cAMP induces co-translational modification of proteins in IPC-81 cells

Randi HOVLAND*, Anne P. DØSKELAND†, Thor S. EIKHOM†, Bernard ROBAYE‡ and Stein O. DØSKELAND*¹

*Department of Anatomy and Cell Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway, †Department of Biochemistry and Molecular Biology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway, and ‡Institute of Interdisciplinary Research, School of Medicine, Free University of Brussels, Route de Lennik 808, B-1070 Brussels, Belgium

An elevated cAMP concentration results in growth arrest and protein synthesis-dependent apoptosis in the promyelocytic leukaemia cell line IPC-81. A comparison of two-dimensional gels of extracts from these cells labelled with [³⁵S]methionine revealed that five distinct protein spots were induced by cAMP in a protein-synthesis-dependent manner. The spots seemed to result from the acidic shift of a precursor protein. The most abundant spot was phospho-actin. The spots induced by cAMP in intact cells were induced by cAMP-dependent protein kinase (cAPK) during the translation *in vitro* of mRNA from the leukaemia cells. The effect of cAPK was strictly co-translational, none of the spots being induced when cAPK was added after translation. This suggested that the protein spots arose by co-translational

phosphorylation catalysed by cAPK. Two of the protein spots, phospho-actin and a protein with a molecular mass of 30 kDa and an isoelectric point of 4.5, were studied further with respect to expression. They were produced during the whole pre-apoptotic period, had cellular half-lives of several hours and were induced by the same concentrations of cAMP analogue that induced apoptosis. It is suggested that the accumulation of co-translationally modified proteins could be important for long-term cAMP signalling.

Key words: apoptosis, cAMP-dependent protein kinase, co-translational phosphorylation, leukaemia; two-dimensional electrophoresis.

INTRODUCTION

cAMP is known to modulate long-term cell processes such as differentiation and death [1–4]. In several cases cAMP-induced cell death accompanies terminal differentiation or retro-differentiation [5–8]. The main effector system of cAMP in mammalian cells is the cAMP-dependent protein kinase (cAPK). The active catalytic subunit of cAPK phosphorylates serine or threonine residues. So far, readily reversible phosphorylation is believed to account for all the observed effects of cAPK activation. The effect of cAPK on long-term processes is believed to be mediated by modulation of gene transcription (reviewed in [9]).

An alternative mechanism of the long-term modulation of cell function by cAPK is through stable phosphorylation. Stable, cAMP-induced co-translational phosphorylation of actin has been reported in S49 lymphoma cells [10]. The appearance of a phospho-actin-like protein has also been observed during cAMP-induced retro-differentiation of Chinese hamster ovary (CHO) cells [11]. This was postulated to be due to the cAMP-induced activation of previously untranscribed mRNA. Either of these mechanisms could act independently of or supplement transcriptional control in long-term cAMP signalling. The present study was aimed at probing whether such mechanisms were operative in cAMP-challenged rat promyelocytic IPC-81 cells. These cells undergo apoptosis within a few hours of the activation of their endogenous cAPK [12] or microinjection of the catalytic subunit of cAPK [13]. The cell death observed in response to treatment with cAMP depends on active protein synthesis [14]. Little is known about which proteins or protein modifications are responsible for the anti-tropic effects of cAPK in IPC-81 cells. It was therefore decided to screen for novel proteins and

charge-shifting modifications of existing proteins in cAMP-treated cells. This was done by two-dimensional (2D) PAGE of extracts from [³⁵S]methionine-labelled cells and of protein translation mixtures *in vitro* by using RNA from non-stimulated cells as template. In such gels one would detect proteins induced through synthesis *de novo* from cAMP-induced transcripts, from the activation of previously quiescent transcripts and through stable or transient phosphorylation of precursor proteins. To our surprise, the major changes in protein spots in cAMP-challenged cells were due not to the translation of novel mRNA but to co-translational events that could be reproduced when cAPK was present during the translation *in vitro*. The present study was aimed at describing the phenomenon, deciding whether it was due to the translational activation of previously inactive mRNA or not, determining the stability of the induced protein species and finding whether they were induced by cAMP concentrations similar to those required to induce apoptosis.

EXPERIMENTAL

Cell culture and incubation

The rat promyelocytic leukaemia cell line IPC-81 was grown in Dulbecco's modified Eagle's medium with 10% (v/v) horse serum [12]. Cells to be labelled with [³⁵S]methionine or [³²P]P_i (SJQ0079 and PBS11 respectively; Amersham, Little Chalfont, Bucks., U.K.) were transferred to methionine-free or phosphate-free medium respectively, at least 1 h before the addition of isotope.

For labelling with [³⁵S]methionine (75 µCi/ml), the IPC-81 cells were incubated for 1 h at 37 °C in the absence or presence of 0.2 mM 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP). The

Abbreviations used: 2D, two-dimensional; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; cAPK, cAMP-dependent protein kinase; CHO, Chinese hamster ovary.

