cAMP inhibits translation by inducing Ca^{2+}/calmodulin-independent elongation factor 2 kinase activity in IPC-81 cells

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Received 6 November 1998

Abstract Treatment of IPC-81 cells led to inhibition of protein synthesis, which was accompanied by an increase in the average size of polysomes and a decreased rate of elongation, indicating that it involved inhibition of peptide chain elongation. This inhibition was also associated with increased phosphorylation of elongation factor eEF2 (which inhibits its activity) and enhanced Ca^{2+}/calmodulin-independent activity of eEF2 kinase. Previous work has shown that phosphorylation of eEF2 kinase by cAMP-dependent protein kinase (cAPK) in vitro induces such activator-independent activity, and the present data show that such a mechanism can occur in intact cells to link physiological levels of cAPK activation with inhibition of protein synthesis.

Key words: cAMP; Translation; Elongation factor 2; eEF2 kinase; IPC-81 cell

1. Introduction

mRNA translation is subject to acute regulation under a wide variety of conditions, and this is thought to involve, primarily, changes in the activity or availability of translation initiation or elongation factors, which in turn are mediated through alterations in their states of phosphorylation [1–4]. The role of cAMP-dependent protein kinase (cAPK) in the regulation of translation in intact cells has not been firmly established [5–7]. Although several translation initiation factors (e.g. eIF3, eIF4A, eIF4B, eIF4G) have been reported to be in vitro substrates for cAPK [1,8], the biological significance of such in vitro phosphorylation is unclear.

There is also a sophisticated system for regulating peptide chain elongation [9] (for reviews, see [1,10]). The best studied example of the acute control of elongation involves inactivation of elongation factor 2 (eEF2), a protein required for translocation of peptidyl-tRNAs from the A-site to the P-site [11–13]. eEF2 can be phosphorylated and inactivated by a dedicated eEF2 kinase (also known as Ca^{2+}/calmodulin-dependent kinase III), which so far appears to have eEF2 as its only substrate [13,14]. This kinase and myosin heavy chain kinase A are so far the only known members of a unique kinase family whose ATP binding site is the only sequence motif shared with other eukaryotic protein kinases [9]. Another pathway for inhibition of eEF2, ADP ribosylation, is also remarkably dedicated to eEF2 regulation [5,15]. The question of whether control of elongation is physiologically important has remained largely unanswered, but it was recently demonstrated that insulin stimulates protein synthesis by enhancing elongation through dephosphorylation of eEF2 [16].

In the present study we have investigated the effects of cAMP on protein synthesis and specifically peptide chain elongation, in intact cells. We were prompted to do this by the observation that, in vitro, cAPK can phosphorylate and activate eEF2 kinase (by rendering its activity partially independent of Ca ions) [17]. It was a high priority to study whether physiological levels of cAPK activation can regulate eEF2 kinase in intact cells and whether this led to modulation of the rate of elongation. For our work, we used the IPC-81 cell line which has been used extensively in studies on the anti-growth effects of cAMP [18]. Furthermore, IPC-81 cell clones expressing a mutant of cAPK which responds poorly to cAMP are available [19]. The data indicate that cAMP regulation of eEF2 kinase operates to regulate peptide chain elongation in intact cells.

2. Materials and methods

2.1. Model system

Rat myeloid IPC-81 cells were cultured at densities of around 5×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% horse serum [20]. The IPC-RI_{D336} cAMP-resistant clone was isolated as described by [19]. Apoptosis-resistant bcl-2-transfected IPC-81 cells (IPC-81_{bcl2}) were used in some experiments. cAPK was activated by adding the cAMP analog 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) to the growth medium.

