This approach (14) has been used successfully to show the involvement of CDK2 in staurosporine- and tumor necrosis factor-α-induced death, among others (19). The IPC-81 cells were infected with recombinant retrovirus containing bicistronically encoded CDK-dn and enhanced GFP (EGFP). Fluorescent cells were considered to co-express CDK-dn and EGFP. The cells transduced with CDK1-dn or CDK2-dn grew more slowly than cells transduced with EGFP alone, and after 20–30 generations, they were completely outgrown by non-transduced cells, as expected from the inhibition of CDK2 in staurosporine- and tumor necrosis factor-α-induced death, among others (19). The IPC-81 cells were infected with recombinant retrovirus containing bicistronically encoded CDK-dn and enhanced GFP (EGFP). Fluorescent cells were considered to co-express CDK-dn and EGFP. The cells transduced with CDK1-dn or CDK2-dn grew more slowly than cells transduced with EGFP alone, and after 20–30 generations, they were completely outgrown by non-transduced cells, as expected from the inhibition of CDK1 or CDK2. The cells were therefore tested for sensitivity to 8-CPT-cAMP-induced apoptosis during the first 5–10 generations after transduction. Cells transduced with CDK1-dn or CDK2-dn showed slightly increased apoptosis in response to 8-CPT-cAMP as compared with either non-fluorescent cells in the same dish (not shown) or with cells transduced with EGFP alone (Fig. 3). This suggested that the protective effect of the inhibitors of CDKs (Figs. 2 and 3).
TABLE I

IC50 values of cdk inhibitors as apoptosis antagonists

| CDK inhibitor     | IC50 apoptosis Cdk1/cyclinB | IC50 apoptosis Cdk2/cyclinA2 | Kinase | Activity | Ref.       |
|-------------------|----------------------------|-----------------------------|--------|----------|------------|
| Alsterpaullone    | 0.2                        | 0.0035                      | >10    | 0.040    | 53, 73     |
| Purvalanol A      | 0.860                      | 0.004                       | 0.07   | 0.075    | 53, 72, 73 |
| Butyrolactone I   | 8.5                        | 1.1                         | 0.68   | 0.15     | 51, 53     |
| Roscovitine       | 7.0                        | 0.45                        | 0.7    | 100      | 50, 53     |
| Olomoucine        | 22                         | 7                           | 7      | >1000    | 53, 54     |
| Isopentenyadenine | 600                        | 55                          | 50     | >100     | 53, 54     |

1 and 2 could not be explained by the inhibition of CDK1 or CDK2.

We have shown previously that IPC-81 cells are sensitive to cAMP-induced death in all phases of the cell cycle but particularly in the late S phase/G2 (7). The slight enhancement of death observed in cells expressing CDK1-dn and CDK2-dn (Fig. 3) could therefore be due to the accumulation of cells in a more vulnerable phase of the cell cycle. A 6-h pulse with the CDK inhibitor roscovitine should lead to an increased proportion of cells in the vulnerable part of the cell cycle and an enhanced sensitivity to cAMP-induced death. This was also demonstrated in cells pretreated with roscovitine, which showed a more rapid apoptotic response to 8-CPT-cAMP than the control cells (Fig. 4).

In conclusion, inhibition of CDK1 and CDK2 could not explain the anti-apoptotic effect of CDK inhibitors. In contrast, inhibition of CDK1 and CDK2 slightly enhanced 8-CPT-cAMP-induced death.

Overexpression of CDK5 Enhanced Apoptosis, whereas Enforced Expression of CDK5-dn Protected against cAMP-induced IPC-81 Cell Apoptosis—Since inhibitors directed against CDK1, CDK2, and CDK5 protected against apoptosis and CDK1-dn and CDK2-dn failed to protect (see above), we presumed that a selective inhibition of CDK5 would result in a reduced rate of apoptosis. To test this hypothesis, a kinase activity-deficient CDK5 (CDK5-T33), demonstrated to have a dominant negative effect on CDK5-dependent cellular processes (30), was expressed in IPC-81 cells. Cells with enforced expression of CDK5-dn (CDK5-T33) were partially protected against cAMP-induced apoptosis, in contrast to the cells with enforced expression of wild-type CDK5, which appeared to have an increased susceptibility to apoptosis (Fig. 5A).