¹ To whom correspondence should be addressed (e-mail stein.doskeland@pki.uib.no).

medium was removed by centrifugation (1000 *g* for 4 min) and the cell pellet was either frozen in liquid N₂ for protein fractionation or precipitated in 10 % (w/v) trichloroacetic acid. For studies of phosphorylation patterns, cells were incubated for 30 min with [³²P]_i (250 µCi/ml) and then for another 45 min in the presence of 0.2 mM 8-CPT-cAMP, 10 µg/ml cycloheximide or 0.2 mM 8-CPT-cAMP plus 10 µg/ml cycloheximide. To study protein turnover, the cells were preincubated with [³⁵S]methionine and 0.2 mM 8-CPT-cAMP for 75 min, resuspended in fresh medium and split into three batches treated as follows: no additive, 10 µg/ml cycloheximide or 0.2 mM 8-CPT-cAMP plus 10 µg/ml cycloheximide. The degree of apoptosis was estimated by phase contrast microscopy and differential interference contrast microscopy [12].

Protein synthesis *in vitro*

RNA from IPC-81 cells was isolated by the procedure of Chomczynski and Sacchi [15]. Total RNA (0.15 µg/µl of translation mixture) was translated in the presence of [³⁵S]methionine (0.8 µCi/µl; SJ1515; Amersham) in a rabbit reticulocyte lysate system (RPN3150; Amersham) with 100 mM potassium acetate/0.5 mM magnesium acetate at 30 °C for 60 min. The translation reactions contained no extra additions, 50 nM of purified regulatory subunit of cAPK (RI α mutated in the B-site for cAMP binding) or 30–180 nM catalytic subunit of cAPK. cAPK-induced post-translational modifications were studied by allowing protein synthesis for 60 min, then stopping it with 5 µg/ml cycloheximide. After 10 min, 300 nM catalytic subunit of cAPK was added together with 1 mM ATP; incubation continued for a further 10 min. The translation mixtures were frozen at –80 °C until separated by 2D PAGE.

Linked transcription and translation *in vitro* of full-length mouse cDNA of β -actin [16] was performed with a linked SP6 *in vitro* transcription and translation system (RPN 3156; Amersham). The translations were performed in either the absence or the presence of 400 nM purified catalytic subunit of cAPK.

Protein fractionation

Frozen cell pellets were homogenized (Kinematika Polytron) in buffer A [50 mM Hepes (pH 7.2)/10 mM benzamidine/5 mM EDTA/3 mM EGTA/2 mM dithioerythritol/120 mM KCl/50 µg/ml soybean trypsin inhibitor/50 µg/ml aprotinin/7 µg/ml chymostatin/6 µg/ml antipain/48 µg/ml leupeptin/14 µg/ml pepstatin/1 µM microcystin-LR (protein phosphatase inhibitor)] and centrifuged at 50000 *g* for 15 min at 4 °C. The supernatant was made 26 % (w/v) in polyethylene glycol. After centrifugation and removal of precipitate, the supernatant was diluted in 2 vol. of buffer B [10 mM Hepes (pH 7.2)/0.3 mM EDTA/0.3 mM EGTA] and mixed end-over-end with hydroxyapatite beads (Biogel-HT; Bio-Rad, Hercules, CA, U.S.A.) for 1 h at 4 °C. The beads were washed in buffer B and the proteins retained on the matrix were eluted with 500 mM K₂HPO₄ in buffer B and concentrated by precipitation in 10 % (w/v) trichloroacetic acid.

Actin was purified by DNase I affinity chromatography. Sepharose 4B bound to DNase I (8 mg DNase I/ml of resin) was prepared in accordance with the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). A control resin was prepared identically but without DNase I. Homogenate supernatant obtained by centrifugation (3000 *g* for 10 min) or translation lysates (40 µl aliquots) diluted 1:10 in 5 mM Tris/HCl, pH 7.5, containing 1 mM dithioerythritol, 1 mM ATP, 0.2 mM EDTA and 0.4 mM CaCl₂ were applied to DNase I-Sepharose

(4 mg of protein/ml of resin). The mixture was mixed end-over-end for 1 h at 4 °C, poured on to a column and washed extensively with the 5 mM Tris/HCl buffer described above. Retained proteins were eluted with 10 M formamide/2 mM Tris/HCl (pH 7.5)/2 mM CaCl₂/0.1 mM dithioerythritol. Both the flow-through fraction and the formamide-eluted fraction were concentrated by precipitation in 10 % (w/v) trichloroacetic acid.