2.2. Determination of protein synthesis

At the start of the experiment, the cells (IPC-81_{wt}, IPC-81_{bcl2} or IPC-RI_{D336}) were shifted to CO_2-equilibrated and methionine-free DMEM containing 2% horse serum. The cAMP agonist 8-CPT-cAMP (100 μM final concentration) or the cAMP antagonist Rp-8-Br-cAMPS (200 μM final concentration) was added to the medium 1 h before cell harvesting. 15 min before harvesting each well was supplemented with 7.5 μCi [^{35}S]methionine (Amersham SJ1515). After centrifugation ($1100\times g$ for 2 min at 4°C) the cell pellets were resuspended and lysed in ice-cold 50 mM Tris-HCl pH 7.5 containing 1% Nonidet-P40, 100 mM NaCl, 2 mM EDTA and a protease inhibitor cocktail (0.7 μg/ml chymostatin, 0.6 μg/ml antipain, 5 μg/ml leupeptin and 1.4 μg/ml pepstatin). The lysates were centrifuged at $16\,000\times g$ for 10 min, and the proteins in the supernatant were precipitated by adding trichloroacetic acid (TCA) to 10% final concentration. The precipitate was filtered through GF-C filters (Whatman), washed three times with 1 ml 10% TCA and finally with 1 ml 96% ethanol. The filters were air-dried and counted in scintillant. Cumulative protein synthesis was measured by a similar procedure, but with

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PII: S0014-5793(99)00039-3

[^{35}S]methionine present in the growth medium from the start of the experiment.

2.3. Polysomal profile analysis and morphometric evaluation of polysome size

Cells were resuspended in 1 ml K buffer (20 mM Tris-HCl pH 7.6, 120 mM KCl and 7 mM $MgSO_4$) and lysed by adding Triton X-100 to 0.4% final concentration. Nuclei and membrane-bound ribosomes were removed by centrifugation at $6000\times g$ for 10 min. Ribosomes from approximately 1.5×10^7 cells were fractionated on a linear sucrose gradient as described [21]. IPC-81 cells were also prepared for electron microscopy [17], and membrane-bound and free polysomes were quantitated by morphometric analysis, as described by Gundersen [22]. Polysomes were studied in series of sections, and particles were sampled using a random plane with identical probabilities, irrespective of particle size.

2.4. Estimation of elongation rate

The effect of cAMP treatment on the ribosomal transit time was measured as described [23]. This involves the separate determination of the cumulative [^{35}S]methionine incorporation into the free ribosome fraction (containing completed protein chains) compared with total cellular protein (containing both completed and unfinished proteins).

2.5. Determination of eEF2 phosphorylation by Western blotting after 2D-PAGE

Cell lysates were prepared using a EMBL-made cell cracker (a metal cylinder with an internal diameter of 8.020 mm containing a metal bead with a diameter of 8.006 mm) with a buffer containing both protease and phosphatase inhibitors (15 mM HEPES pH 7.5, 2.3 mM $MgCl_2$, 120 mM KCl, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM sodium orthovanadate, 1 mM ATP, 1 mM pyrophosphate, 1 μM microcystin and the protease inhibitor cocktail (see above)). Before isoelectric focusing (first dimension protein separation), samples were diluted 1:4 in 2D buffer containing 9.8 M urea, 100 mM 1,4-dithioerythritol, 1.5% v/v Pharmalyte (Pharmacia) pH 3.5–10, 0.5% v/v Pharmalyte pH 5–6, 4% w/v CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and 0.2% w/v SDS. The two-dimensional sample separation was done as described [24] using a linear immobilized pH 3–10 gradient (Pharmacia) for the first dimension run and 7.5% SDS-PAGE for the second dimension run. After completion of the gel run, the proteins were transferred to nitrocellulose membranes by electroblotting for 70 h at 15 V and 4°C. Blots were incubated with anti-eEF2 antibody [25] followed by alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega). The blots were developed using BCIP/NBT (Sigma) as substrate.

2.6. eEF2 kinase assay

The Ca^{2+}/calmodulin-dependent and -independent activities of eEF2 kinase in lysates from cAMP-stimulated cells were measured as previously described [16,26]. Reactions were stopped in standard SDS-PAGE sample buffer, and the proteins were separated on a 10% SDS gel followed by autoradiography. Control assays were also performed in which the eEF2 was omitted. The radioactivity associated with the band corresponding to eEF2 was quantified using an HP Instant Imager.

