Volume 233, number 2, 233–238 FEB 05940 June 1988

Isolation and characterization of two novel calcium-dependent phospholipid-binding proteins from bovine lung

Catherine M. Boustead, John H. Walker and Michael J. Geisow*

Department of Biochemistry, University of Leeds, Leeds LS2 9JT and **Delta Biotechnology, 59 Castle Boulevard, Nottingham NG7 1FD, England*

Received 4 March 1988

Two calcium-dependent proteins of apparent M_r 32000 and 34000 were isolated from bovine lung. Approx. 70 mg/kg of each was obtained. Two-dimensional gel electrophoresis in the presence of 8 M urea showed their apparent p*I* values to be 5.1 and 5.0, respectively. Both proteins are related immunologically to calelectrin from *Torpedo marmorata*. They also have very similar amino acid compositions to calelectrin. Partial sequence information shows that both proteins contain the highly conserved sequence described for the annexins, a new family of calcium-dependent membrane-binding proteins. In common with other members of this family, the new proteins bind to acidic phospholipids in a calcium-dependent manner.

Annexin; Calpactin; Endonexin; Lipocortin; Ca^{2+}-dependent phospholipid-binding protein; (Bovine lung)

1. INTRODUCTION

Calcium plays a key role in cell regulation, and its effects are mediated by calcium-binding proteins, such as calmodulin [1]. Recently, a new family of calcium-binding proteins has been identified in a wide range of tissues, and termed the annexins [2,3]. The members of this group so far identified include calelectrin from the electric ray *Torpedo marmorata* [4,5] and the mammalian proteins calpactin I (a substrate of the tyrosine kinase $pp60^{src}$ [6,7], lipocortin I (a substrate of the EGF receptor tyrosine kinase) [7,8], endonexin [9,10], protein II [11,12], endonexin II [13] and p70 [9,11,14]. These proteins share the property of binding to acidic phospholipids in the presence of micromolar calcium concentrations [5,9,10,13, 15–18]. One of the annexins, lipocortin I, has been identified as an inhibitor of phospholipase A_2 [8,19,21], and calpactin I [20,21], p70 [21] and endonexin II [13] have been shown to share this property.

Correspondence address: C.M. Boustead, Department of Biochemistry, University of Leeds, Leeds, LS2 9JT, England

Three of the mammalian annexins (endonexin, p70 and calpactin I heavy chain) were shown to cross-react with an antiserum to *Torpedo* calelectrin [9,22]. Subsequently, a 17-amino-acid consensus sequence was identified in *Torpedo* calelectrin, endonexin, protein II, calpactin I heavy chain [10], p70 [23] and endonexin II [13]. Complete sequence data are now available for calpactin I heavy chain [20,24,25], lipocortin I [8] and protein II [12]. A high degree of homology was found between all three proteins. Each one is made up of a variable NH_2-terminal tail followed by four internal repeats of about 70 amino acids, including the 17-amino-acid consensus sequence.

The total number of mammalian proteins which share the characteristics of the annexins is so far unknown. Thirteen proteins from bovine adrenal medulla (chromobindins) have been identified which bind to chromaffin granule lipids in a calcium-dependent manner but only four (identified as calpactin I, lipocortin I, endonexin and p70) are so far known to contain the consensus sequence [17,26]. We describe here the isolation and characterization of two new proteins isolated from bovine lung which share with annexins the proper-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

ties of calcium-dependent binding to acidic phospholipids, immunological similarity to *Torpedo* calelectrin and possession of the highly conserved sequence.

2. MATERIALS AND METHODS

2.1. *Isolation of annexins*

Bovine lung was obtained from a local abattoir and stored frozen at −70°C until required. Tissue (50 g) was homogenized in a Waring blender with 250 ml of 0.15 M NaCl/10 mM Hepes/5 mM EGTA (pH 7.4) and centrifuged for 30 min at 40000 × *g*. The supernatant was removed, and $CaCl_2$ added to it to a final concentration of 6 mM (1 mM excess). After 15 min on ice, the fraction was centrifuged for 30 min at 40000 × *g*. The pellet was washed first with 100 ml of 0.15 M NaCl/10 mM Hepes/1 mM $CaCl_2$ (pH 7.4) and then with 100 ml of 10 mM Hepes/1 mM $CaCl_2$ (pH 7.4). The pellet was then resuspended in 15 ml of 10 mM Hepes/10 mM EGTA (pH 7.4) and centrifuged for 30 min at 100000 × *g*. Phenylmethylsulphonyl fluoride (0.25 mM) was present throughout, and all steps were performed at 4°C.