2D PAGE

Samples were either (1) rabbit reticulocyte lysate, cell pellets or protein fractions or (2) cell suspensions precipitated with trichloroacetic acid. All trichloroacetic acid-precipitated samples were washed in 5 % (w/v) trichloroacetic acid and extracted with diethyl ether. The samples were dissolved in 2D sample buffer [consisting of 9.8 M urea, 100 mM dithioerythritol, 1.5 % (v/v) Pharmalyte pH 3.5–10, 0.5 % (v/v) Pharmalyte pH 5–6 and 4 % (w/v) CHAPS].

The 2D sample separation for Figures 1(A) and 1(B) was done as described in [17] with a non-linear pH 3–10 gradient for the first dimension of separation and SDS/PAGE with a 6–16 % (w/v) gel gradient for the second dimension. The other gels were prepared by the procedure described in [18] with a linear immobilized pH 4.0–7.0 gradient (Pharmacia Biotech) for the first dimension and SDS/PAGE [13.8 % (w/v) gel] for the second dimension. The gels containing [³⁵S]methionine-labelled protein were soaked in En³Hance (100 ml per gel) for 1 h before being dried. The exposed autoradiographic films were scanned on an Agfa Arcus II flatbed scanner and the densities of the spots were quantified with NIH Image version 1.54 or Phoretix 2D version 4.00. For NIH Image, six spots whose intensity seemed unchanged after treatment served as 'benchmarks' against which the changes in intensity after treatment were measured. The background was subtracted as an average of three encircled areas. The data analysed by Phoretix were presented as average percentages between the presumed precursor and the cAMP-induced spot in the related spot pairs. The average of boundary background subtraction was used. Molecular mass/pI coordinates were determined by averaging results from seven gels calibrated with standard proteins (2D SDS/PAGE standards; Bio-Rad).

RESULTS

2D electrophoretic detection of protein spots induced during the pre-apoptotic period in cAMP-stimulated leukaemia cells

The induction of apoptosis in cAMP-challenged IPC-81 cells depends on active protein synthesis during the pre-apoptotic period. To search for novel protein species induced during this period, cells were incubated with cAMP analogue and [³⁵S]methionine, the proteins were separated by 2D PAGE and labelled proteins were detected as radioactive spots. Two novel protein spots (a and b in Figure 1B) were detected by conventional 2D PAGE based on the use of ampholytes and a non-linear pH 3–10 gradient in the first dimension. Eight additional spots (including c, d, e and n in Figure 1D) were detected by using a longer (18 cm), shallower (pH 4–7) linear Immobiline gradient in the first dimension. The same proteins were induced whether cAPK of the IPC-81 cells had been activated by the cAMP analogue 8-CPT-cAMP or by agents elevating the endogenous cAMP, such as prostaglandin E₂ and cholera toxin. Only five of the spots (n, o, p, q and r) were induced in cells labelled with [³⁵S]methionine and subsequently treated with cAMP in the presence of the protein synthesis inhibitor cycloheximide (results not shown). The remaining five spots (a–e) were therefore not

Figure 1 Effect of cAMP on the two-dimensional protein pattern from IPC-81 cells

IPC-81 cells were labelled with [35 S]methionine in the absence (A, C, E) or presence (B, D, F) of 0.2 mM 8-CPT-cAMP. The cells were labelled for 10–30 min (A, B) or 0–60 min (C–F) after addition of the cAMP analogue or vehicle. The proteins were either separated on non-linear pH 3–10 tube gels in the first dimension and SDS/PAGE in a linear 6–16% (w/v) gradient of polyacrylamide in the second dimension (A, B) or separated on an immobilized pH gradient (pH 4–7) by SDS/PAGE [13.75% (w/v) gel] (C–F). The samples were either extracts of trichloroacetic acid-precipitated cells (A–D) or high-speed supernatant of IPC cell homogenate fractionated by precipitation with polyethylene glycol and by hydroxyapatite chromatography (E, F). The circles in (A) and (C) indicate spots whose intensity was altered after treatment with cAMP analogue. The solid arrowheads point to spots whose intensity increased, the open arrowheads point to spots showing a decrease in intensity and the broken circles indicate spots appearing only in (C) or (D). The molecular masses and pI values of protein standards are indicated on the ordinate and abscissa respectively: actin (43 kDa, pI 5.0), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa, pI 4.5). Further details are given in the Experimental section.