3. Results

3.1. Activation of cAMP kinase in cells inhibits translation elongation

Protein synthesis in IPC-81 wild type cells immediately started to decline after 8-CPT-cAMP challenge (data not shown), and the maximal 27% inhibition of protein synthesis was seen after 1 h (Fig. 1A). A similar effect of 8-CPT-cAMP was noted in apoptosis-resistant bcl-2-transfected cells. To exclude the possibility that the effect observed could be due to inhibition of amino acid uptake by cAMP, continuous [^{35}S]methionine labelling experiments were also performed. In both untreated and cAMP-treated cells the rate of [^{35}S]methionine incorporation was linear during the first hour, but in cAMP-treated cells the degree of labelling was reduced by one third compared to the untreated cells (data not shown).

Table 1
Morphometric assessment of polysome size on electron micrographs of cells treated with 8-CPT-cAMP for 1 h and control IPC-81 cells

	Fraction of polysomes with *n* ribosomes		
	$n=3$–4	$n=5$–6	$n>7$
Control	0.52 ± 0.04	0.33 ± 0.10	0.15 ± 0.07
cAMP	0.25 ± 0.07	0.31 ± 0.03	0.44 ± 0.07

The standard error is based on three experiments.

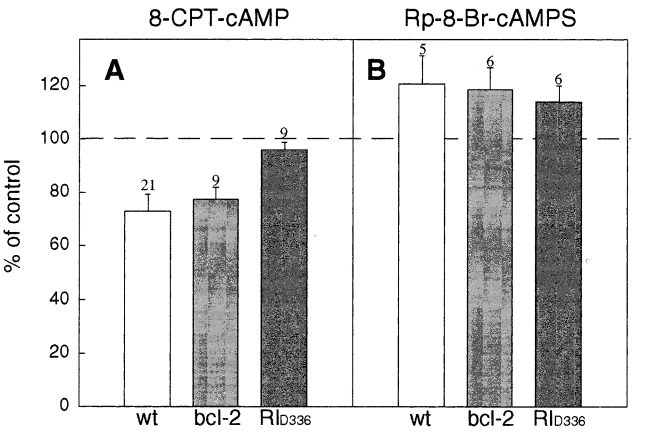

Fig. 1. The effect of a cAPK agonist (100 μM 8-CPT-cAMP, panel A) and a cAPK antagonist (200 μM Rp-8-Br-cAMPS, panel B) on total protein synthesis in IPC-81_{wt} cells, apoptosis-resistant IPC-81_{bcl2} cells and cAMP-subresponsive IPC-RI_{D336} cells. The S.E.M. and *n* are indicated. In Fig. 1B the level of protein synthesis with 95% confidence intervals was as follows: IPC-81_{wt} cells 121 ± 21%, IPC-81_{bcl2} cells 118 ± 16% and IPC-RI_{D336} cells 114 ± 12%. The level of protein synthesis in control cells is indicated by the broken line.

In order to assess the degree of cAPK activation required to regulate protein synthesis, experiments were performed using the cAPK inhibitor Rp-8-Br-cAMPS. This is the most potent and specific inhibitor known for the type I isozyme of cAPK which is the predominant type of cAPK in IPC-81 cells, and is able to depress cAPK activity below the basal resting level [24]. Rp-8-Br-cAMPS appeared to enhance protein synthesis (Fig. 1B) in the absence of added cAMP analogue, indicating that even the basal level of cAPK activity was sufficient to exert some tonic inhibition of protein synthesis.

