The EMBO Journal vol.8 no.1 pp.111 – 116, 1989

Cloning and sequencing of a calcium-binding protein regulated by cyclic AMP in the thyroid

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Communicated by R.Thomas

p24 is a thyroid protein (M_r 24 000) identified by two-dimensional gel electrophoresis on the basis that its synthesis and phosphorylation are up-regulated by thyrotropin and cyclic AMP agonists. p24 cDNA was cloned from a λgt11 cDNA library using a polyclonal antibody raised against the protein recovered from a Western blot spot. The encoded polypeptide (189 residues) displays a putative target-site for phosphorylation by cyclic AMP-dependent protein kinase and belongs to the superfamily of proteins binding Ca^{2+} through 'EF hand' domains. It presents four such domains of which two agree closely with the consensus. The ability of p24 to bind Ca^{2+} has been directly confirmed on Western blots. p24 was detected in many tissues including the salivary glands, the lung and the brain. The ubiquitous nature of p24, together with its regulatory and sequence characteristics suggest that it constitutes an important target common to the cyclic AMP and Ca^{2+}-phosphatidylinositol cascades.

Key words: calcium-binding protein/cyclic AMP phosphorylation/sequence homology/thyroid regulation

Introduction

Two main pathways are involved in the regulation of cell function, proliferation and differentiation: the cyclic AMP (cAMP) cascade and the calcium–phosphatidylinositol cascade. Cross-regulations allow precise coordination between these pathways. These cross-regulations may involve the modulation of the receptor–G protein–cyclase or phospholipase C complexes, the modulation of the intracellular signal disposal systems or reciprocal controls on the target enzyme systems. Proteins regulated by both calcium and cAMP are therefore of major interest.

Here we present the cloning and sequencing of a protein with an apparent M_r of 24 000 and pI 5.4 (p24) identified previously by two-dimensional gel electrophoresis of dog thyroid cell proteins (Lecocq *et al.*, 1979). This protein (p24) binds calcium and is phosphorylated in intact cells in response to thyrotropin (TSH) and cAMP; its synthesis is up-regulated in cultured cells by these same agents which promote expression of the differentiated thyrocyte phenotype. It is down-regulated by dedifferentiating agents such as epidermal growth factor (EGF) and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA).

Results

Characteristics of p24 revealed by two-dimensional gel electrophoresis

As shown in Figure 1, p24 is phosphorylated in response to TSH in dog thyroid slices. This effect is already observed after 10 min incubation with this hormone, persists as long as TSH is present in the incubation medium and is reproduced by all agents which increase the level of cAMP (Lecocq *et al.*, 1979; Lamy *et al.*, 1984). Silver staining of the proteins after electrophoresis reveals two partially overlapping spots, as phosphorylated p24 contains additional negative charge, suggesting that a sizeable proportion of p24 is phosphorylated (data not shown). Antiserum against the protein purified on a two-dimensional gel was raised in rabbits. It proved to be monospecific, detecting only p24 in immunoblots of total dog thyroid proteins separated on two-dimensional gels (data not shown). Figure 2 shows that in primary cultures of dog thyroid cells, p24 synthesis is stimulated by TSH but is decreased in response to EGF and phorbol esters (TPA) (Lamy *et al.*, 1986). In this system, the position of p24 on the two-dimensional gel pattern was determined by using anti-p24 antiserum (Figure 2, panels E and F).

Selection and sequencing of a full-length cDNA clone for p24

A cDNA library was constructed in the expression vector λgt11 with poly(A) mRNA from dog thyroids. The library was screened using a 1/250 dilution of the anti-p24 antiserum. A total of six positive clones were detected out of 5.6×10^5; they were purified to homogeneity and the size of their inserts was determined: these ranged from 950 to 1280 bp. The inserts from the six positive clones all contained one internal *Eco*RI site and cross-hybridized. The longest of these clones (clone 5A) was selected for subcloning in M13 and completely sequenced (Sanger *et al.*, 1977).