derived from the post-translational modification of pre-existing protein. The apparent molecular masses (kDa)/pI values of the spots on the basis of standards were 43/5.00 (a), 29/4.55 (b), 32/4.80 (c), 25/4.75 (d), 23/5.00 (e), 22/4.90 (n), 50/5.50 (o), 33/5.80 (p), 32/5.70 (q) and 21/5.30 (r). Spot b was close to another spot existing in extracts from non-stimulated cells. This contaminating protein could be removed by precipitation with polyethylene glycol. It was then demonstrated that protein b was completely cAMP-dependent (Figures 1E and 1F). It was observed that some protein spots consistently decreased in intensity after cAMP treatment. These proteins (a'–e' and n'–r') generally had similar molecular masses but higher isoelectric points than the induced proteins. The mechanism of cAMP induction of spots a–e might be enhanced transcription, enhanced translation of previously quiescent mRNA, or a co-translational

modification of nascent polypeptides resulting in altered migration on 2D gels. Inhibition of transcription by actinomycin D (10 μ g/ml) had no effect on the cAMP-induction of protein spots a–e (results not shown). The mRNA coding for these proteins therefore either was present in cells not stimulated with cAMP or was stabilized by cAMP [19]. To distinguish between these possibilities, RNA isolated from non-stimulated cells was used as a template for protein synthesis *in vitro*.

2D PAGE analysis of proteins synthesized *in vitro* with leukaemia cell mRNA as template

The mRNA from unstimulated IPC-81 cells was tested in rabbit reticulocyte lysate for the ability to direct the synthesis of the

Figure 2 2D gels of [^{35}S]methionine-labelled proteins translated *in vitro* in the presence of cAPK

RNA from non-stimulated IPC cells was translated *in vitro* with rabbit reticulocyte lysate. The translations were performed for 60 min with no additions (**A, C, D, F**), in the continuous presence of 50 nM regulatory subunit (RI α A335D) of cAPK (**B**) or in the continuous presence of the catalytic subunit (C) at 30 nM (**E, G, H**) or 180 nM (**I**). For (**C**), cycloheximide (5 $\mu\text{g}/\text{ml}$) was added to the lysate after translation; 10 min later, 300 nM catalytic subunit of cAPK and 1 mM ATP were added and the incubation was continued for another 10 min. To enhance the resolution in the regions of spots a and c, the sample loading was decreased by 50%. This allowed a clearer separation of spot a (**E, G**) and a clearer distinction between spot c and a spot (x) not induced by cAPK (**E, F**). The molecular mass and pI standards, symbols and spot designations are as described in the legend to Figure 1.

proteins induced in intact cells by cAMP stimulation. When translation *in vitro* was performed in the presence of active cAPK (30–180 nM), protein spots a–e were all detected (Figures 2E, 2G, 2H and 2I); they were not additionally increased when mRNA from cAMP-stimulated cells was used as template for the synthesis *in vitro* (results not shown). This indicated that mRNA able to code efficiently for protein spots a–e was already present in non-stimulated cells. To detect some of the weaker spots (such as e) the gels had to be overloaded with respect to the detection of spot a, which migrated close to the actins. The resolution between spot a and bulk actins was improved by a lower loading. It was then clear that even as little as 30 nM cAPK induced spot a (Figures 2D and 2E). The additional resolution gained by a lower loading also helped to resolve spot c from another cAMP-

independent spot with a slightly slower migration in the second dimension (Figures 2F and 2G).

Spots a–e were not observed when cAPK was added to the lysate after the completion of translation (Figure 2C). This supported the conclusion from the intact cell studies that the induction of spots a–e was not due to a post-translational event. Some other spots, including spot n, obviously arose by post-translational modification, because they were also observed when cAPK was added after protein synthesis had been arrested by cycloheximide (Figure 2C).

Translational activation [20,21] is the commonest explanation for the increased synthesis of specific proteins in the absence of an increased mRNA level. It is believed to be mediated through the interaction of *trans*-acting proteins with *cis*-acting elements

Figure 3 Pairs of proteins whose intensities were inversely regulated by cAPK during translation *in vitro*

RNA from non-stimulated cells was translated *in vitro* as described in the legend to Figure 2. The incubations were performed with no additions, with 300 nM catalytic subunit added post-translationally or with 30, 60 or 180 nM catalytic subunit present co-translationally. The proteins were separated by 2D PAGE and the autoradiographic densities of the spots were quantified with Phoretix 2D software. Results are presented as percentages between the presumed precursor (open bars) and the cAMP-induced spot (filled bars) in the related spot pairs (means $\pm$ S.E.M., numbers of experiments are indicated). The positions on the 2D gels of spots n and n' (A), b, b', and b2' (B), c and c' (C), d and d' (D), and e and e' (E) are shown in Figure 2.

