A protein with homology to the C-terminal repeat sequence of *Octopus* rhodopsin and synaptophysin is a member of a multigene family in *Dictyostelium discoideum*

Angelika A. Noegel, Günther Gerisch, Friedrich Lottspeich and Michael Schleicher

Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

Received 9 April 1990

Monoclonal antibodies were raised against a protein with a molecular mass of 24 kDa that has been described as a membrane-associated, actin binding protein from *Dictyostelium discoideum* [(1985) J. Cell Biol. 100, 727–735]. Using these monoclonal antibodies we isolated from a λgt11 expression library cDNA clones coding for this protein. The cDNA deduced amino acid sequence revealed the presence of an unusual carboxy-terminus which has homologies to the C-termini of *Octopus* rhodopsin and synaptophysin. This part of the protein sequence contains 5 direct repeats with the motif GYP (P)Q(P). Southern and Northern blots showed that this sequence is present in a series of *Dictyostelium* genes transcribed in all stages of development.

Actin-membrane interaction; Actin binding protein; *Dictyostelium*; *Octopus* rhodopsin; Synaptophysin

1. INTRODUCTION

The interaction of plasma membranes with the underlying actin filament network regulates cell motility, adhesion, receptor distribution, endocytosis and other activities of eukaryotic cells. In *Dictyostelium* several small proteins have been isolated that are candidates for mediating the assembly of actin at the membrane. Ponticulin, an integral membrane glycoprotein, binds laterally to actin filaments [1]; hisactophilin, which is found both in the cytoplasm and in association with membranes, binds to actin in a pH-dependent manner [2]. Stratford and Brown [3] have studied the binding of actin to membranes by applying radiolabelled, cross-linked filamentous action to sedimentation and gel overlay assays. Using these assays a 24 kDa protein, p24, was purified from membranes which bound to both monomeric and filamentous actin.

We have isolated cDNA clones that code for p24 using a specific monoclonal antibody. Sequence analysis revealed that p24 contains a repeated sequence motif at its carboxy-terminus which, as indicated by Southern and Northern blot analysis, is present in several *Dictyostelium* genes. A similar C-terminal sequence motif has been reported for *Octopus* rhodopsin [4] and synaptophysin [5–7].

Correspondence address: M. Schleicher, Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

2. MATERIALS AND METHODS

2.1. *Growth and development of* Dictyostelium discoideum

D. discoideum strain AX2-214 was grown axenically at 23°C [8] for protein and nucleic acid preparation. For development, AX2 cells were washed in 17 mM Soerensen phosphate buffer, pH 6.0, and deposited on nitrocellulose filters type HABG (Millipore) [9]. The filters were incubated at 21°C and cells were harvested at different stages of development for RNA isolation.

2.2. *Protein purification and production of monoclonal antibodies*

For purification of p24, a plasma membrane-enriched fraction was prepared using the polyethylene glycol/dextran two-phase system of Brunette and Till [10] as described [11]. The membranes were extracted in consecutive steps with 0.5%, 2% and 5% sodium deoxycholate (DOC) at 4°C. p24 was specifically solubilized in a second extraction with 5% sodium deoxycholate at 40°C essentially as described [3]. The p24-fraction was concentrated and the detergent removed by precipitation of the protein with 10% trichloroacetic acid (TCA) and subsequent washes with 80% ethanol.

Monoclonal antibodies were obtained by immunizing Balb/c mice with resuspended p24 from the TCA precipitate using Freund's adjuvant. Spleen cells were fused with X63-Ag8.653 myeloma cells. Monoclonal IgG was purified from hybridoma culture supernatants by chromatography on protein A-Sepharose and labeled with ^{125}I by the chloramine-T method.