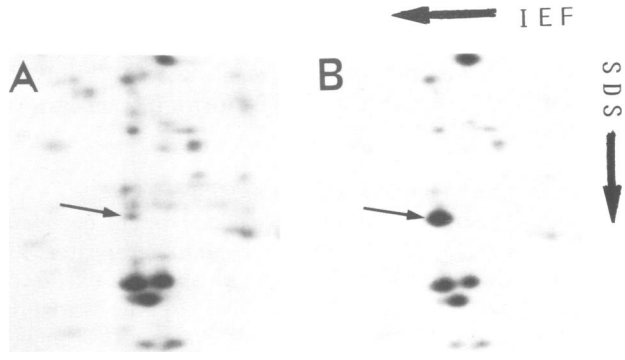


Fig. 1. Autoradiographic details of the two-dimensional pattern of [^{32}P]phosphate-labelled polypeptides from (**A**) untreated dog thyroid slices or (**B**) slices treated with TSH at 10 mU/ml. The slices were incubated for 1 h with [^{32}P]phosphate as described in Materials and methods. The arrowhead indicates the position of p24.

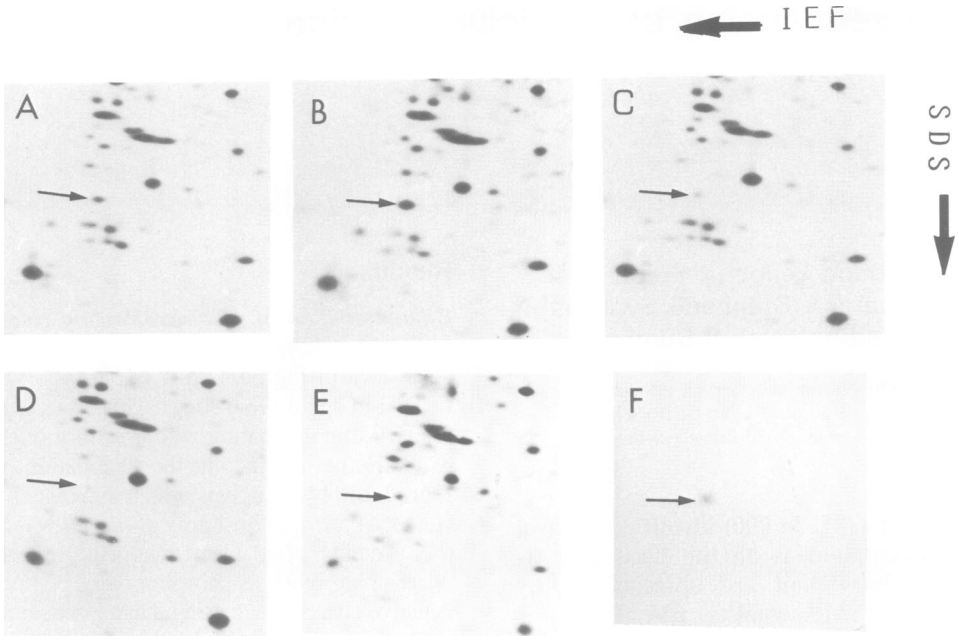


Fig. 2. Autoradiographic details of the two-dimensional pattern of [^{35}S]methionine-labelled polypeptides from dog thyroid cells in primary culture. Effect of TSH, EGF and TPA on p24 synthesis, (**A**) control; (**B**) TSH, 1 mU/ml; (**C**) EGF, 25 ng/ml; (**D**) TPA, 10 ng/ml. The arrowheads indicate the position of p24 revealed by immunodetection; (**E**) autoradiography of the Western blot before immunodetection; (**F**) autoradiography of the same Western blot after specific immunodetection against p24. Only ^{125}I radiation has been recorded.

A 30mer oligonucleotide corresponding to the 5′ end of clone 5A (position 13−42) was used to isolate longer cDNA clones from the λgt11 library. The p24 sequence was found to be present in 0.1% of the clones. Six clones were used for sequencing after subcloning in M13. The sequencing strategy is summarized in Figure 3 (upper panel). Sequencing through the internal *Eco*RI site revealed the respective positions of the two fragments. The nucleotide sequence together with the deduced amino acid sequence of p24 cDNA is shown in Figure 3 (lower panel). The sequence extends over 1322 nt. It terminates in a poly(A) tail preceded by the canonical polyadenylation signal (ATAAA). An open reading frame of 567 nt is found between positions 1 and 567. It delimitates a 5′-non-translated region of at least 87 nt (positions −87 to −1) and a 3′-non-translated segment of 668 nt (positions 568−1235). The first ATG of the sequence was chosen as the initiator codon on the basis of its agreement with Kozak's consensus (Kozak, 1984) (CCAGCATGG versus CC(G/A)CCATGG) and the presence of an in-frame TGA nine bases upstream. Using the cDNA insert (clone 5A) as a hybridization probe, a single species of mRNA (1.4 kb in size) was detected by Northern blot analysis of dog thyroid mRNA (Figure 4).