located in the 5' or 3' untranslated region of a particular mRNA, leading to the derepression of translation [22,23]. For the mRNA from IPC-81 cells the appearance of spots a–e *in vitro* was dependent on added cAPK, because the spots were not observed when cAPK had not been added (Figures 2A, 2B, 2D and 2F). This suggested that an eventual mRNA 'silencing' protein [24] had to be present both in unstimulated IPC-81 cells and in rabbit reticulocyte lysate and that its action was repressed by cAPK. To determine whether the putative 'silencer' could be inactivated by a limited period of incubation with cAPK, the rabbit reticulocyte lysate was preincubated with cAPK for 10 min. The cAPK activity was then inhibited by excess regulatory subunit; [35 S]methionine was then added. None of the cAMP-induced proteins (a–e) were found (results not shown). These results suggested that cAPK must be active continuously to enhance the appearance of spots a–e. To exclude the possibility that any observed effect was due to a basal cAPK activity in the rabbit reticulocyte lysate, translations were performed in the presence of dominant-negative regulatory subunit (RIz mutated in the B site for cAMP binding) of cAPK. No differences were detected in the protein pattern when the regulatory subunit was added (Figure 2B). The above experiments did not give any positive indication of translational activation of mRNA species and made us consider that the cAPK-induced protein spots arose by co-translational modification.

Evidence that cAPK acts co-translationally to induce an acidic shift of proteins

Co-translational modification is a hitherto little-recognized reason for the translation-dependent appearance of new protein spots. In this case the appearance of the modified protein should correlate with the depression of the unmodified precursor protein. Such an inverse precursor–product relationship will not occur if the appearance of a novel protein is because of the activation of previously translationally quiescent mRNA: it is therefore diagnostic of protein modification. It was found that each of spots b–e had a product–precursor-like relationship to another, slightly more basic, protein of similar molecular size. This was noted after the analysis of cAMP-stimulated cells (Figure 1) as well as of proteins translated *in vitro* (Figure 2). In each case the decrease in a protein spot (b', c', d', e') coincided with an increase in a more acidic protein (b, c, d, e).

To obtain quantitative data on the relationship between b and b', c and c', d and d', and e and e', the spot intensities were determined in gels from experiments with various concentrations of cAPK during translation *in vitro*, using Phoretix software to detect and quantify protein spots. Figure 3 shows the relative spot intensities (as percentages) of each product and presumed precursor. Increasing the concentration of cAPK during translation led to a concerted decrease in the presumed precursor (b'–e') and an increase in the presumed product (b–e). It is concluded that cAPK acts during protein translation to induce acidic shifts of proteins b'–e'. It was noted that spot c' was more sensitive to cAPK-induced acidic shifts than spots d' and e'. An acidic shift of protein n' was observed whether cAPK was present co-translationally or after translation (Figure 3A). This fits with the fact that spot n was observed in intact cells whether or not protein synthesis was blocked.

Further scrutiny suggested two isoelectric variants of spot b' (b' and the less abundant b2') in an extract from translation mixtures *in vitro* without added cAPK (Figure 2). Whereas b2' seemed to be converted completely to more acidic forms at very low cAPK concentration, b' was converted only partly, even at high cAPK concentrations (Figure 3B). A trace amount of b2'

Figure 4 2D protein pattern of ^{32}P -labelled proteins from IPC-81 cells

The IPC-81 cells were exposed for 30 min to ^{32}P and then for 45 min to vehicle (control) (A), 0.2 mM 8-CPT-cAMP (B), 10 $\mu\text{g}/\text{ml}$ cycloheximide (C) or 8-CPT-cAMP plus cycloheximide (D). The panels show the part of the gel in the region of spot a. The position of the standard protein, actin (43 kDa), is indicated.

could also be detected in an extract from unchallenged IPC-81 cells (Figure 1C).

Translation-dependent appearance of phospho-actin spot a in cAMP-stimulated leukaemia cells

The magnitude of the acidic shift observed for proteins b'–e' was compatible with the introduction of one extra negative charge to the precursor protein. Protein phosphorylation is the most common inducible modification introducing an extra negative charge. To test for this, IPC-81 cells were incubated with ^{32}P to label ATP and thereby any protein phosphorylated by kinases. A cAMP-induced phosphoprotein was detected in a similar position to that of spot a (Figure 4B). The phosphate incorporation was dependent on continuing protein synthesis, because no cAMP-induced phosphoprotein was observed when protein synthesis was blocked by cycloheximide (Figure 4D). Protein spot a was more abundant than spots b–e and was the only cAMP-induced spot that was silver-stained in gels from extracts of cells treated for 90 min or more with cAMP analogue (results not shown). The lack of ^{32}P -labelling of spots b–e therefore does not exclude the possibility that they were phosphorylated.

Figure 5 Western blots of 2D gels probed with anti-actin antibody

Proteins from control cells (A) and cells treated with 8-CPT-cAMP for 1 h (B) were separated by 2D PAGE and probed with anti-actin antibody. The two blots were aligned by using background spots. The cAMP analogue induced spot, a, is indicated with an arrowhead.

