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Cyclic AMP-dependent protein kinase-induced vimentin filament disassembly involves modification of the N-terminal domain of intermediate filament subunits

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The intermediate filament protein vimentin was phosphorylated with cAMP-dependent protein kinase under conditions that induce filament disassembly. Digestion of phosphorylated vimentin with lysine-specific endoprotease and subsequent tryptic peptide mapping indicated that a 12 kDa N-terminal fragment contained all the phosphorylation sites found in the intact molecule. Analysis of cyanogen bromide digests indicated that two phosphorylated peptides were produced, with the major ^{32}P-labeled species representing amino acid position 14–72, and a minor ^{32}P-labeled peptide representing amino acid positions 1–13. These results demonstrate that phosphorylation of sites within the N-terminal head domain of vimentin are associated with phosphorylation induced filament disassembly.

Intermediate filament; Phosphorylation; cAMP-dependent kinase

1. INTRODUCTION

The intermediate filament protein vimentin is one of the most prominent cellular phosphoproteins in animal cells of mesenchymal origin [1–3]. The precise effect of phosphorylation on intermediate filaments in intact cells is unknown. However, the phosphate turns over more rapidly than the protein itself [1,4–5], and there are temporal associations between increased vimentin phosphorylation and alterations in filament organization [6–9]. These observations have led to speculation that phosphorylation plays an important role in regulating the organization of the intermediate filament component of the cytoskeleton.

Vimentin contains structural domains that are characteristic of all non-epithelial intermediate filament proteins [10,11]. A central α-helical 'rod' domain is flanked by non-α-helical terminal domains [10–12]. The central domain appears to be required for maintaining the coiled-coil interactions between subunit proteins. Based on sensitivity to specific proteolysis, the N-terminal 'head' and C-terminal 'tail' domains appear to be located at the periphery of filaments [14–16]. The N-terminal domain appears to be required for filament assembly, but may not be required to maintain assembled filaments [16–19]. The C-terminal domain does not appear to be required for the assembly of intermediate filaments [17]. Although the functional significance of these terminal domains remains speculative, a number of in vitro interactions with specific molecules have been reported [18–24].

Vimentin is a substrate in vivo [25–27] as well as in vitro [28] for cAMP-dependent protein kinases. Recently Inagaki et al. [29] reported that in vitro phosphorylation of vimentin filaments with cAMP-dependent kinase resulted in filament disassembly, while phosphorylation with C-type kinase had no effect on filaments. Peptide mapping studies indicated that this phosphorylation-induced filament disassembly was site specific [29].

The present studies were conducted to determine if modification of specific structural regions of

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vimentin are involved in phosphorylation specific filament disassembly. These studies indicate that the sites of vimentin phosphorylation by the catalytic subunit of cAMP-dependent kinase are within the N-terminal or head domain of the molecule.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Hamster BHK-21 cells were maintained in a monolayer culture in a 1:1 mixture of F-12/Dulbecco's modified essential medium supplemented with 5% fetal bovine serum.

2.2. *Purification of vimentin*

BHK-21 cell vimentin was purified from Triton-insoluble cytoskeletons made essentially as described by Zackroff and Goldman [30]. The Triton-insoluble proteins were then separated on SDS-polyacrylamide gels [31]. Following electrophoresis the gels were stained and destained as previously described [32]. The stained vimentin band was excised from the gel and eluted in a solution containing 0.1% SDS, 0.05 M ammonium bicarbonate. The eluted protein was precipitated in 9 vols of acetone/ammonium hydroxide 5:0.1 (v/v) at −20°C for 30 min. The precipitated protein was recovered by centrifugation at 10000 × *g* for 30 min. The precipitate was dried under vacuum and then resuspended in 5 mM Tris-HCl, pH 8.8. Filaments were assembled in vitro by dialysis against 0.1 M NaCl, 30 mM Tris-HCl, pH 7.0. Insoluble vimentin filaments were collected by centrifugation at 12000 × *g*.

2.3. *In vitro phosphorylation*

cAMP-dependent protein kinase was purified from bovine heart as described by Beavo et al. [33]. The catalytic subunit was then dissociated and purified by the method of Bechtel et al. [34]. [γ-^{32}P]ATP was produced according to Johnson and Walseth [35] from carrier free [^{32}P]phosphoric acid. Purified vimentin was phosphorylated essentially under conditions described by Inagaki et al. [29]. A solution containing 5 μM vimentin, 3.5 μg/ml catalytic subunit of cAMP-dependent protein kinase, 0.05 mM ATP (200 μCi [γ-^{32}P]ATP), 30 mM Tris-HCl, pH 7.0, 120 mM NaCl and 1 mM $MgCl_2$, was incubated for 30 min at 25°C. An equal volume of SDS sample buffer was then added and the ^{32}P-labeled vimentin repurified by SDS gel electrophoresis as described above.