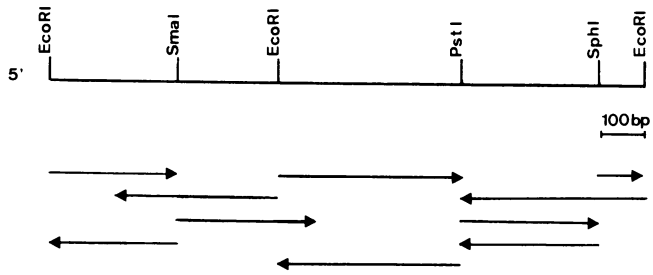
Analysis of p24 amino acid sequence

The open reading frame of 189 codons would encode a polypeptide of M_r 21 104 which is close to the apparent M_r of p24 on SDS−PAGE (24 000). There is an excess of acidic amino acids (35 glutamic and aspartic acids) in comparison to basic amino acids (25 lysine and arginine) which accounts for the acidic properties of p24.

The stimulation of [^{32}P]phosphate incorporation into p24 by TSH in dog thyroid slices (Lecocq *et al.*, 1979) suggests that this protein could be a substrate for cAMP-dependent protein kinases. Since most physiological substrates for this enzyme contain at least two basic residues N-terminal to the phosphorylatable serine or threonine (Cohen, 1988), it is suggested that p24 might be phosphorylated at serine 40.

A computer search of the Protein Identification Resource databank (Release 15.0, December 1987) using the FASTP program (Lipman and Pearson, 1985) revealed significant similarities between p24 and most of the calcium-binding proteins containing EF hands and, in particular, with calmodulin. The calcium-binding domains of these proteins have characteristic structures consisting of 29 amino acid residues arranged in a helix-loop-helix conformation (EF hand) (Kretsinger, 1980). The homology between p24 and calmodulin is characterized by an optimized score of 96. When the p24 sequence is compared to 1000 randomly shuffled calmodulin sequences, the average aligned score is 33.6 ± 7.4 (SD). The score of the p24−calmodulin comparison is thus situated at 8.4 SD above the mean which is considered significant (Lipman and Pearson, 1985). Figure 5 shows the best alignment between p24 and calmodulin. Provided an insertion of 17 residues is introduced at position 160, the sequence similarity extends through the four calcium-binding domains of calmodulin. An analysis of these sequences in p24 reveals the expected pattern of residues conserved in EF hand structures. The flanking α-helices in calcium-modulated proteins have been proposed to be amphipathic (Kretsinger, 1980). In p24 those residues predicted to be on the hydrophobic side of the helices (labelled n on Figure 5) agree with the consensus. In the potential calcium-binding loops, the residues found at the positions predicted to bind calcium are all amino acids with oxygen-containing side chains. The glycine at position 15, which is believed to cause a sharp bend in the calcium-binding loop, is conserved in p24 except in domain 1 where an arginine residue is found. It is noteworthy that this arginine is part of the putative phosphorylation site at serine 40.

Such sequence and structural similarities clearly make p24 a member of the calmodulin superfamily. They indicate

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                -87          AGAGCAGGCTGTGTGGCACCGGACCACAGCCTGGTCAGTCGAGTGGGAATCCTTCCTCAAACTCTCCCCCAGCCAGTCTGACCCAGC

 1 ATG GAT GCC GTG GAC GCC ACT GTG GAG AAG CTC CGG GCA CAG TGC CTG TCC CGA GGG GCC TTG GGC ATC CAG GGC CTG GCC AGG TTT TTC
   Met Asp Ala Val Asp Ala Thr Val Glu Lys Leu Arg Ala Gln Cys Leu Ser Arg Gly Ala Leu Gly Ile Gln Gly Leu Ala Arg Phe Phe
     1                                 10                                  20                                  30

91 CGC CGC CTG GAC CGG GAC AGG AGC CGA TCC CTG GAC TCC AGG GAA CTC CAG CGG GGC CTG GCT GAG CTG GGA CTG GTG CTG GAC ACG GCC
   Arg Arg Leu Asp Arg Asp Arg Ser Arg Ser Leu Asp Ser Arg Glu Leu Gln Arg Gly Leu Ala Glu Leu Gly Leu Val Leu Asp Thr Ala
                                    40                                  50                                  60