2.3. *Amino acid sequence analysis*

Purified p24 was dissolved in 70% formic acid and incubated with 10% (w/v) cyanogen bromide (Sigma) for 16 h at 21°C. After removal of the cyanogen bromide and formic acid, the sample was subjected to SDS-gel electrophoresis on 18% acrylamide gels. After Coomassie blue staining one major fragment with an apparent molecular mass of 9 kDa was detected and used for amino-terminal sequence determination. The fragment was blotted onto a siliconized glass fiber sheet (Glassybond, Biometra, Göttingen, FRG) and se-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

quenced on a gas-phase sequenator 470A from Applied Biosystems (Foster City, CA) using the program 02NPTH [12,13].

2.4. *Isolation of cDNA clones and DNA sequencing*

A λgt11 cDNA expression library [14] was screened with iodinated monoclonal antibody 115-266-10 specific for p24. Several clones were recognized by this antibody. The inserts were subcloned into pUC19 [15] and their sequences determined by the dideoxynucleotide chain termination method using uni and reverse primers as well as a sequence specific DNA primer [16,17]. The sequence was analysed with programs of the University of Wisconsin, Genetic Computer Group, UWGCG [18].

2.5. *DNA and RNA isolation from* D. discoideum *and hybridization analysis*

Chromosomal DNA was isolated from partially purified nuclei [19]. DNA was digested with restriction enzymes, the fragments were separated in 0.7% agarose gels in Tris-phosphate buffer, pH 7.8 [20], transferred to nitrocellulose filters (BA85, Schleicher and Schuell, 3354 Dassel, FRG) and probed with nick-translated cDNA inserts as described [19]. Total cellular RNA isolated from strain AX2-214 was purified by several phenol/chloroform extractions. For Northern blot analysis RNA was separated in 1.2% agarose gels in the presence of 6% formaldehyde [20]. Hybridization was performed as described [21].

2.6. *Expression of p24 encoding sequences in* E. coli

The cDNA inserts of the two longest clones, λcDRS17 and λcDRS31, which both contained the complete coding sequence, were cloned into the *Eco*RI site of plasmid pT7-7 provided by Dr S. Tabor [22]. *E. coli* K38 cells harboring these plasmids were tested for expression of a fusion protein with mAb 115-266-10. Expression was induced by incubation for 20 min at 42°C followed by 2 h at 37°C in the presence of 100 μg/ml rifampicin.

2.7. *Electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [23], and immunoblotting using the method of Towbin et al. [24].

3. RESULTS

3.1. *Isolation and analysis of cDNA clones coding for p24*

Several cDNA clones were isolated from a λgt11 library [14] using the monoclonal antibody 115-266-10. Clones λcDRS17 and λcDRS31 harbored the longest inserts with a size of about 0.7 kb. An open reading frame extended in both inserts from an ATG initiation codon at position 26 to a TAA termination codon at position 581 of the sequence shown in Fig. 1. The 3′ noncoding region is extremely AT-rich as it is typical for *D. discoideum*. This region varied in length in the different clones isolated, which could be due to annealing of the oligo dT primer for the reverse transcriptase reaction at different positions in this 3′ region. The inserts of λcDRS17 and -31, both containing the complete coding region, were subcloned into *E. coli* ATG expression vectors and the protein products identified by the

```
   1 atatactcaatagaaaaaacatataatggaattattaagacaaggtgaacacttacattc  60
   1                          M  E  L  L  R  Q  G  E  H  L  H  S   12

  61 aacctctagatctactttagaatcaaaatgcagaaaatataaacttattatgcaaaatga 120
  13  T  S  R  S  T  L  E  S  K  C  R  K  Y  K  L  I  M  Q  N  D   32

 121 tggtaatttagtattatatattggtagtcttaaaagccaatctgatgaatattgtttatg 180
  33  G  N  L  V  L  Y  I  G  S  L  K  S  Q  S  D  E  Y  C  L  W   52

 181 gtcttcagcttcatgtggtaagggacatggtccatacagactttccatgcaagaagatgg 240
  53  S  S  A  S  C  G  K  G  H  G  P  Y  R  L  S  M  Q  E  D  G   72

 241 aaatcttgtaatttatgattcaagaaattctgcaatatgggcttcaggaactatgggtca 300
  73  N  L  V  I  Y  D  S  R  N  S  A  I  W  A  S  G  T  M  G  H   92

 301 tggagttagaggtcactattcaatgaaattaagatcaagtggtcaaattgttgtttatga 360
  93  G  V  R  G  H  Y  S  M  K  L  R  S  S  G  Q  I  V  V  Y  D  112
                               ------------------------------------

 361 taaatataagcaaatcttatactcatcaaagccttgcactagagaccacctcctttcact 420
 113  K  Y  K  Q  I  L  Y  S  S  K  P  C  T  R  D  H  L  L  S  L  132
      ----

 421 cccttgtgccaaaccatcaggacatccacaaagtgcttatccaccacaacaacctggata 480
 133  P  C  A  K  P  S  G  H  P  Q  S  A  Y  P  P  Q  Q  P  G  Y  152

 481 tggttatccagctcaaccaggttacccaccacaaccaggctacccaccacaacatggtta 540
 153  G  Y  P  A  Q  P  G  Y  P  P  Q  P  G  Y  P  P  Q  H  G  Y  172

 541 tccaccacaacatggttacccacaacaaccaggatattattaaagtaatgaaacttttt 600
 173  P  P  Q  H  G  Y  P  Q  Q  P  G  Y  Y  *                   185

 601 ttttaaaaaaaaaaaaaaaaaaatattattattaataaaatgtaaattaaaaacttttt 660

 661 ttaataaaaaaaaaaaaaataaaataaaataaataatatataaa 704
```