To get additional proof that cAMP acted via cAPK to depress protein synthesis, experiments were performed using the cAMP-resistant IPC-RI_{D336} cells. These cells express both normal and mutated RIα subunit of cAPK, the mutation having a dominant-negative effect [19]. The dominant RI_{D336} is expressed in a high enough quantity to sequester 65% of the catalytic subunit of cAPK, leaving about a third of the cAPK free for normal regulation by cAMP [19]. Even in these cells, Rp-8-Br-cAMPS enhanced protein synthesis (Fig. 1B), suggesting that protein synthesis was regulated at levels of cAPK activation less than 35% of maximal. Protein synthesis in IPC-RI_{D336} cells was little affected by concentrations of 8-CPT-cAMP in the range 0–100 μM (Fig. 1A), which is the range where activation of cAPK beyond 35% is blocked efficiently by RI_{D336}. RI_{D336} requires about 30-fold more 8-CPT-cAMP to release the catalytic subunit than is the case for wild

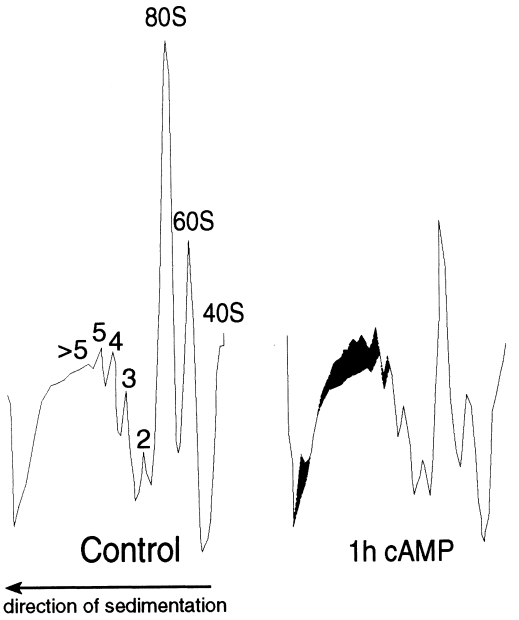

Fig. 2. A typical polysome sedimentation profile from control cells and cells 1 h after cAMP challenge, measured by absorbance at 260 nm, and representing one of four experiments. The shaded area illustrates the increase in longer polysomes seen after a direct overlay of the two profiles. The position of free ribosomal subunits and the smaller polysomes (with 2–6 ribosomes per mRNA) is indicated.

type RI, and its effect can be relieved by millimolar concentrations of 8-CPT-cAMP in the intact cell [19]. In fact, at 3 mM 8-CPT-cAMP, protein synthesis in the mutant cells was as inhibited as at 100 μM 8-CPT-cAMP for the wild type cells (data not shown). It can be concluded that cAPK is responsible for the observed effects of the cAMP analog and that cAPK modulates protein synthesis at low, physiological degrees of activation.

Having shown that cAPK inhibited translation in the intact cell, the next step was to find out if this inhibition occurred at the level of initiation or elongation. As an initial investigation, polysomal profiles were studied (Fig. 2). A recruitment of free ribosomes into polysomes was found, and the number of very long polysomes in particular increased (Fig. 2), a finding which was confirmed by morphometric evaluation of the number of ribosomes per polysome on electron micrographs (Table 1). The combination of accumulation of polysomes and diminished protein synthesis strongly suggests inhibition of elongation.

It was important to determine if the extent of protein synthesis inhibition estimated above could be explained by elongation inhibition alone, i.e. if the degree of elongation inhibition corresponded to the degree of protein synthesis inhibition in pulse-labelling experiments. For this, the actual elongation rate had to be measured. Such measurements were performed in control cells and in cells pre-treated for 1 h with cAMP analog (Fig. 3). The average mRNA transit time in control cells was around 3 min. We found that cAMP treatment increased the average transit time from 3 to 5 min, i.e. it reduced the average elongation rate by approximately 40% (Fig. 3). However, our data are insufficient to give a statistically significant difference in transit time between control and cAMP-treated cells. This reduction in elongation rate can fully explain the reduction of protein synthesis found in pulse-labelling experiments (Fig. 1) and extended labelling experiments (data not shown).