To estimate the expression of CDK5-dn and CDK5-wt, subclones of such cells were selected by flow cytometry and cell sorting based on the fluorescence of the co-expressed GFP gene product and expanded. As demonstrated by immunoblot analyses, there was a robust overexpression of CDK5 in the expanded clones (Fig. 5, B and C). It appeared that cells overexpressing CDK5-wt had a shorter latency time before the onset of apoptosis (Fig. 5B) and required lower concentrations of 8-CPT-cAMP to die (Fig. 5C). The opposite was demonstrated also for cells overexpressing CDK5-dn. Based on these results, we therefore conclude that active CDK5 was necessary for cAMP-induced apoptosis.

The CDK5-overexpressing cells exhibited normal growth rate and low frequency of spontaneous apoptosis despite having enhanced cAMP-induced apoptosis (Fig. 5 and data not shown). This observation suggested that other cAMP-induced factors, besides CDK5, were required together with CDK5 to commit the cells to apoptosis. The experiments shown in Fig. 6 were undertaken in part to know whether CDK activity was essential for the 8-CPT-cAMP induction of such factors. Cells preincubated with 8-CPT-cAMP and roscovitine during the first 2 h and then with only 8-CPT-cAMP had nearly as high apoptosis after 6.5 h (Fig. 6C) as cells incubated continuously with...
The effect of cdk inhibitors on okadaic acid- (OA), daunorubicine- (DR), and staurosporine (SP)-induced apoptosis

IPC-81 leukemia cells were treated with 0.5 μM OA (9 h), 0.5 μM DR (6 h), or 0.1 μM SP (4 h) in the absence or presence of 15 μM roscovitine (RCV) or 15 μM butyrolactone-1 (BRL). Apoptotic cell death was scored at the end of treatment (indicated by %) for each compound tested as described under “Experimental Procedures.” Data represent the mean ± S.E. from six different experiments.

| OA   | DR  | SP  | RCV | BRL | Apoptosis | S.E. |
|------|-----|-----|-----|-----|-----------|------|
| μM  | μM  | μM  | μM  | μM  | %         | n    |
| 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 81.1      | 2  |
| 0.5 | 0.5 | 0.5 | 0.5 | 85.3 | 2.3       | 3  |
| 0.5 | 0.5 | 0.5 | 0.5 | 84.4 | 1.6       | 4  |
| 0.5 | 0.5 | 0.5 | 0.5 | 49.4 | 1.9       | 1  |
| 0.5 | 0.5 | 0.5 | 0.5 | 38.2 | 1.2       | 1  |
| 0.5 | 0.5 | 0.5 | 0.5 | 32.7 | 3.0       | 1  |
| 0.5 | 0.5 | 0.5 | 0.5 | 91.9 | 2.4       | 1  |
| 0.5 | 0.5 | 0.5 | 0.5 | 94.2 | 1.2       | 1  |
| 0.5 | 0.5 | 0.5 | 0.5 | 93.7 | 1.2       | 1  |

8-CPT-cAMP (Fig. 6A) and considerably more than cells preincubated with vehicle only (Fig. 6B). This suggested that factors essential for cell death were induced independently of CDK5 activity, at least during the first 2 h of 8-CPT-cAMP treatment.

To examine the specific time point prior to the death onset that CDK5 had to be active, roscovitine was present during the first 3, 4, 5, or 6 h of 8-CPT-cAMP treatment. The cells were then washed and incubated with 8-CPT-cAMP alone to make the total incubation time 6.5 h. The release of CDK inhibition after 6 h did not lead to any enhanced death (Fig. 6C). Cells preincubated for 5, 4, or 3 h with roscovitine showed gradually higher rates of apoptosis with half-maximal protection being noted when the release from roscovitine occurred about 2.3 h before the assessment of death, i.e., after slightly more than 4 h of preincubation with roscovitine (Fig. 6D). This suggested that an average of 2–2.5 h was required for CDK5 to phosphorylate or interact with relevant substrates and/or for the latter to induce apoptosis in cells primed with cAMP.