2.4. *Filament disassembly*

Purified vimentin was assembled in vitro as described above. Insoluble filaments were rinsed in 120 mM NaCl, 30 mM Tris-HCl, pH 7.0, and centrifuged at 3000 × *g* for 5 min. Approx. 15 μg of insoluble vimentin was resuspended in a total volume of 50 μl for phosphorylation with the catalytic subunit as described in the previous section. In some experiments, 900 cpm of ^{35}S-labeled vimentin was phosphorylated with unlabeled ATP. At various times after the addition of kinase, the reaction mixture was centrifuged at 12000 × *g* for 2 min and the supernatant recovered for SDS gel analysis. Following electrophoresis, the gels were stained, destained and the vimentin band excised. The gel pieces were rinsed in water and then incubated in 1 M sodium salicylate for 20 min. ^{35}S Radioactivity of gel pieces was determined in 4 ml scintillation fluid.

2.5. *Lysine-specific endoprotease and V-8 protease digestion*

Purified ^{32}P-labeled vimentin was resuspended in 50 μl of a solution containing 0.05% SDS, 0.05 M ammonium bicarbonate, pH 8.0. 0.3 U of lysine-specific endoprotease (Calbiochem) in 10 μl of the same buffer was added. The digestion was carried out for 4 h at 37°C. An additional 0.15 U of lysine-specific protease was added, and the mixture incubated for 4–5 more h at 37°C.

2.6. *Tryptic phosphopeptide mapping*

^{32}P-labeled vimentin nor N-terminal peptide were digested with trypsin and the resulting phosphopeptides were analyzed on thin layer cellulose by the method of O'Connor [36] as previously described [37].

2.7. *Cyanogen bromide cleavage of the N-terminal peptide*

For the isolation of vimentin and the 12 kDa N-terminal peptide for cyanogen bromide cleavage, all gel electrophoresis was conducted as described above except with the addition of 0.5 mM thioglycolic acid to the cathode running buffer. The 12 kDa N-terminal peptide of vimentin, produced following lysine-specific protease digestion as described above, was identified in unstained gels by autoradiography and excised from the gel using the autoradiograph as a guide. The peptide was eluted from the gel, acetone precipitated and dried under vacuum. Approx. 50 μg of purified peptide was resuspended in 200 μl of 70% formic acid containing 200 μg cyanogen bromide. The sample was incubated under nitrogen at 4°C for 24 h, dried under nitrogen, resuspended in 50 μl of water and lyophilized. Peptides were resuspended in 0.1% trifluoroacetic acid (TFA) and separated by reversed-phase chromatography on a 4.6 mm × 12.5 cm Whatman Partisphere C_8 column (Whatman). All analyses were performed with a Gilson gradient HPLC system (Gilson Medical Electronics). The column was run at room temperature with a flow rate of 1 ml/min. Samples were applied to the column and run in aqueous 0.1% TFA, 10% acetonitrile for 2 min. Retained material was eluted with a linear gradient of 10–80% acetonitrile, 0.1% TFA for 50 min. 0.5 ml fractions were collected and ^{32}P radioactivity determined by Cerenkov counting.

2.8. *Amino acid analysis*

Peptides from reversed-phase HPLC were dried under nitrogen and then hydrolyzed in vacuo at 105°C in constant boiling HCl. The acid was then removed under vacuum and the amino acids analyzed as the phenylthiocarbamyl (PTC) derivatives. Derivatization with phenylisothiocyanate and reversed-phase HPLC of the PTC-amino acids was carried out as by the method of Heinrikson and Meridith [38] as previously described [39].