3.2. *cAMP treatment of cells causes Ca^{2+}/calmodulin-independent activation of eEF2 kinase*

A molecular mechanism for cAMP-induced inhibition of elongation has already been suggested by Redpath and Proud [17]. They found that the catalytic subunit of cAPK phosphorylated and activated purified eEF2 kinase, making its activity Ca^{2+}/calmodulin-independent. To investigate if this was also the case in intact cells, lysates from control and cAMP-stimulated IPC-81 cells were prepared and assayed for eEF2 kinase activity, using purified eEF2 as substrate (Fig. 4A). After autoradiography, the [^{32}P]phospho-eEF2 was quantified by direct measurement of radiolabelling. In lysates from unstimulated cells, the activity with 1 mM EGTA was about 10% of that in the presence of Ca^{2+}/calmodulin. This Ca^{2+}/calmodulin-independent activity increased in lysates from cells treated with cAMP for 15 min or longer to reach half the level observed with Ca^{2+}/calmodulin (Fig. 4A). The maximal activity of eEF2 kinase measured in the presence of Ca^{2+}/calmodulin was similar at all time points studied, indicating that the total amount of enzyme did not change.

Activation of eEF2 kinase is expected to lead to increased phosphorylation of the cellular eEF2. This was investigated on Western blots prepared after 2D gel electrophoresis of cytoplasmic proteins probed with rabbit anti-eEF2 antibodies (Fig. 4B). Care was taken to avoid proteolysis and dephosphorylation during preparation of the cell lysates. Various forms of eEF2 can be separated by isoelectric focusing [27]

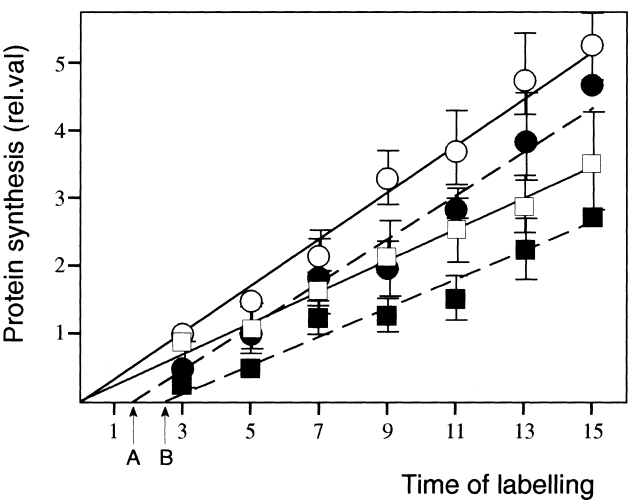

Fig. 3. Estimation of average ribosomal transit time. The short-term cumulative incorporation of [^{35}S]methionine was measured in the combined polysomal and free fraction (open symbols), and the free protein fraction only, i.e. the fraction with completed protein chains (filled symbols), in control cells (○) and cAMP-stimulated cells (□). The data points represent an average of three experiments and the S.E.M. are indicated. Linear lines showing the rate of [^{35}S]methionine incorporation in the total and free fractions were drawn and extrapolated to the abscissa to find the half average transit time. In this case, the half average transit time was 1.5 min for control cells (A) and 2.5 min for cAMP-stimulated cells (B). For the theoretical basis for such calculations, see [12].