The Critical CDK5-dependent Step Occurred Immediately Upstream of Caspase Activation—To pinpoint more closely the CDK5-dependent step in the cAMP-induced death program, the CDK inhibitor roscovitine was added at various time points to IPC-81 cells continuously treated with 8-CPT-cAMP. Delaying the time point of roscovitine addition from 1 to 3 h after the onset of 8-CPT-cAMP treatment resulted in a progressive decline in protection against apoptosis (Fig. 7A). Half-maximal protection was observed when the CDK activity was blocked about 155 min after the addition of 8-CPT-cAMP (Fig. 7, A and C). An analogous experiment was performed with the pan-caspase inhibitor zVAD-fmk. This inhibitor could protect the cells even longer, half-maximal protection being noted 180 min after the addition of 8-CPT-cAMP (Fig. 7, B and C). Similar results were obtained with another broad-acting caspase inhibitor, Boc-fmk (data not shown). This suggested that the CDK5-dependent commitment step occurred before the caspase-dependent commitment step. The protein kinase inhibitor KN-93 did not mimic the effect of the CDK inhibitor. KN-93 has specificity toward the multifunctional Ca2+/calmodulin-dependent protein kinases I, II, and V (55) and inhibits okadaic acid-induced cell death (56). Experiments similar to those for roscovitine and zVAD-fmk were conducted with the protein synthesis inhibitor cycloheximide. This compound could protect 50% of the cells when given 140 min after 8-CPT-cAMP (data not shown). This suggested that CDK activity was required until shortly after the requirement for protein synthesis disappeared and prior to the activation of zVAD-inhibited caspases. These data suggested that CDK activity was required upstream of caspase activity, and it could also be required for caspase activation. We therefore compared the cleavage of pro-caspase-3 in cells treated with 8-CPT-cAMP in the absence and presence of roscovitine. The CDK inhibitor prevented cleavage of pro-caspase-3 (Fig. 8), demonstrating that the critical CDK5-dependent step occurred upstream of caspase activation.

Cdk5 Is Induced through CRE during the Preapoptotic Phase of cAMP-induced Leukemic Cell Death—Overexpression of CDK5 accelerated the cAMP-induced death response (Fig. 5), indicating that elevation of CDK5 above its basal level could enhance apoptosis. For this reason, it was of interest to know whether the induction by cAMP of CDK5 mRNA observed in the gene arrays (see above) was translated into increased levels of CDK5 protein. Western blot analysis showed increase pro-
tein level of CDK5, using either polyclonal (data not shown) or monoclonal (Fig. 9A) antibodies against CDK5. The protein expression of CDK2 (Fig. 9B) and CDK1 (Fig. 9C) was also analyzed. Each blot was re-probed with anti-β-actin monoclonal antibody as an internal control of protein loading (data not shown). The densitometrically determined intensity of CDK1, CDK2, and CDK5 was divided by the corresponding intensity of β-actin. The relative CDK/actin values in cell extracts from cells treated with and without 8-CPT-cAMP were plotted as a function of the time of treatment. The CDK5/actin ratio was significantly (p < 0.003) increased in cells treated from 90 to 180 min with 8-CPT-cAMP. No significant effect was noted for CDK1 and CDK2 (Fig. 9E). The presence of roscovitine did not affect the level of CDK1, CDK2, or CDK5 (data not shown). When using antibodies specific for time with 200 μM 8-CPT-cAMP (B) or for 20 h with various concentrations of 8-CPT-cAMP (C). The presence of roscovitine did not affect the level of CDK1, CDK2, or CDK5 (data not shown). When using antibodies specific for protein level of CDK5, using either polyclonal (data not shown) or monoclonal (Fig. 9A) antibodies against CDK5.

The protein expression of CDK2 (Fig. 9B) and CDK1 (Fig. 9C) was also analyzed. Each blot was re-probed with anti-β-actin monoclonal antibody as an internal control of protein loading (data not shown). The densitometrically determined intensity of CDK1, CDK2, and CDK5 was divided by the corresponding intensity of β-actin. The relative CDK/actin values in cell extracts from cells treated with and without 8-CPT-cAMP were plotted as a function of the time of treatment. The CDK5/actin ratio was significantly (p < 0.003) increased in cells treated from 90 to 180 min with 8-CPT-cAMP. No significant effect was noted for CDK1 and CDK2 (Fig. 9E). The presence of roscovitine did not affect the level of CDK1, CDK2, or CDK5 (data not shown). When using antibodies specific for