3. RESULTS

As previously reported by other investigators, vimentin is an in vitro substrate for cAMP-dependent protein kinases [28,29]. SDS gel analysis of the phosphorylation mixture after

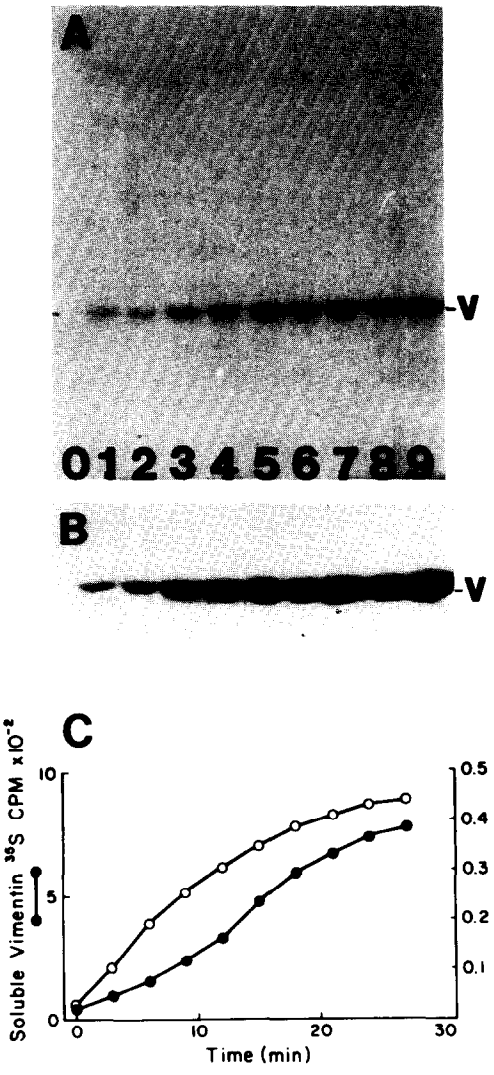

Fig.1. Phosphorylation-induced vimentin filament disassembly. Purified vimentin filaments were assembled in vitro and phosphorylated with the catalytic subunit of cAMP-dependent protein kinase as described in section 2. (A) ^{32}P-autoradiograph of SDS gel of the phosphorylation reaction with time. The position of vimentin is indicated (V). (B) ^{35}S-autoradiograph of SDS gel electrophoresis of soluble vimentin released with time in the presence of kinase and ATP. Gel lanes 0–9 correspond to 3 min intervals between 0 and 27 min. (C) The vimentin bands were excised from gels as shown in A and B and radioactivity determined to indicate the release of soluble vimentin with time of phosphorylation. Individual points represent the average of triplicate experiments.

30 min indicated that vimentin was the major phosphorylated protein (fig.1A). Similar experiments with ^{35}S-labeled filamentous vimentin indicated a time-dependent release of soluble vimentin in the presence of catalytic subunit and ATP (fig.1B). Incubations in the absence of kinase or ATP failed to produce soluble vimentin (not shown). A comparison of the time course for vimentin phosphorylation and the release of soluble vimentin (fig.1C) indicates that both phosphorylation and filament disassembly approach maximal values within 30 min. To determine if a specific region of vimentin was phosphorylated under disassembly conditions, ^{32}P-labeled vimentin was eluted from SDS gels and treated with lysine-specific endoprotease. Digestion with lysine-specific protease produces a 12 kDa N-terminal fragment which represents the N-terminal 93 amino acids and a number of smaller fragments from the remainder of the molecule [13]. The identity of this fragment has been verified by amino acid analysis [39]. SDS gel analysis of the fragments produced by this digestion indicated that the predominant phosphorylated peptide was the 12 kDa N-terminal peptide (fig.2).

Tryptic phosphopeptide mapping studies have indicated that vimentin is phosphorylated at multiple serine sites [36,37,41]. To demonstrate that essentially all the phosphorylation that occurs in the intact molecule is localized in the 12 kDa N-terminal fragment, tryptic phosphopeptide mapping studies were carried out on vimentin and the N-terminal peptide. As shown in fig.3, the pattern of multiple tryptic phosphopeptides produced from intact vimentin and the N-terminal peptide are virtually identical.