181 GAA GCG GAG GGC GTG TGC AGG CGC TGG GAC CGT GAT GGC AGC GGG ACG CTG GAC CTG GAG GAG TTC CTG AGG GCA CTG CGG CCC CCC ATG
    Glu Ala Glu Gly Val Cys Arg Arg Trp Asp Arg Asp Gly Ser Gly Thr Leu Asp Leu Glu Glu Phe Leu Arg Ala Leu Arg Pro Pro Met
                                    70                                  80                                  90

271 TCC CAG GCC CGG GAG GCG GTC ATT GCA GCT GCA TTT GCC AAG CTG GAC CGC AGC GGG GAT GGT GTG GTG ACC GTG GAT GAC CTC CGG GGG
    Ser Gln Ala Arg Glu Ala Val Ile Ala Ala Ala Phe Ala Lys Leu Asp Arg Ser Gly Asp Gly Val Val Thr Val Asp Asp Leu Arg Gly
                                   100                                 110                                 120

361 GTG TAC AGT GGC CGC ACC CAC CCC AAG GTA CAA AGT GGG GAG TGG ACC GAG GAG GAG GTG CTC CGC CGC TTC CTG GAC AAC TTC GAC TCC
    Val Tyr Ser Gly Arg Thr His Pro Lys Val Gln Ser Gly Glu Trp Thr Glu Glu Glu Val Leu Arg Arg Phe Leu Asp Asn Phe Asp Ser
                                   130                                 140                                 150

451 TCC GAG AAG GAC GGG CAG GTC ACA CTG GCT GAA TTC CAG GAC TAC TAC AGT GGT GTG AGC GCC TCC ATG GAC ACA GAT GAG GAG TTT GTG
    Ser Glu Lys Asp Gly Gln Val Thr Leu Ala Glu Phe Gln Asp Tyr Tyr Ser Gly Val Ser Ala Ser Met Asp Thr Asp Glu Glu Phe Val
                                   160                                 170                                 180

541 GCC ATG ATG ACC AGT GCC TGG CAG CTG TGAGCCATCATGCACGTTTGCTCAGCCAGCCCCACTGTAGCGTAGGACCCCCACCCTGGCCCTCTCTCACCTAGGCAGCCCCC
    Ala Met Met Thr Ser Ala Trp Gln Leu

AAGGCGGAGGCCACAGGACCAGCTGGGCCAGGTCTCAGAGGACCTGGCTTGGCCACTATATGCAAGTAGGACAAAGGTTCCGAGAATGGGTCACTGGCTCAGGACCCCAGGGAGCAAGGC

CCTCCCAGTCCATGCTGTGCAGACCTCAGGCCTCACAAGCTCTGCAGTTGCCCCTCCTCACTAGGCATCCTCTTGGTTCTGCTCAGTGAATCCTTGGCCCTGCTAGCACCCCCACCTACC

CTTCCCACCACTTGAGATTGCACCCCCACCCTAAAGCCAGGGGCCGGGAAGCAAACAGCTCTTCTGTGAAGCAAAGCTCAGCATGGAGGCGGCTAGTCTTGGAAGGCAGCCAAGCTCGGT

CACCTGCCTGGTGGACATGGAGTGTGGGGCAGGGGCCAGGGGCCCACAGACAGGCCAGGCTGCTCTGGGAGGTGGAGGCTATTCCTGGCCAGGCTGTCCAGCTTCCTGAGGCCCCTGGGG

CATGCAGGAGGCCAGGGTTTTAGTTAAAAGTTTTAATGTAGTTCCCAAATACATTTCATATGACAATCTTACATAAATGTTCCAAAACAAAAAAAAAAAAAAAAA 1235
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Fig. 3. (**Upper**) Restriction map of 5A cDNA and strategy followed for sequencing. (**Lower**) Nucleotide and predicted amino acid sequence of p24. Nucleotides and amino acids are numbered from the presumed initiator ATG. The putative polyadenylation signal in the 3'-non-coding region is underlined. The putative phosphorylation site in p24 is boxed.

strongly that p24 would be capable of binding one or several Ca^{2+} ions (see below).