FIG. 5. Enforced expression of CDK5-dn counteracted apoptosis in IPC-81 cells, and overexpression of CDK5-wt showed enhanced apoptosis. Cells were transduced with retrovirus carrying a bicistronic expression vector for expression of EGFP alone, expression of CDK5-dn and EGFP, or expression of CDK5-wt and EGFP (see "Experimental Procedures"). A shows the apoptotic score of EGFP-expressing cells treated with 200 μM 8-CPT-cAMP for 3 or 5 h. The cells were studied 48 h after infection, and EGFP expression was detected by fluorescence microscopy. For the experiments shown in B and C, cells with enforced expression of either EGFP alone (○), CDK5-dn and EGFP (●), or CDK5-wt and EGFP (△) were selected by flow cytometry with cell sorting and expanded. They were then treated for various periods of

FIG. 6. The effect of release of CDK inhibition on apoptosis. Cells were pretreated for various periods of time with 200 μM 8-CPT-cAMP (A), vehicle (B), or a combination of 20 μM RCV and 200 μM 8-CPT-cAMP (C). The drugs were removed by washing, and the cells were further incubated with 200 μM 8-CPT-cAMP for a total incubation time of 6.5 h, at which time the cells were fixed for the scoring of apoptosis. The data represent the mean ± S.E. from four different experiments. The presence of RCV during the first 2 h had no significant effect on apoptosis, but the substitution of 8-CPT-cAMP with vehicle had a significant (p = 0.004, Student's t test) effect. The effect of RCV during the first 3 h was highly significant (p < 0.001). D shows a plot of the results in C and of additional similar experiments. It shows the effect of the release of CDK inhibition (by removing roscovitine) on apoptosis after 6.5 h of treatment. The horizontal arrows show during which period of time RCV was present.
the neuronal CDK5 activator p35/p25, no p35/p25 was detected in IPC-81 cell extracts, in contrast to a clear expression in mouse brain extracts run in parallel (data not shown).

A putative CRE element has been reported in the promoter region of the mouse cdk5 gene (57). Such elements are activated by CREB/CREM transcription factor family members, whose actions are counteracted by the inhibitor protein of cAMP-responsive element, ICER (12, 58). Treatment with 8-CPT-cAMP of IPC-81 cells with enforced stable expression of ICER (13) failed to increase CDK5 protein (Fig. 9, D and F) and CDK5 mRNA on gene arrays (data not shown) or Dot Blots (Fig. 10 A). A moderate increase of CDK5 mRNA was observed in wild-type cells treated with 8-CPT-cAMP by Dot Blot and Northern blot analysis. The increase was highly significant as judged by Wilcoxon paired analysis (p < 0.001). No increase was observed for β-actin mRNA (data not shown). The CDK5/β-actin mRNA ratio showed a peak between 1 and 2 h of 8-CPT-cAMP-treatment (Fig. 10 B). In conclusion, the 8-CPT-cAMP induction of CDK5 in preapoptotic IPC-81 leukemic cells was mediated by CREB/CREM-dependent activation of

the pan-caspase inhibitor zVAD-fmk (zVAD; △). RCV or zVAD was added after various time periods of 8-CPT-cAMP–incubation, as indicated by the vertical arrows, and scored for apoptosis after a total of 6 h. For further details, see “Experimental Procedures.” Data represent the mean ± S.E. from five different experiments. C represents a replot of data similar to those presented in A and B. Cells were treated with 200 μM 8-CPT-cAMP for 6 h and scored for apoptosis. At the time points indicated on the abscissa, the cells received 100 μM zVAD-fmk (△), 30 μM RCV (○), or 20 μM KN93 (□). Further details are described in the description of panel A. The ordinate represents the percentage of cells that were apoptotic after 6 h incubation as compared with cells receiving 8-CPT-cAMP alone. Each point represents the mean ± S.E. of six separate experiments. The temporal difference in the degree of protection provided by zVAD and RCV was highly significant (p < 0.003, judged by the Wilcoxon signed-rank test).
DISCUSSION

The cAMP-stimulated IPC-81 cells undergo unusually rapid programmed cell death, 50% apoptosis being observed within 4 to 5 h after the onset of cAMP challenge. The cAMP level needs to be elevated during the first 2 h, and only during this time can transcriptional inhibitors abrogate death (7, 9). The present study shows that protein synthesis is required during the first 2.5 h of cAMP stimulation to achieve 50% death, whereas caspase activity is required for the first 3 h. By adding cell-permeable cyclin-dependent protein kinase inhibitors at various time points during the preapoptotic period, CDK activity was shown to be required prior to the caspase-dependent step.
i.e. until ~2.6 h after the onset of cAMP stimulation (Fig. 7). The complete recovery of cells co-incubated with CDK inhibitor and the agonistic cAMP analog 8-CPT-cAMP and then washed (Fig. 1H) argues that the CDK-dependent step was upstream of the irreversible part of the death execution pathway.