Figure 6 cAPK induction of protein spot a during the synthesis of pure β -actin *in vitro*; co-adsorption of protein a and actin to DNase I-Sepharose

A linked transcription–translation assay *in vitro* was conducted in the presence of ^{35}S methionine with mouse β -actin cDNA as template. The assay was performed in the absence (A) or presence (B) of 400 nM of the catalytic subunit of cAPK during translation. The actin produced was affinity-purified with immobilized DNase I before being subjected to 2D PAGE and autoradiography. In an intact-cell experiment, IPC-81 cells were incubated with ^{35}S methionine and 0.2 mM 8-CPT-cAMP, then homogenized. The proteins in the supernatant were precipitated with trichloroacetic acid and subjected to 2D PAGE directly (C) or after pre-absorption by exposure to DNase I-Sepharose, which binds actin (D). The dotted line marks the region of actin. Note the co-migration of the major induced actin spot (protein a) in the assay *in vitro* (B) and the cell experiment (C).

Because protein a migrated as expected for a phosphorylated, and thereby more acidic, form of actin we tested whether anti-actin antibodies recognized spot a along with the other actin forms on a Western blot of 2D gels of an extract from cells treated with cAMP analogue. There was a clear acid shift of actin as a result of treatment with cAMP (Figure 5), suggesting that spot a was a modified form of actin.

To prove that spot a was phospho-actin, cDNA for β -actin was used as template for protein synthesis *in vitro* in a linked transcription/translation assay. Three charge variants of β -actin were resolved by 2D electrophoresis when the translation was performed in the absence of cAPK and the sample had been affinity-purified with immobilized DNase I (Figure 6A). The resolution was better with affinity-purified actin, presumably because much smaller amounts of protein and nucleic acids were loaded on the first dimension gels. The improved resolution allowed the determination of a clear precursor–product relationship between each of the three actin variants in control lysates (Figure 6A) and novel spots appearing in lysates treated with the pure catalytic subunit of cAPK during translation (Figure 6B).

Figure 7 Stability of the co-translationally induced protein spots a-d

The turnover of the protein spots a-d was studied by labelling IPC-81 cells in the presence of cAMP analogue and [35 S]methionine for 75 min, followed by a chase with medium containing unlabelled methionine and cycloheximide or cycloheximide plus 8-CPT-cAMP. The cell pellets were dissolved in 2D sample buffer and the proteins were separated by 2D PAGE. (A) The protein pattern of extract from cells just before the chase; (B) the pattern after a 1.5 h chase with cycloheximide; (C) the pattern after 6 h with cycloheximide plus 8-CPT-cAMP. Molecular masses and pI values of protein standards are indicated on the ordinate and abscissa respectively (see the legend to Figure 1 for the standards used). Protein spots a-c and their presumed precursors (a'-c') are indicated by circles.

The major β -actin variant produced by the co-translational presence of cAPK *in vitro* migrated exactly like protein a in lysate from cAMP-stimulated cells (Figure 6C). As when total RNA was used as template (Figure 2), cAPK was able to produce spot a only when present during translation; its effect was completely prevented by the inhibitory regulatory subunit of the kinase. cAPK seemed not to act by promoting the activation of (for example) another kinase because preincubation of the lysate with cAPK did not result in any production of protein a (results not shown). This suggests a direct phosphorylating action of cAPK.

A final proof that protein a is actin was provided by the fact that it was removed together with the other actin spots by preincubation of IPC-81 cell extracts with immobilized DNase I (Figure 6D). It is concluded that protein a is phospho- β -actin.

Figure 8 Correlation between the induction of the co-translationally induced proteins a and b and apoptosis

The IPC-81 cells were labelled with [35 S]methionine and exposed to 8-CPT-cAMP at concentrations ranging from 5 to 200 μ M for 1 h. The proteins were separated by 2D PAGE; the autoradiographic density of spots a ($\square$) and b ($\blacksquare$) (see Figure 1 for definition of the spots) was quantified by computer analysis (NIH Image). (A) Relative spot intensity as a function of cAMP analogue concentration; (B) degree of apoptosis as a function of cAMP analogue concentration, as determined by morphological assessment after 9 h of incubation. The insets show electron micrographs of an IPC cell that has not been exposed to cAMP analogues and one that has undergone apoptosis after treatment with 200 μ M 8-CPT-cAMP. Scale bar, 1 μ m.

Correlation between co-translational protein modification and the induction of apoptosis in cAMP-stimulated leukaemia cells

A prolonged activation of cAPK is required for the induction of apoptosis in IPC-81 cells [12]. It was therefore important to determine whether the cAMP-induced protein spots were expressed during the whole pre-apoptotic period. This was studied by pulse-labelling the cells with [35 S]methionine at different time points after stimulation with cAMP and determining the autoradiographic intensity of the protein spots on the 2D gels. The spots arising from co-translational modification (see above), such as spots a, b and c, were expressed at near-constant level compared with bulk proteins during the period (15–180 min) tested (results not shown). Apoptosis began between 3 and 5 h after the onset of cAMP challenge; all spots were

more weakly labelled in autoradiographs from cells pulse-labelled from 4 to 5 h after addition of cAMP analogue (Figure 7). Nevertheless, spots a and c were evident. It is concluded that co-translational modification occurred during the whole pre-apoptotic period. This suggested that co-translational modified proteins could accumulate, provided that they were stable in the cell.