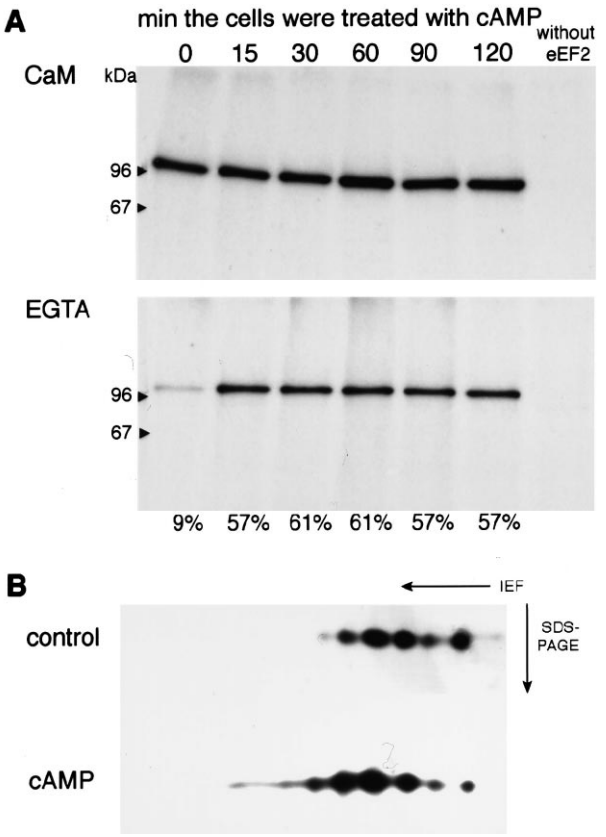

Fig. 4. A: The Ca^{2+}/calmodulin-independent activity of eEF2 kinase in IPC-81 lysates from untreated and cAMP-treated cells (lower panel) compared to the Ca^{2+}/calmodulin-dependent (total) eEF2 kinase activity (upper panel). Lysates were prepared from control cells and cells treated with cAMP for 15 min to 2 h, and assayed for eEF2 kinase activity using purified rabbit reticulocyte eEF2 as substrate. The positions of molecular weight markers are shown on the left. The extent of radiolabel incorporated into the substrate (eEF2) was quantified by a HP Instant Imager. The percentage of Ca^{2+}/calmodulin-independent activity related to an averaged total activity is indicated below the panel. B: Western blots of gels with proteins from control (upper panel) and 8-CPT-cAMP-treated (lower panel) cells separated by two-dimensional gel electrophoresis and probed with anti-eEF2 antibodies. eEF2 isoforms in a control cell lysate are directly compared with eEF2 isoforms in a lysate from cells treated with cAMP for 1 h. The two blots were aligned according to the background spots.

or by 2D gel electrophoresis [28]. In theory, six different mobility forms of eEF2 can be expected, i.e. eEF2 with and without the His-714 to diphthamide modification in un-, mono- and biphosphorylated forms. This was also what we observed. In the control cell lysates, eEF2 antibodies recognized five spots, and in the lysates from cAMP-treated cells, a sixth even more acidic spot appeared (Fig. 4B). There was a clear shift towards the more acidic forms of eEF2 with cAMP treatment, and after such treatment the less acidic (unphosphorylated) forms constituted only a small fraction of the total. It should also be noted that a substantial portion of eEF2 appeared to be phosphorylated even in control cell lysates, indicating that IPC-81 cells may have a basal eEF2 kinase activity that is higher or a phospho-eEF2 phosphatase activity which is lower than what has previously been found in reticulocyte lysates, Swiss 3T3 cells, Chinese hamster ovary cells, hepatocytes [27] or human amnion cells [28].

4. Discussion

We report that activation of cAMP-dependent protein kinase inhibited protein synthesis in leukemia cells through retardation of the rate of polypeptide chain elongation (Figs. 1–3). The degree of inhibition of elongation (40%, Fig. 3) corresponded with the degree of inhibition of protein synthesis (Fig. 1), and was accompanied by increased phosphorylation of eEF2 (Fig. 4B). This prompted us to study eEF2 kinase, which is usually dependent on Ca^{2+}/calmodulin for its activity [29], but which shows Ca^{2+}/calmodulin-independent activity in vitro after incubation with purified cAPK [17]. The present study showed that cAMP stimulation did indeed lead to eEF2 kinase becoming partially independent of Ca^{2+}/calmodulin in intact cells (Fig. 4A), indicating that eEF2 kinase is a biologically relevant target for cAPK. The phosphorylation of eEF2 kinase did not require massive stimulation of cAPK, since the enzyme appeared to be partially activated in control cell lysates under calcium-free conditions (Fig. 4A). This correlates with our data showing that even the basal cAPK activity was enough to exert some tonic inhibition of protein synthesis, as revealed by experiments showing enhancement of protein synthesis when the basal cAPK activity was decreased by Rp-8Br-cAMPS (Fig. 1B). This may also explain why the basal average ribosomal transit time (Fig. 3) was somewhat longer than what has generally been reported [23].