So far, two mechanisms of action have been reported for inhibitors of cell cycle-related CDKs, such as CDK1 and CDK2, to protect against apoptotic cell death. The first is by arresting cycling cells (14, 50) and thereby preventing them from entering parts of the cell cycle in which they are vulnerable to specific apoptogens (22, 59–61). This appears to be an unlikely explanation for the protective effect of CDK inhibitors in the present study since they were efficient when given less than 2 h before cell death. During such a short period (<2h), only a small fraction of the cells can accumulate in any position of the cell cycle, making it unlikely that the CDK inhibitors protected IPC-81 cells through cell cycle arrest. Furthermore, IPC-81 cells pulsed with CDK inhibitor for 4 h showed enhanced, rather than inhibited, apoptosis in response to 8-CPT-cAMP (Fig. 4). This is opposite of what was expected if the CDK inhibitors acted to arrest cells in a less vulnerable part of the cell cycle.

A second mechanism by which CDK inhibitors can protect against apoptosis is by blocking the unscheduled activity of CDK1 or CDK2 (19–23, 62). A number of apoptogens, including staurosporine (1a), okadaic acid (1b), and tumor necrosis factor-a (1c), induce caspase activation (2) and activation of CDK1 or CDK2 (3), leading to apoptosis (5) via unknown routes (4). B shows the possible pathways involving CDK5 in cAMP-induced IPC-81 leukemic cell death. The increase of cellular cAMP (1) results in translocation of the catalytic subunit (C) of the cAMP-dependent kinase A to the nucleus (2) and the activation of CRE-governed gene transcription (3). This, in turn, leads to increased expression of CDK5 mRNA and protein (4 and 5) and possibly of CDK5 activator (x) or substrate (y). Caspase activation (7) and apoptosis (8) requires CDK5 phosphorylation of either the induced (γ) protein substrate or of a preformed (x) protein substrate (6).

The involvement of CDK5 is further strengthened by the observation of up-regulation by 8-CPT-cAMP to death. The CDK5 mRNA level increased during the first 2 h of 8-CPT-cAMP stimulation, when cAMP-dependent gene transcription is critical for the cell death program to be initiated (8, 13). Furthermore, the CDK5 protein level increased in the preapoptotic time window when protein synthesis was required for death. Cells expressing an inhibitor (ICER) of CRE-mediated gene transcription were blocked with respect to both up-regulation of CDK5 (Fig. 2) and death (13). Finally, cells with enforced overexpression of CDK5 showed a more rapid apoptotic response in response to 8-CPT-cAMP.

Cdk5 is not the only cAMP-dependent factor required for IPC-81 cell death since overexpression of CDK5 to a level above that obtained in 8-CPT-cAMP-treated cells did not induce death by itself. The results of experiments where 8-CPT-cAMP was co-incubated with CDK5 inhibitor, which was subsequently removed, suggested that CDK5, in order to be proapoptotic, had to co-exist with other cAMP-induced factors. Such
factors can be CDK5 activators that post-transcriptionally modify CDK5, CDK5 substrates, or enhances of CDK5 substrate actions. Some of these options are depicted in Fig. 11B. We were unable to detect significant levels of the known CDK5 activator p35 in either untreated or 8-CPT-cAMP-treated IPC-81 cells, and the identity of the CDK5 activator(s) in IPC-81 cells is still unknown, as is the apoptosis-relevant substrate for CDK5. We do know, however, that the CDK-dependent step occurred about 25 min prior to the point when cells could no longer be protected by caspase inhibitors and that an additional couple of hours elapsed from the CDK-dependent commitment point until the cells became morphologically apoptotic with disarranged cytoskeleton.

Hyperphosphorylation of the intermediate filament Tau with subsequent effects on the cytoskeleton has been described in cases when CDK5 has been activated (27, 32, 33, 53, 69, 70). The present study strongly supports this notion.