The stability of the induced protein forms was studied by using cells labelled with [35 S]methionine during the first 75 min of treatment with cAMP analogue. The turnover of labelled protein spots was determined by using a chase with medium containing unlabelled methionine. The chase was performed under three conditions: medium alone, medium with cycloheximide (10 μ g/ml) and with 8-CPT-cAMP (0.2 mM) plus cycloheximide. It should be noted that most of the cells were apoptotic after 3 h of chase in the presence of 8-CPT-cAMP alone, preventing the accurate determination of half-lives longer than about 3 h in the presence of this compound alone. Samples for 2D PAGE analysis were removed every 90 min for 6 h. The half-lives of proteins a and d seemed to be at least 3 h and those of proteins b and c at least 2 h. The 2D pattern of proteins from cells before chase is shown in Figure 7(A). The patterns obtained after treatment with cycloheximide for 90 min, or with 8-CPT-cAMP plus cycloheximide for 6 h are shown in Figures 7(B) and 7(C) respectively. An important finding was that the decline in intensity of spots a–d was accompanied by a decline in spots a'–d' (Figure 8). This means that the half-life of the cAMP-induced co-translationally modified proteins was comparable with that of the unmodified precursors, indicating (1) that the modification had not substantially decreased the protein stability and (2) that there was no reversal of the modification to produce the precursor protein. The turnover of the protein spots seemed not to be affected by cycloheximide or by cycloheximide plus cAMP analogue. It is concluded that spots a–d had half-lives long enough to allow considerable accumulation in the intact cell.

To examine further whether the co-translational modification could be associated with apoptosis, cells were exposed to increasing amounts of cAMP analogue; the intensities of spots a and b (the most prominent spots) were quantified and compared with the degree of induction of apoptosis. There was a close correlation between the induction of the spots and apoptosis (Figure 8).

DISCUSSION

For cAMP elevation to commit IPC-81 cells to apoptotic death, the cAMP stimulus must have a duration of at least 2 h [12]. This suggests that substrates of cAPK must be actively phosphorylated during at least the first 2 h of the pre-apoptotic period. It is also known that active protein synthesis is required during the first 2 h after cAMP challenge [14,25]. The present study was undertaken to search for both the appearance of novel proteins and the phosphorylation of existing proteins during the pre-apoptotic period in cAMP-stimulated IPC-81 cells. 2D PAGE is the most powerful method for this purpose, and a long, shallow (pH 4–7), immobilized pH gradient was found to yield the optimal resolution of the proteins in the most common pI range. Surprisingly few of the proteins in the pI range studied showed either consistent induction, disappearance or change of mobility in cells incubated for 0.5–3 h with an apoptogenic concentration of cAMP analogue. Of the 10 consistently observed new protein spots, none was coded for by novel, cAMP-induced mRNA because their presence was independent of transcription and they could be produced by translation *in vitro* from mRNA isolated from non-

stimulated IPC-81 cells. Five of the protein spots (n–r) could be ascribed to post-translational acidic shift of a precursor protein, presumably due to cAMP-induced phosphorylation. The remaining five protein spots (a–e) thus had to be ascribed to either translational activation of protein synthesis or co-translational modification leading to altered migration on the gels. That so many proteins are induced or migration-modulated in a co-translational manner in response to cAMP is unprecedented: it was therefore investigated in more detail.

The translational activation of previously silent mRNA was first considered as a possible explanation, as it has been described in a number of cell systems (reviewed in [26]), and was recently reported to be responsible for the increased synthesis of approx. 100 proteins in activated T-cells [21]. The mechanisms for translational activation are not yet known in detail, but altered subcellular compartmentation of mRNA can have a part, as can suppressors interacting with specific sequences in the 3' and/or 5' region of mRNA [24,27]. All the protein spots induced by cAMP in intact IPC-81 cells were reproduced in an *in vitro* rabbit reticulocyte translation assay in the continuous presence of active cAPK. This argued against any IPC-cell-specific compartmentation of mRNA or any IPC-cell-specific modulator of mRNA translation as being required for their induction. The attention was therefore directed towards the cAMP-induced co-translational modification of proteins as being responsible for the induction of protein spots a–e. In that case the induction of the modified protein should be accompanied closely by a decline in the non-modified precursor protein. In fact, all of the cAMP-induced spots (a–e) seemed to be derived from more basic precursor proteins, indicating co-translational modification rather than the translation of previously silenced mRNA.