The hydropathy profile of p24, calculated according to the method of Kyte and Doolittle (1982), shows a highly hydrophilic character with no indication of transmembrane(s) segment(s) (data not shown). A preliminary immunohistochemical study suggests a cytoplasmic location for this protein (unpublished results).

Calcium-binding properties of p24

To show that p24 actually binds Ca^{2+}, total thyroid proteins were subjected to two-dimensional gel electrophoresis, transferred to a nitrocellulose membrane and incubated in a buffer containing $^{45}Ca^{2+}$. After autoradiography, the nitrocellulose membrane was extensively washed and submitted to immunodetection with the anti-p24 antiserum. Autoradiography of the nitrocellulose membrane revealed the presence of a calcium-binding protein (Figure 6A), the position of which coincided exactly with the immunodetected p24 spot (Figure 6B).

Tissue distribution of p24

The tissue distribution of p24 was tested in various dog tissues using immunodetection by anti-p24 antiserum on

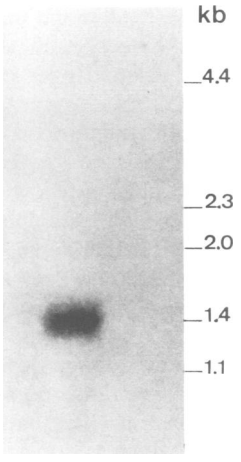

Fig. 4. Northern blot hybridization analysis of dog thyroid p24 mRNA. Blotting was carried out as described in the text.

Western blots of SDS–PAGE-separated proteins (Figure 7). p24 is present at least in the thyroid, the salivary gland, the lung, the brain and the cerebellum. These results were

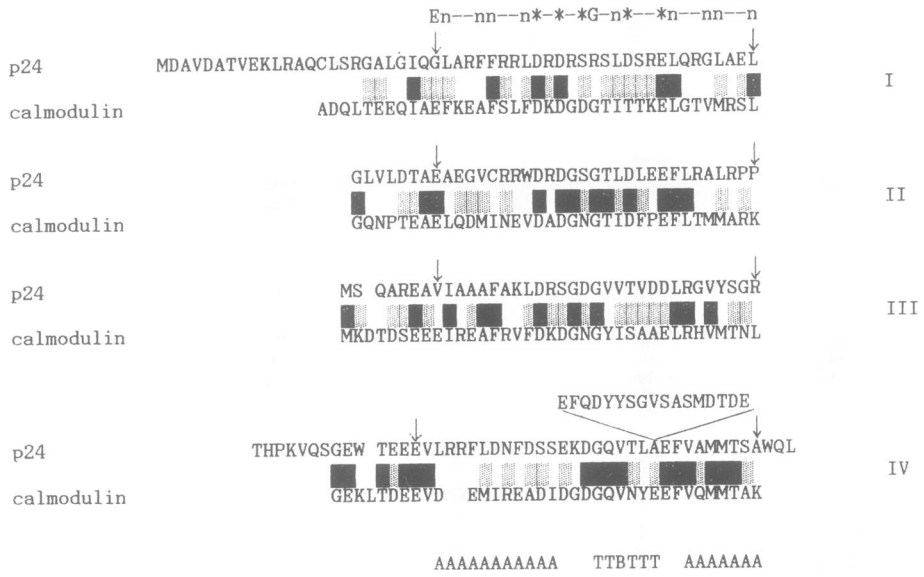

Fig. 5. Comparison of the amino acids sequences of bovine calmodulin and p24. Black symbols: identical residues; stippled symbols: conservative replacements (positive score in PAM 250 matrix) (Dayhoff *et al.*, 1978). The consensus structure of the EF hand domain is represented above (E, glutamine; n, hydrophobic residue; G, glycine; *, oxygen-containing residue; −, any residue). Roman numerals to the right denote the four calcium-binding domains in calmodulin. The amino acids that constitute the EF hand calcium-binding domains lie between the residues identified with an arrow. The arrowhead indicates the insertion site of fragment 161−177. The predicted secondary structure according to Garnier *et al.* (1978) is represented below the sequence (A, α helix; B, β sheet; T, turn; blank, random coil).

confirmed by two-dimensional gel electrophoresis (data not shown).