Fig. 1. cDNA and derived amino acid sequence of p24 from *D. discoideum*. The sequence of both DNA strands was determined by the dideoxy chain termination method. The sequence from nucleotides 1–676 was derived from cDRS17, whereas nucleotides 677–704 were derived from other p24 specific cDNA clones. cDRS31 extends from position 13–688. The N-terminal amino acid sequence of a CNBr-peptide determined by Edman degradation is underlined.

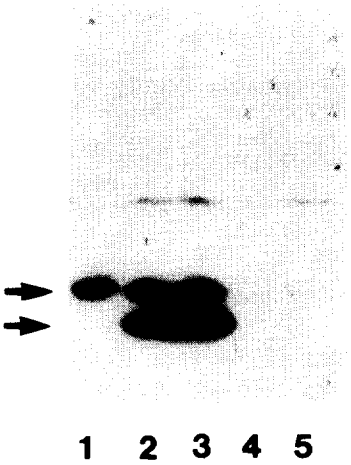

Fig. 2. Expression of p24 in *E. coli* and *D. discoideum*. *E. coli* K38 harboring plasmid pT7-7 that carried either cDNA insert cDRS17 (lanes 2 and 4) or cDRS31 (lanes 3 and 5) were employed to compare the sizes of the cDNA-encoded polypeptides with the size of p24 as produced in *D. discoideum*. Cell homogenates of induced (lanes 2 and 3) and uninduced (lanes 4 and 5) cultures were subjected to SDS-PAGE in 15% gels and assayed with mAB 115-266-10. Homogenate of strain AX2 (lane 1) was run in parallel. cDRS17 contained an in frame stop codon in front of the ATG initiation codon, therefore translation must start at the p24-specific start codon.

monoclonal antibody (Fig. 2). In addition to an immunoreactive polypeptide with the same mobility as authentic p24, a peptide with an apparent molecular mass of 21 kDa was detected by the antibody. This peptide could either represent a breakdown product of p24 in bacteria or could be the result of reinitiation of translation at an ATG within the p24 coding region [25].

3.2. *Identification of the p24 mRNA and of genomic fragments coding for p24*

With the cDNA inserts cDRS17 and cDRS31 as probes, 7 different RNA-species could be distinguished on Northern blots (Fig. 3A). These RNAs with sizes ranging from 0.65 kb to 1.9 kb were present throughout the development of *D. discoideum*, reaching a maximum of accumulation during the early aggregation stage. The two major RNA species differed slightly in the accumulation pattern during development. RNA species 2 (0.8 kb) reached a maximum level at 6 h after the beginning of starvation, and species 5 (1.65 kb) at 3 h.