The 12 kDa N-terminal peptide contains the entire N-terminal or head domain of hamster vimentin, which represents amino acid positions 1–71, plus the first 24 amino acids of the α-helical rod domain [13,40]. To determine if the sites of phosphorylation were exclusively within the N-terminal domain, cyanogen bromide fragments of the 12 kDa peptide were prepared. The N-terminal peptide contains methionine at positions 13 and 72 [40]. Consequently, 2 of the 3 resulting cyanogen bromide peptides represent the complete N-terminal domain. Reversed-phase HPLC analysis of cyanogen bromide-digested 12 kDa N-terminal peptide revealed two distinct ^{32}P-labeled species, designated in order of elution CNBr 1 and 2 (fig.4). The major ^{32}P-labeled species, CNBr 2,

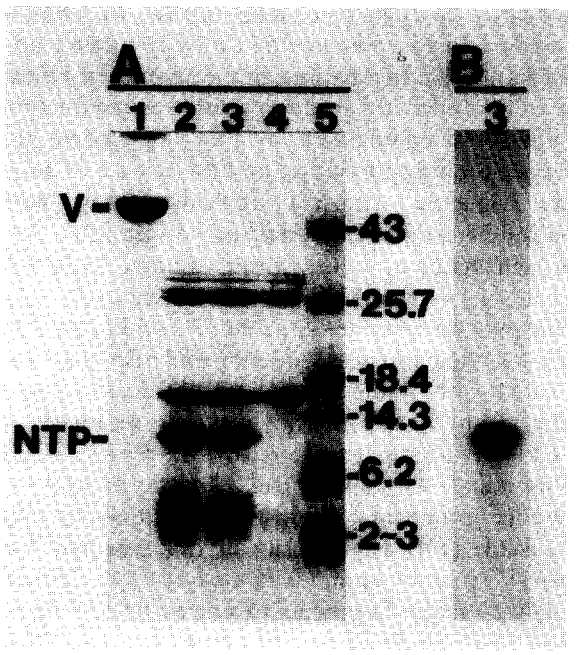

Fig.2. Lysine-specific protease digestion of vimentin phosphorylated by the catalytic subunit of cAMP-dependent kinase. Vimentin was repurified following in vitro phosphorylation, digested with lysine-specific protease, and analyzed on a 15% SDS gel. (A) Protein stain, (B) ^{32}P-autoradiograph. Gel lanes: (1) 15 μg undigested vimentin, (2) 15 μg unphosphorylated vimentin plus protease, (3) 15 μg phosphorylated vimentin plus protease, (4) protease only, (5) protein standards: ovalbumin, 43 kDa; chymotrypsinogen, 25.7 kDa; lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa; trypsin inhibitor, 6.2 kDa; and insulin A and B chains, 2.3–3.4 kDa (unresolved).

migrates as a 7 kDa peptide in SDS gel electrophoresis (fig.5) and the amino acid composition is consistent with this peptide representing amino acid positions 14–72. The minor ^{32}P-labeled peptide, CNBr 1, was too small to be resolved on SDS gels, but amino acid analysis revealed that the peptide contained serine, homoserine, threonine, arginine and tyrosine, indicating that it is the N-terminal 13 amino acids of vimentin. These results indicate that all detectable phosphorylation occurred within the N-terminal 72 amino acids, or within the N-terminal 'head' domain.

4. DISCUSSION

These experiments agree with the observation of Inagaki et al. [29] that phosphorylation of vimentin filaments with cAMP-dependent kinase results in filament disassembly and demonstrate that this phosphorylation occurs exclusively in the N-terminal 'head' domain of the molecule. Together, these observations indicate that modification of

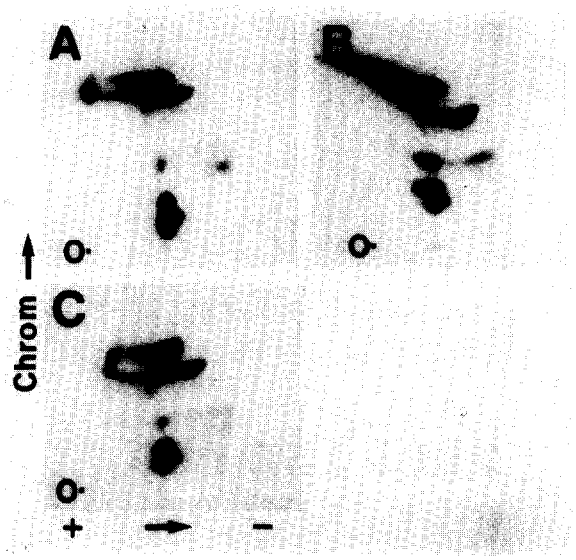

Fig.3. Phosphotryptic peptide analysis of phosphorylated vimentin and the 12 kDa N-terminal peptide. Electrophoresis at pH 1.9 was performed in the first dimension followed by ascending chromatography in the second dimension. (A) Vimentin, (B) 12 kDa N-terminal peptide, (C) mix of phosphopeptides from A and B. Origins are denoted O.