Discussion

p24 protein is subjected to multiple regulations in the dog thyroid cell. (i) It is rapidly phosphorylated during the full functional activation of the cell by TSH through the cyclic AMP cascade; it is not phosphorylated in response to acetylcholine which activates the Ca^{2+}-phosphatidylinositol cascade and has some similar but mostly opposite effects to TSH (Lecocq *et al.*, 1979; Lamy *et al.*, 1984). (ii) Its synthesis is enhanced by TSH and cyclic AMP analogues which trigger cell proliferation and maintain expression of the differentiated thyrocyte phenotype. It is decreased by EGF and TPA which also trigger cell proliferation but repress expression of differentiation (Lamy *et al.*, 1986).

Because of these interesting properties, the cDNA of this protein has been cloned and sequenced. The primary structure of the encoded polypeptide indeed presents characteristics compatible with an important regulatory role: it contains a site which could correspond to a target for cAMP-dependent protein kinase; it clearly belongs to the calmodulin superfamily of proteins binding Ca^{2+} ions through 'EF hand' (Kretsinger, 1980) (Figure 5). Its ability to bind Ca^{2+} has been directly confirmed (Figure 6). Two of the putative Ca^{2+}-binding domains of p24 show deviation from the consensus 'EF hand'. The first lacks a glycine (position 39) which is considered essential to the shape of calcium-binding domains. The replacement arginine belongs to the putative target site for phosphorylation by cAMP-dependent protein kinase (Figure 3). It is tempting to speculate that this characteristic reflects the existence of some kind of cross-talk between regulation of p24 by the cAMP and the Ca^{2+}-phosphatidyl inositol cascades respectively. The fourth domain displays an insertion of 17

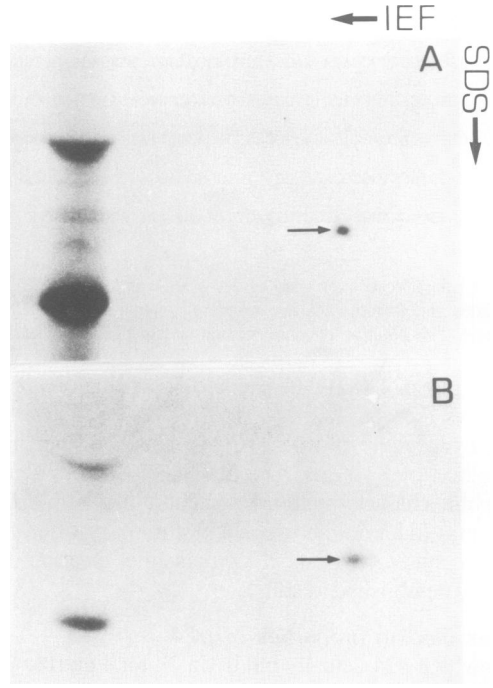

Fig. 6. Calcium-binding properties of p24: autoradiography of a section of a Western blot around the p24 location. **(A)** Autoradiography of the blot after $^{45}Ca^{2+}$ binding (4 days exposure). **(B)** Autoradiography of the same blot after $^{45}Ca^{2+}$ washing followed by immunodetection with p24-specific antiserum (1 days exposure). The arrowheads show the position of p24; mol. wt markers (carbonic anhydrase 30 000 and trypsin inhibitor 21 500) are shown on the left.

residues at position 160 (Figure 5), which disrupts its structure and, most probably, makes it non-functional. Interestingly, this insertion is located precisely at a place corresponding to the position of a conserved intron in the

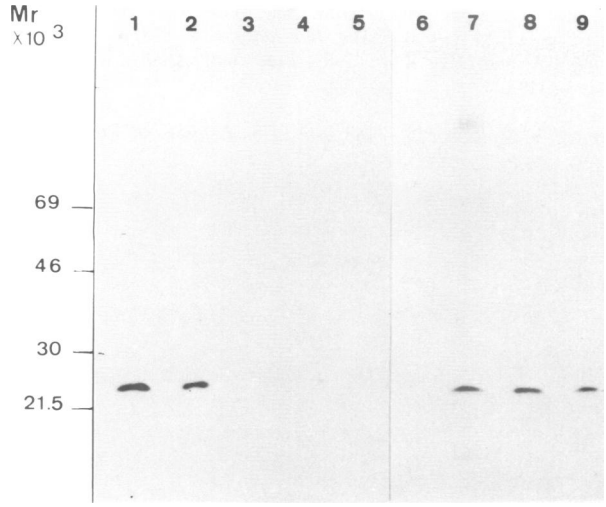

Fig. 7. Tissue distribution of p24 revealed by Western blotting and immunodetection with anti-p24 antiserum of 100 μg total proteins separated by SDS−PAGE from dog (**1**) thyroid (**2**) salivary gland (**3**) pancreas (**4**) stomach (**5**) spleen (**6**) heart (**7**) lung (**8**) brain (**9**) cerebellum.

calmodulin gene family (Wilson *et al.*, 1988). This strongly suggests that it originated from an 'exonization' process (Parma *et al.*, 1987).