The most obvious explanation of the present findings is cAPK-catalysed co-translational phosphorylation of the five proteins, because they all were induced by cAPK added to a translation mixture *in vitro*. The cAPK could act on the nascent chain during protein elongation or in the period before the completed peptide assumes its final conformation. It could be that the phosphorylation site becomes inaccessible when the protein has achieved the correct folding. Phosphorylation of unfolded or misfolded proteins is not a new concept. In 1975 Bylund and Krebs reported that certain proteins were more susceptible to phosphorylation by cAPK after denaturation [28]. Finally, cAPK could act immediately after completion of polypeptide synthesis but before the phosphorylation site becomes inaccessible owing to stable association of the synthesized protein with other protein(s). It should be noted that both fatty acid conjugation and N-terminal acetylation of proteins would give an acidic shift on a 2D gel. Either of these modifications can occur co-translationally [29,30], but has so far not been reported to be regulated by any signal transduction pathway. Regulated, C-terminal acetylation of transcription factors has recently been reported but occurs in a post-translational manner [31,32].

There is limited precedence for co-translational phosphorylation, and all published cases that we are aware of involve cAPK. Recombinant cAPK can autophosphorylate in *Escherichia coli* in an apparently co-translational reaction [33] but it is unknown whether this reaction occurs in mammalian cells. In cAMP-stimulated cells only three proteins have been reported to possibly undergo co-translational phosphorylation. In cAMP-stimulated pinealocytes, a small fraction of malate dehydrogenase is stably phosphorylated in a protein-synthesis-dependent manner [34]. In adrenal cells, stimulation with cAMP induced the cycloheximide-sensitive phosphorylation of a protein (28 kDa, pI = 6.5). The phosphorylation was reversed in a few minutes [35]. This differs from the situation in the currently studied IPC-81 cells, in which

the induced protein forms were stable for hours and could therefore influence cellular processes long after the cAMP stimulus had been turned off.

The third protein believed to undergo co-translational phosphorylation is actin. Co-translational induction by cAMP of stable phospho-actin or a very similar protein has been described in S49 lymphoma cells [10] and CHO-K1 cells [11] respectively. The present study showed that actin (spot a) was co-translationally phosphorylated in cAMP-stimulated IPC-81 cells in a process that could be reproduced when cAPK was added to a protein translation reaction *in vitro*. It is of interest that S49 lymphoma cells undergo death after approx. 1–2 days of stimulation with cAPK [1,2], but the mechanism is obscure. The reverse transformation of CHO-K1 cells, accompanied by a decreased proliferation rate, requires 1–3 days of stimulation with cAMP [7]. The IPC-81 cells undergo relatively rapid growth arrest and apoptosis in response to activation with cAPK when transcription is intact [12]. When cAMP-dependent transcription through cAMP-response element is blocked by overexpression of the transcriptional inhibitor ICER, the induction of apoptosis is slower [25], but it is still as rapid as in S49 lymphoma cells. It therefore seems that co-translationally phosphorylated actin can be associated with anti-tropic effects of prolonged cAMP stimulation. This notion is supported by the tight correlation between actin phosphorylation and apoptosis in cAMP-challenged IPC-81 cells (Figure 7). Continuing experiments in our laboratory (R. Hovland and S. O. Døskeland, unpublished work) show that denatured actin is easily phosphorylated by cAPK and is readily dephosphorylated by protein phosphatase 2A, whereas phospho-actin isolated from IPC-81 cells is not dephosphorylated. This suggests that the phosphate group in co-translationally phosphorylated actin is inaccessible to protein phosphatases, explaining its stability in the intact cell. It is of interest that the actin-like phosphoprotein induced by cAMP in CHO-K1 cells was not dephosphorylated even after prolonged incubation with acid phosphatase from potato [11].

In distinction from classical post-translational protein phosphorylation, the co-translational modification seems to be of limited reversibility. This allows a build-up of stable co-translationally modified proteins in cells exposed to a prolonged tonic or intermittent cAMP stimulation, allowing a kind of cellular memory. The slow turnover of the co-translationally modified proteins argues against the possibility that they are produced by 'accidental phosphorylation' of denatured protein, because misfolded protein would be expected to be cleared rapidly from the cell. Another finding supporting the potential biological significance of the co-translational modification is the fact that nearly 100 % of proteins b2' and c' were modified. This contrasts with the situation for the previously recognized substrates, actin and malate dehydrogenase, for which only a fraction of the newly synthesized protein became modified. The final striking finding of the present study is that cAMP-induced co-translational modification is not restricted to one or two proteins but affects at least five distinct proteins in a single cell type. This suggests that this phenomenon can have broader implications than hitherto realized. Protein isolation and microsequencing is in progress to determine the identity of the co-translationally modified proteins and site of the co-translational modification.

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