Southern blot analysis revealed several bands that were recognized by the cDNA inserts. In *Hin*dIII-digested genomic DNA, 3 bands of >10, 5.6 and 4.6 kb hybridized to the cDNA probe. In *Eco*RI-digested genomic DNA, bands of 9.5 and 4.9 kb were recognized (Fig. 3B). Both enzymes have no recognition sites within the cDNA. The results of the Southern blot and Northern blot analyses indicate that the p24 encoding gene carries sequences that are also present in other genes, and is therefore a member of a multigene family. To investigate the relationship between p24 and the other members of this family, we isolated *Alu*I fragments from clone cDRS31 to produce two subclones, one containing the internal sequence from position 189–491, the other containing the carboxy-terminal region from position 492–688. Hybridization of these cDNA fragments to RNA showed that the internal fragment specifically recognized the 0.8 kb transcript, while the fragment containing the carboxy-terminus hybridized to all RNA species recognized by the complete cDNA, indicating that this part is com-

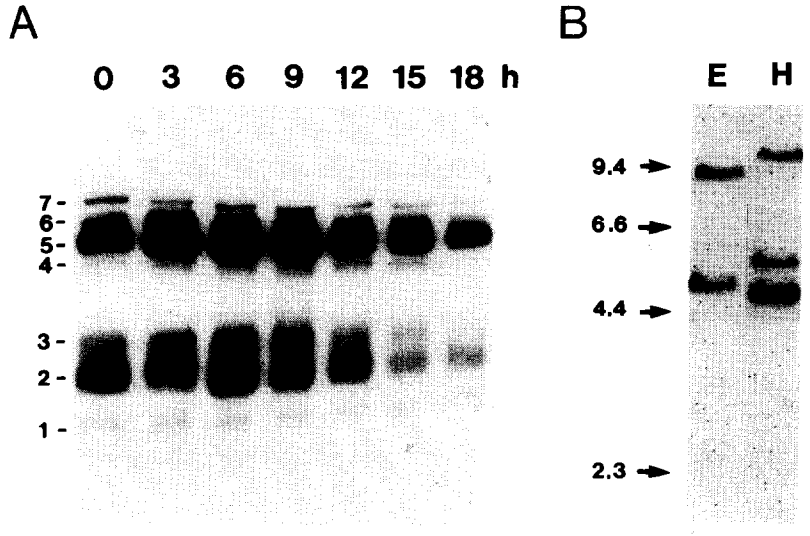

Fig. 3. Northern and Southern blot analysis using a cDNA probe comprising the entire p24 coding region. (A) For expression of p24-specific mRNA, RNA was isolated from different developmental stages of *D. discoideum* strain AX2 as indicated in hours after starvation. 10 μg of total RNA per lane were separated on a 1.2% agarose gel in the presence of 6% formaldehyde, transferred to a nitrocellulose filter and hybridized with cDNA probe cDRS31. The 12 h stage of development corresponded to aggregation, the 18 h stage to slug formation. The different mRNAs recognized are numbered. (B) 10 μg of *Eco*RI and *Hin*dIII digested genomic DNA were separated on a 0.7% agarose gel, blotted and hybridized as above. Sizes of fragments of *Hin*dIII digested λ DNA are given in kb.