The cloning of p24 protein illustrates the power of the strategy in which interesting proteins revealed by two-dimensional gel electrophoresis are used to generate antibodies which are used, in turn, to screen a cDNA expression library.

We have used an anti-p24 antiserum to isolate cDNA recombinants capable of expressing p24 epitopes. In addition to the observation that all λgt11 clones recognized by the anti-p24 antiserum correspond to a single cDNA species, the authenticity of the cDNA is supported by several criteria. (i) The M_r of 21 104 given by the sequence of the recombinant cDNA is compatible with the M_r of p24 (24 000) determined by two-dimensional gel electrophoresis and the relative excess of acidic amino acids would account for the acidic isoelectric point (pI ~5.4) of p24. (ii) The cDNA sequence of p24 shows that this protein contains as expected a putative cAMP dependent phosphorylation site. (iii) The deduction of the calcium-binding properties from the cDNA sequence was confirmed by experimental Ca^{2+}-binding properties of p24 separated on two-dimensional gel electrophoresis from total dog thyroid proteins (Figure 6). This establishes a clear identity relationship between the recombinant protein and p24.

The *in vitro* translation product of p24 mRNA is identical in size to the *in vivo* synthesized protein as determined by immunoprecipitation and co-migration in SDS−PAGE (data not shown). This indicates that p24 polypeptide is not processed post-translationally in a major fashion.

The role of p24 is, as yet, unknown. As p24 is found in several tissues (Figure 7), it is not a protein involved in the specialized iodine metabolism of the thyrocyte. Its synthesis is not increased by mitogenic agents (EGF, TPA) other than TSH; it is therefore not an obligatory signal in the proliferation-regulating cascades. The direct control of p24 by calcium and post-translational phosphorylation in response to cAMP is reminiscent of phosphorylase kinase (Shenolikar *et al.*, 1979). However, in this latter case the dual regulatory mechanisms involve cAMP-dependent phosphorylation of subunits (α,β) distinct from the subunit (δ=calmodulin) which binds calcium. p24 thus represents a paradigm for immediate target molecules of both the cAMP and the Ca^{2+} branch of the Ca^{2+}-phosphatidylinositol cascades. It suggests that these two regulatory pathways do converge to control some functional process of the differentiated thyrocyte. The wide range of tissues containing this protein suggests a rather general target system such as ion transport or cell motility.

We propose to call p24 which binds *cal*cium and which is regulated by *cy*clic AMP through *phos*phorylation: calcyphosine.

Materials and methods

Labelling of dog thyroid slices with [^{32}P]phosphate for two-dimensional gel analysis (Lamy et al., 1984)

Thyroid slices of ~0.2 mm thickness were incubated at 37°C, under 95% O_2/5% CO_2 in 1 ml of the low phosphate (0.5 μM) Krebs−Ringer bicarbonate buffer containing 0.5 mCi carrier-free [^{32}P]phosphate with or without TSH (10 mU/ml).

Labelling of dog thyroid cells with [^{32}S]methionine for two-dimensional gel analysis (Lamy et al., 1986)

Dog thyroid cells were obtained as described (Roger *et al.*, 1983). Cells were grown in Dulbecco's-modified Eagle's medium (DMEM) + Ham's F 12 medium + MCDB 104 medium (2:1:1, by vol.) in the presence of 5 μg/ml insulin without serum. After 4 days of culture, the different mitogens were added to the culture medium; 12 h later, 100 μCi/ml [^{35}S]methionine was added to the culture medium and the culture was pursued for another 10 h.

Two-dimensional gel electrophoresis

The procedures were described by Lamy *et al.* (1986). Briefly, the first dimension separations (isoelectric focusing) were performed on 4% (w/v) polyacrylamide rod gels containing 4% Servalytes (3.2% pH 5−7; 0.8% pH 2−11). The second dimension separations were performed in linear gradients (6−16%) polyacrylamide slab gels.