In conclusion, cAMP-induced IPC-81 leukemia cell death appears to depend on CDK5 activity and is accompanied by CDK5 up-regulation mediated through CRE-dependent transcrip-

tion. This may be the clearest example so far of an extra-

neuronal effect of CDK5 and represents the first example of a proapoptotic CDK action upstream of caspase activation. This CDK-dependent programmed cell death system in IPC-81 cells offers a unique opportunity to identify apoptosis-associated CDK substrates upstream of caspase activation and to study CDK5 regulation outside the nervous system.

Acknowledgments—We are grateful to Dr. David Ucker for providing pCMV-CDK1-dn/CDK2-dn and for careful reading of the manuscript, Dr. Zahara Zakeri for providing pCMV-CDK5-T33, Dr. Laurent Meijer for providing pcDNA-3.1-CDK5-wt, Dr. Ole Eriksson for providing pcDNA-3.1-CDK5-dn, Dr. John Eriksson for providing pcDNA-3.1-CDK5-wt, Dr. Ole Eriksson for providing pcDNA-3.1-CDK5-dn, and Dr. Laurent Meijer for providing pCMV-CDK1-dn/CDK2-dn and for careful reading of the manuscript.

REFERENCES

1. Vaux, D. L., and Strassler, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2229–2234.
2. Strasser, A., O'Connor, L., and Dixit, V. M. (2000) Annu. Rev. Biochem. 69, 217–245.
3. Schneider, P., and Tschopp, J. (2000) Pharoa. Acta Hsul 74, 281–286.
4. Walczak, H., and Krammer, P. H. (2000) Exp. Cell Res. 255, 56–66.
5. Nichols, D. W. (1999) Cell Death Differ. 6, 1028–1042.
6. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052.
7. Gerstjens, B. T., Cressy, L. I., Ruchaud, S., Houe, G., Lanoette, M., and Deslandes, S. O. (1994) J. Cell. Sci. 107, 3363–3377.
8. Lanotte, M., Riviere, J. B., Hermouet, S., Houe, G., Vintervy, O. K., Gerstjens, B. T., and Deslandes, S. O. (1991) J. Cell. Physiol. 146, 73–80.
9. Ruchaud, S., and Lanotte, M. (1997) Biochem. Soc. Trans. 25, 410–415.
10. Gerstjens, B. T., Millgren, G., Otten, A., Maronde, E., Genieser, H. G., Jastorff, B., Vintervy, O. K., McKnight, G. S., and Deslandes, S. O. (1995) J. Biol. Chem. 270, 20599–20607.
Rocque, W. J., Shewchuk, L., Veal, J. M., Walker, D. H., and Kuyper, L. F. (2001) Science 291, 134–137

Choi, K. S., Eom, Y. W., Kang, Y., Ha, M. J., Rhe, H., Youn, J. W., and Kim, S. J. (1999) J. Biol. Chem. 274, 31775–31783

Yao, S. L., McKenna, K. A., Sharkis, S. J., and Bedi, A. (1996) Cancer Res. 56, 4551–4555

Kim, S. G., Kim, S. N., Jong, H. S., Kim, N. K., Hong, S. H., Kim, S. J., and Bang, Y. J. (2001) Oncogene 20, 1254–1265

Li, W., Fan, J., and Bertino, J. R. (2001) Cancer Res. 61, 2579–2582

Jin, Y. H., Yoo, K. J., Lee, Y. H., and Lee, S. K. (2000) J. Biol. Chem. 275, 30256–30263

Gervais, J. L., Seth, P., and Zhang, H. (1998) J. Biol. Chem. 273, 19207–19212

Tang, D., Lee, K. Y., Qi, Z., Matsuura, I., and Wang, J. H. (1996) Biochem. Cell Biol. 74, 419–429

Zhang, J., and Johnson, G. V. (2000) J. Neurochem. 75, 2346–2357

Munoz, J. P., Alvarez, A., and MacCioni, R. B. (2000) Neuroreport 11, 2733–2738

Yin, M. B., Toth, K., Cao, S., Gus, B., Frank, C., Slocum, H. K., and Rustum, Y. M. (1999) Int. J. Cancer 85, 341–348

Gray, N., Dettavaud, L., Doerig, C., and Meijer, L. (1999) Curr. Med. Chem. 6, 859–875

Gray, N. S., Wodicka, L., Thunnissen, A. M., Norman, T. C., Kwon, S., Espinoza, F. H., Morgan, D. O., Barnes, G., Leclerc, S., Meijer, L., Kim, S. H., Lockhart, D. J., and Schultz, P. G. (1998) Science 281, 533–538