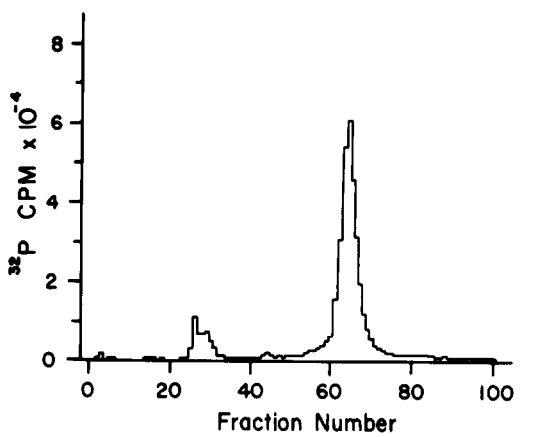

Fig.4. Cyanogen bromide cleavage of ^{32}P-labeled 12 kDa N-terminal peptide. Purified N-terminal peptide prepared from phosphorylated vimentin was digested with cyanogen bromide and the peptides separated by reversed-phase HPLC on a C_8 column. The flow rate was 1 ml/min and 0.5 ml fractions were collected.

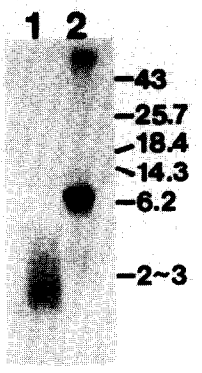

Fig.5. SDS gel analysis of cyanogen bromide phosphopeptides derived from the 12 kDa N-terminal region. Autoradiograph of HPLC purified cyanogen bromide peptides CNBr 1 (lane 1) and CNBr 2 (lane 2). The positions of protein standards (in kDa) are indicated.

the N-terminal region of vimentin filaments can dramatically affect filament subunit interactions under in vitro conditions.

Examination of the amino acid sequence of hamster vimentin indicates that it is not surprising that this domain of the molecule is phosphorylated by cAMP-dependent kinase. The N-terminal domain is a positively charged, serine rich region [13,40]. It contains 7 sites with serines located within one or two positions on the C-terminal side of arginine residues, Arg-X-Ser or Arg-X-X-Ser, a characteristic feature of certain other substrates for cAMP-dependent kinase [42].

In intact cells, there are temporal associations between changes in filament phosphorylation and alterations in filament organization. When cells undergo mitosis, there is a dramatic reorganization of intermediate filaments that is associated with increased subunit phosphorylation [4,6–9,41]. The kinase or kinases that are involved in this process have not yet been characterized, but the mitosis-specific increase in phosphorylation of vimentin also involves serine sites within the 12 kDa N-terminal fragment [39].

While there is accumulating indirect evidence that phosphorylation can affect intermediate filament organization, a direct extrapolation of in vitro assembly/disassembly studies must be viewed with some caution. In particular, Soellner et al. [43] have reported that the small soluble pool of vimentin in cells is not more highly phosphorylated than filamentous vimentin. In addition, the increased phosphorylation of vimentin in mitotic cells does not appear to result in a highly phosphorylated soluble form of the molecule [6,7]. More likely, phosphorylation induced filament disassembly is an indication of the importance of the N-terminal 'head' domain on modifying filament subunit interactions and a reflection of a more subtle mechanism in living cells.