Polyclonal antisera against p24

200 μg of total proteins from dog thyroids were mixed with a small proportion of [^{32}P]phosphate-labelled proteins from dog thyroid slices which had been incubated in the presence of TSH and [^{32}P]phosphate. They were separated by two-dimensional gel electrophoresis and transferred on nitrocellulose membranes PH79 (Schleicher and Schuell, Dassel, FRG). The area of the nitrocellulose corresponding to the position of p24 was revealed by autoradiography and cut out. Fifteen such nitrocellulose pieces were solubilized in 0.5 ml dimethyl sulphoxide (DMSO) and mixed with an equal volume of complete Freund's adjuvant before s.c. injection into a rabbit. Two subsequent injections were done at 15 day intervals with incomplete Freund's adjuvant (Knudsen, 1985).

Blotting and immunodetection of proteins

After separation by gel electrophoresis, proteins were transferred to a nitrocellulose membrane for 16 h at 60 V and 4°C (Towbin *et al.*, 1979). Polyclonal antiserum against p24 was used at a 1/250 dilution. [^{125}I]Protein A (Amersham, 5 × 10^6 d.p.m./ml) was used as a secondary reagent for development by autoradiography (Burnette, 1981). When the proteins were labelled with [^{35}S]methionine, autoradiography before immunodetection was carried out by direct contact of the nitrocellulose membrane with Kodak XAR5 film. After immunodetection, a screen was placed between the nitrocellulose membrane and the Kodak XAR5 film in order to prevent spot formation by [^{35}S]methionine-labelled proteins but to allow the recording of spots due to [^{125}I]protein A complexes.

Dog thyroid cDNA expression library

Poly(A) mRNA was purified by the thiocyanate guanidinium method (Chirgwin *et al.*, 1979) and poly (U)−Sepharaose 4B chromatography (Pharmacia) from dog thyroids. Its integrity was tested by an *in vitro* translation assay in rabbit reticulocyte lysate (Amersham) and it was then copied into cDNA using a cDNA synthesis kit (BRL) (Gubler and Hoffman, 1983). A λgt11 cDNA library containing 1.2 × 10^7 individual clones was constructed according to standard procedures (Young and Davis, 1983; Maniatis *et al.*, 1982).

Screening of the cDNA library

The library was screened following the method of Young and Davis (1983) using a 1/250 dilution of the anti-p24 antiserum. Positive clones were plaque purified and the size of their inserts was determined. Cross-hybridization of inserts was tested by standard Southern blotting (Maniatis *et al.*, 1982). To select full-length p24 cDNA clones, the library was screened by hybridization (Maniatis *et al.*, 1982) using a 30mer oligonucleotide (Applied Biosystem 381A) corresponding to the 5′ end of the longest clone (clone 5A) obtained by antibody screening. cDNA inserts were subcloned in M13 and sequenced on both strands by Sanger's method (Sanger *et al.*, 1977).

Calcium-binding properties of p24

Approximately 400 μg of total protein from dog thyroid slices were separated on two-dimensional gels and transferred to nitrocellulose membrane. Calcium-binding proteins were detected by incubation with $^{45}Ca^{2+}$ and autoradiography as described by Maruyama *et al.* (1984). In order to re-use the same blot for immunodetection, $^{45}Ca^{2+}$ was washed off by incubating the transfer membrane for 30 min in a 100 mM NaCl solution. The efficiency of the washing was controlled by re-exposure of the blot.

Northern blot analysis

After denaturation using glyoxal according to the procedure of McMaster and Carmichael (1977), 5 μg of poly(A) mRNA isolated from dog thyroid was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Pall Biodyne A). The blot was hybridized with probe 5A labelled with [α-^{32}P]dATP by the random priming procedure (Feinberg and Vogelstein, 1983) to a specific radioactivity of $\sim 10^8$ c.p.m./μg.

Acknowledgements

We thank Marc Parmentier for helpful discussions, Christiane Christophe for the synthesis of oligonucleotides and Ann Hepburn for critical review of the manuscript. This study was benefitted from grants from the following organizations: Sciences de la vie (Ministère de la Politique Scientifique), Solvay, FRSM, CGER, IRSIA and ARBD (asbl).

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Received on September 28, 1988; revised on October 14, 1988