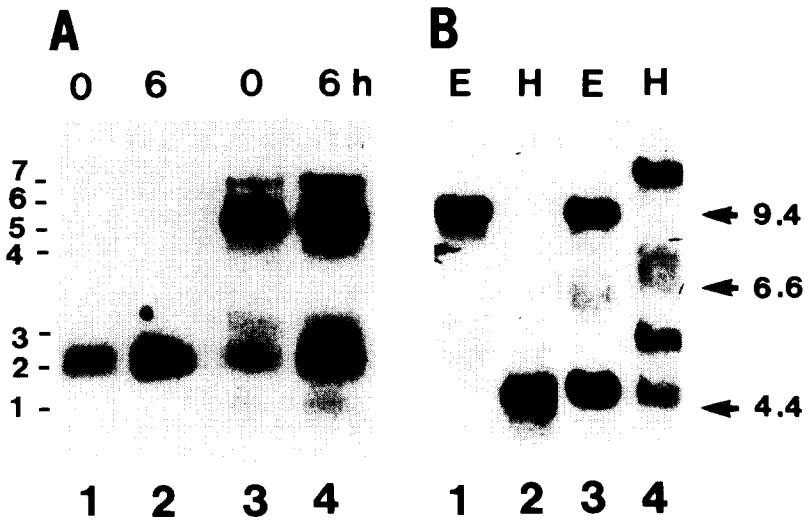

Fig. 4. Northern blot (A) and Southern blot (B) analysis using the p24-specific fragment including positions 189–491 of the cDNA sequence shown in Fig. 1 (lanes 1 and 2) or the 3'-terminal fragment comprising positions 492–688 (lanes 3 and 4). For the Northern blots RNA from either growing (0 h) or aggregating (6 h) cells was used. In (B) the sizes of fragments of *Hin*dIII digested λ DNA are given in kb.

mon to all of them (Fig. 4A). In agreement with these data, the internal fragment recognized on Southern blots one 9.5 kb fragment in *Eco*RI digested and one 4.6 kb fragment in *Hin*dIII digested genomic DNA, indicating that these fragments contain the p24 encoding gene. The probe containing the carboxy-terminal sequences hybridized to all bands that were recognized by the cDNA insert cDRS31 covering the entire coding region (Fig. 4B).

3.3. *Deduced amino acid sequence*

The cDNA-derived sequence indicates a protein consisting of 185 amino acids. Its calculated molecular mass of 20 697 Da is close to the one obtained for p24 in SDS-PAGE. To confirm that the cDNA-derived sequence corresponded to that of p24, sequence information was obtained from the protein as purified from *D. discoideum* cells. Since its N-terminus was blocked, a CNBr-peptide comprising about half of its size was isolated. The N-terminal sequence of this fragment was identical with positions 101–113 of the cDNA-derived sequence (Fig. 1).

p24	Octopus rhodopsin	synaptophysin
GYPAQP	GYPPP	GYGQGPG
GYPPQP	GYPPQG	GYGPQD
GYPPQH	AYPPPQ	SYGPQG
GYPPQH	GYPPQ	GYQP
GYPQQP	GYPPQ	DYGQPASGGG
GYY	GYPPQ	GYGPQG
	GYPPQ	DYGQQ
	GAPPQ	GYGQQ

Fig. 5. Comparison of repeats in the C-terminal regions of *Dictyostelium* p24 (residues 153–185), *Octopus* rhodopsin (residues 395–436) [4] and rat synaptophysin (residues 250–297) [5].

4. DISCUSSION

p24 has been isolated as an actin binding protein from plasma membranes of *D. discoideum* cells [3]. Comparison of its sequence to that of other actin binding proteins did not reveal any significant homologies. However, the actin binding sites are not related to each other in different families of actin binding proteins [26]. The p24 sequence contains no obvious hydrophobic regions that could represent transmembrane domains nor is a hydrophobic leader sequence present. Therefore, p24 does not seem to be an integral membrane protein. The way it interacts with membranes remains to be elucidated.

The predicted amino acid composition of p24 is that of a very basic protein. Amino acids 25–38 are repeated in positions 64–77 with slight modifications. The carboxy-terminus is distinguished by the presence of a repetitive amino acid sequence rich in proline, glycine, tyrosine and glutamine. p24 shares this carboxy-terminus with two other membrane proteins, *Octopus* rhodopsin [4] and synaptophysin [5–7]. *Octopus* rhodopsin is distinguished by this sequence from all other known rhodopsins. It has been suggested that synaptophysin is linked through its carboxy-terminus to extravesicular proteins [6]. A proline-, glycine-, tyrosine-, glutamine-rich sequence was also found in a sea urchin glycoprotein [27], in this case not at the C-terminus but as an internal repeat. Neither in *Octopus* nor in vertebrate cells has this repeat structure yet been identified as a common element of a multigene family. We are currently isolating the other members of this gene family in *D. discoideum* and trying to elucidate their location and function within the cells.