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REFERENCES

[1] Cabral, F. and Gottesman, M.M. (1979) J. Biol. Chem. 254, 6203–6206.
[2] Gard, D.L., Bell, P.B. and Lazarides, E. (1979) Proc. Natl. Acad. Sci. USA 76, 3894–3898.
[3] O'Connor, C.M., Balzer, D.R. and Lazarides, E. (1979) Proc. Natl. Acad. Sci. USA 76, 816–823.
[4] Fey, S.J., Mose-Larsen, P. and Celis, J.E. (1983) FEBS Lett. 157, 251–256.
[5] McTavish, C.F., Nelson, W.J. and Traub, P. (1983) FEBS Lett. 154, 251–256.
[6] Celis, J.E., Mose-Larsen, P., Fey, S.J. and Celis, A. (1983) J. Cell Biol. 97, 1429–1434.
[7] Evans, R.M. and Fink, L.M. (1982) Cell 29, 43–52.
[8] Tolle, H.-G., Weber, K. and Osborn, M. (1987) Eur. J. Cell Biol. 43, 35–47.
[9] Westwood, J.T., Church, R.B. and Wagenaar, E.B. (1985) J. Biol. Chem. 260, 10308–10313.
[10] Steinert, P.M., Steven, A.C. and Roop, D.R. (1985) Cell 42, 411–419.
[11] Weber, K. and Giesler, N. (1984) in: Cancer Cells 1, The Transformed Phenotype, pp.23–54, Cold Spring Harbor Laboratory.
[12] Steinert, P.M. and Parry, D.A.D. (1985) Annu. Rev. Cell Biol. 1, 41–65.
[13] Geisler, N., Plessmann, U. and Weber, K. (1983) FEBS Lett. 163, 22–24.
[14] Nelson, J.W. and Traub, P. (1983) Mol. Cell. Biol. 3, 1146–1156.
[15] Steinert, P.M., Idler, W.W. and Goldman, R.D. (1980) Proc. Natl. Acad. Sci. USA 77, 4534–4538.
[16] Giesler, N., Kaufmann, E. and Weber, K. (1982) Cell 30, 277–286.
[17] Kaufmann, E., Weber, K. and Giesler, N. (1985) J. Mol. Biol. 185, 733–739.
[18] Traub, P. and Vorgias, C.E. (1983) J. Cell Sci. 63, 43–67.
[19] Traub, P. and Vorgias, C.E. (1984) J. Cell Sci. 65, 1–20.
[20] Traub, P. and Nelson, W.J. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 575–592.
[21] Traub, P., Nelson, W.J., Kuhn, S. and Vorgias, C.E. (1983) J. Biol. Chem. 258, 1456–1466.
[22] Traub, P., Perides, G., Schimmel, H. and Scherbarth, A. (1986) J. Biol. Chem. 261, 10558–10568.

[23] Georgatos, S.D., Weaver, D.C. and Marchesi, V.T. (1985) J. Cell Biol. 100, 1962–1967.
[24] Georgatos, S.D. and Blobel, G. (1987) J. Cell Biol. 105, 117–126.
[25] Gard, D.L. and Lazarides, E. (1982) Mol. Cell. Biol. 2, 1104–1114.
[26] O'Connor, C.M., Balzer, D.R. and Lazarides, E. (1979) Proc. Natl. Acad. Sci. USA 76, 819–823.
[27] Spruill, W.A., Steiner, A.L., Tres, L.L. and Kierszenbaum, A.L. (1983) Proc. Natl. Acad. Sci. USA 80, 993–997.
[28] O'Connor, C.M., Gard, D.L. and Lazarides, E. (1981) Cell 23, 135–143.
[29] Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M. and Sato, C. (1987) Nature 328, 649–652.
[30] Zackroff, R.V. and Goldman, R.D. (1979) Proc. Natl. Acad. Sci. USA 76, 6226–6230.
[31] Laemmli, U.K. (1970) Nature 227, 680–685.
[32] Evans, R.M., Ward, D.C. and Fink, L.M. (1979) Proc. Natl. Acad. Sci. USA 76, 6235–6239.
[33] Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 299–308.
[34] Bechtel, P.J., Beavo, J.A. and Krebs, E.G. (1977) J. Biol. Chem. 252, 2691–2697.
[35] Johnson, R.A. and Walseth, T.F. (1979) Adv. Cyclic Nucleotide Res. 10, 135–167.
[36] O'Connor, C.M., Gard, D.L., Asai, D.J. and Lazarides, E. (1981) Cold Spring Harbor Conf. Cell Proliferation 8, 1157–1169.
[37] Evans, R.M. (1984) J. Biol. Chem. 259, 5372–5375.
[38] Heinrikson, R.L. and Meridith, S.C. (1984) Anal. Biochem. 136, 65–74.
[39] Evans, R.M. (1988) Eur. J. Cell Biol., in press.
[40] Quax, W., Egberts, W.V., Hendricks, W., Quax-Jeuken, Y. and Bloemendal, H. (1983) Cell 35, 215–223.
[41] Zieve, G.W. (1985) Ann. NY Acad. Sci. 455, 720–723.
[42] Engstrom, L., Ekman, P., Humble, E., Ragnarsson, U. and Zetterqvist, O. (1984) Methods Enzymol. 107, 130–154.
[43] Soellner, P., Quinlan, R.A. and Franke, W.W. (1985) Proc. Natl. Acad. Sci. USA 82, 7929–7933.