When searching the protein sequence of eEF2 kinase for a consensus substrate sequence for cAPK, residues 147–155 (RTKKLSNFL) were found to represent a potential phosphorylation site [30]. This sequence is identical in eEF2 kinase from rat, mouse and man, and in *Caenorhabditis elegans* eEF2 kinase a similar sequence (RLKKCSKHG) is found with conservation of both the upstream basic residues (underlined) and the serine residue. Two other potential cAPK phosphorylation sites were suggested by Redpath et al. [30], but they lack conservation of either the serine residue or the upstream arginine residue. When assayed as a substrate for the purified catalytic subunit of cAPK, the K_m of the synthetic peptide RTKKLSNFL was found to be 610 μM and the V_{max}=2.8 s^{-1} (data not shown). This points to Ser-152 as the most probable site for the 1:1 stoichiometric phosphorylation of eEF2 kinase previously observed. Although the K_m for the peptide was 80-fold higher than for purified eEF2 kinase, the V_{max} was higher [17]. This indicates that the phosphorylation site may be less accessible in eEF2 kinase than in the synthetic peptide, and that eEF2 kinase provides additional contacts improving the affinity for the catalytic subunit of the cAPK.

The teleological significance of cAMP-induced inhibition of elongation is uncertain. Translation was inhibited by concentrations of cAMP which were too low to induce apoptosis, inhibition occurred well before the onset of apoptosis, and was also observed in bcl-2-transfected cells protected from cAMp-induced apoptosis (Fig. 1). Any major change in the pattern of proteins observed after two-dimensional gel electrophoresis has not been found (data not shown). Since cAMP induces growth arrest of IPC-81 cells [18,31], it is possible that the decline in protein synthesis represents an adjustment to a lower rate of proliferation. cAMP-induced inhibition of elongation has also been found in glucagon-stimulated rat liver cells, where the average polypeptide completion time increased from 1.5 min to 2.4 min after intraperitoneal injection of

glucagon [32]. In this situation a transient reduction in protein synthesis may make more amino acids available as precursors for gluconeogenesis. Whatever the purpose, it is clear that cAMP-induced elongational inhibition is not restricted to myeloid leukemia cells. In this setting it may seem puzzling that cAMP decreased, rather than increased, the activity of eEF2 kinase in PC12 cells, but this decrease was due to loss of enzyme, as assayed in the presence of Ca^{2+}/calmodulin, and was a late occurring phenomenon [33,34]. In the IPC-81 cells no loss of total eEF2 kinase activity was noted (Fig. 4A).

It is of interest that the trophic hormone insulin, which antagonizes the action or effects of cAMP with respect to a number of cell functions [3,18], also has the opposite effect to cAMP on the control of elongation. Notably, insulin decreased eEF2 kinase activity in CHO cells in a rapamycin-sensitive manner [16]. A coupling between the retardation of elongation and anti-trophic states of cells was also observed in excitotoxic induction of delayed neuronal cell death. This was preceded by a persistent inhibition of protein synthesis which, at least initially, was caused by eEF2 kinase activation by Ca^{2+} influx [35]. In this model system for apoptosis, the eEF2 kinase activity remained strictly Ca^{2+}/calmodulin-dependent. However, a linkage between elongation retardation and anti-trophic states is not always observed since eEF2 kinase activation coupled with elongational inhibition has also been reported in mitogen-stimulated cells and in proliferating glioma cells [36,37].

In conclusion, the present work adds cAMP-dependent protein kinase to the list of modulators of translation in intact cells, which exerts its effect on the level of elongation. Thus, in addition to calcium, cAMP can act as an alternative second messenger activator of eEF2 kinase and hence of the phosphorylation and inactivation of eEF2. The physiological importance of regulating elongation is not known.

Acknowledgements: The technical assistance of Erna Finsås and Nina Lied Larsen was highly appreciated. This work was supported by the Norwegian Research Council, the Novo Nordic Foundation, Astri and Edvard Riisøens foundation and The Norwegian Cancer Association.

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