Acknowledgments: We thank S. Rapp, I. Lindner and T. Henkel for DNA sequencing, D. Rieger and B. Grabatin for production of monoclonal antibodies and cell culture. We are grateful to Dr S. Tabor for providing the *E. coli* expression system and Drs M.-L. Lacombe and R. Kessin for sharing with us their cDNA library. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Wuestehube, L.J. and Luna, E.J. (1987) J. Cell Biol. 105, 1741–1751.
[2] Scheel, J., Ziegelbauer, K., Kupke, T., Humbel, B.M., Noegel, A.A., Gerisch, G. and Schleicher, M. (1989) J. Biol. Chem. 264, 2832–2839.
[3] Stratford, C.A. and Brown, S.S. (1985) J. Cell Biol. 100, 727–735.
[4] Ovchinnikov, Yu.A., Abdulaev, N.G., Zolotarev, A.S., Artamonov, I.D., Bespalov, I.A., Dergachev, A.E. and Tsuda, M. (1987) FEBS Lett. 232, 69–72.
[5] Leube, R.E., Kaiser, P., Seiter, A., Zimbelmann, R., Franke, W.W., Rehm, H., Knaus, P., Prior, P., Betz, H., Reinke, H., Beyreuther, K. and Wiedenmann, B. (1987) EMBO J. 6, 3261–3268.
[6] Südhof, T.C., Lottspeich, F., Greengard, P., Mehl, E. and Jahn, R. (1987) Science 238, 1142–1144.
[7] Buckley, K.M., Floor, E. and Kelly, R.B. (1987) J. Cell Biol. 105, 2447–2456.
[8] Claviez, M., Pagh, K., Maruta, H., Baltes, W., Fisher, P. and Gerisch, G. (1982) EMBO J. 1, 1017–1022.
[9] Newell, P.C., Telser, A. and Sussman, M. (1969) J. Bacteriol. 100, 763–768.
[10] Brunette, D.M. and Till, J.E. (1971) J. Membr. Biol. 5, 215–224.
[11] Stadler, J., Bordier, C., Lottspeich, F., Henschen, A. and Gerisch, G. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 771–776.
[12] Eckerskorn, C., Mewes, W., Goretzki, H. and Lottspeich, F. (1988) Eur. J. Biochem. 176, 509–519.
[13] Eckerskorn, C., Klose, J., Mewes, W., Lottspeich, F. and Jungblut, P. (1988) Electrophoresis 9, 830–838.
[14] Lacombe, M.-L., Podgorski, G.J., Franke, J. and Kessin, R.H. (1986) J. Biol. Chem. 261, 16811–16817.
[15] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5468.
[17] Chen, E.Y. and Seeburg, P.H. (1985) DNA 4, 165–170.
[18] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[19] Noegel, A., Welker, D.L., Metz, B.A. and Williams, K.L. (1985) J. Mol. Biol. 185, 447–450.
[20] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Noegel, A., Metz, B.A. and Williams, K.L. (1985) EMBO J. 4, 3797–3805.
[22] Tabor, S. and Richardson, C.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074–1078.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[25] Maeda, K., Sczakiel, A., Hofmann, W., Menetret, J.-F. and Wittinghofer, A. (1989) J. Mol. Biol. 205, 269–273.
[26] Hartmann, H., Noegel, A.A., Eckerskorn, C., Rapp, S. and Schleicher, M. (1989) J. Biol. Chem. 264, 12639–12647.
[27] Parr, B.A., Parks, A.L. and Raff, R.A. (1990) J. Biol. Chem. 265, 1408–1413.