2.2. *Ion-exchange chromatography*

The final supernatant was dialysed against 20 mM Hepes (pH 7.4) and applied to a 1.0 × 5.0 cm column of DEAE-cellulose (DE52, Whatman, Maidstone, England) equilibrated in the same buffer. After elution of unabsorbed material, a convex exponential gradient was applied, formed from 40 ml of 20 mM Hepes (pH 7.4) in the mixing vessel and 0.6 M NaCl/20 mM Hepes (pH 7.4) as the limiting buffer [27]. In some cases, in order to separate the two proteins, a shallower gradient was formed from the same buffers by using a mixing volume of 60 ml. The column was run at 12 ml/h and fractions (1 ml) were collected.

2.3. *Gel filtration*

Protein was applied to a 1.6 × 70 cm column of Ultrogel AcA 44 (LKB, Bromma, Sweden) in 0.1 M NaCl/50 mM Tris-HCl/1 mM EGTA/1 mM NaN_3 (pH 7.4) and run at a rate of 6 ml/h. Fractions of 0.8 ml were collected.

2.4. *Protein assay*

Protein concentrations were measured using BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.5. *Electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [28]. Two-dimensional gel electrophoresis was according to O'Farrell [29] as modified by Anderson and Anderson [30]. Ampholines (Pharmalytes; Pharmacia, Uppsala) were a 4:1 ratio of pH 5–8:pH 3–10. Gels were stained with Coomassie brilliant blue. Immunoblotting was performed essentially as described [31], using a 1:100 dilution of antiserum for 1 h and visualising with peroxidase-conjugated second antibody and diaminobenzidine. Rabbit antiserum to *Torpedo* calelectrin has been characterized previously [4].

2.6. *Amino acid analysis*

Proteins were freed of salts by precipitation with 3 vols acetone. Precipitated protein (100 μg) was redissolved in water, dried into hydrolysis vials and acid hydrolysed with 5.7 N HCl in the vapour phase for 24 h. Amino acids were quantitated using an LKB Alpha-Plus analyser.

2.7. *Amino acid sequencing*

Peptides were produced either by trypsin or lys-C digestion at an enzyme to substrate ratio of 1:200 (w/w) for 24 h. Peptides were isolated by reverse-phase HPLC using Brownlee aquapore C_8 columns and a gradient of 0–70% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Peptides selected at random were sequenced using an Applied Biosystems model 477A amino acid sequencer.

2.8. *Phospholipid binding*

Phospholipid vesicles were made in the presence of 240 mM sucrose from phosphatidylcholine (PC) alone, or equimolar mixtures of PC and phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or phosphatidic acid (PA) (Sigma, Poole, England) [32]. The liposomes were harvested by adding 2 vols buffer A [100 mM KCl/2 mM $MgCl_2$/1 mM EGTA/20 mM Hepes (pH 7.4)], buffer B (buffer A plus 1.1 mM $CaCl_2$ to give 100 μM free Ca^{2+}) or buffer C (buffer A plus 2 mM $CaCl_2$ to give 1 mM free Ca^{2+}) and centrifuging for 10 min at 12000 × *g*. Following two further washes in buffer A, B or C, binding of the proteins to liposomes was measured. 10 μg protein was incubated for 15 min at 20°C with liposomes (150 μg phospholipid) in a total volume of 500 μl of either buffer A, B or C. After centrifugation for 10 min at 12000 × *g*, the supernatants were removed. The pellets were washed once in 500 μl buffer A, B or C, extracted with 300 μl acetone at −20°C for 30 min, and centrifuged for 10 min at 12000 × *g*. Equal proportions of the supernatants and pellets were analyzed on 10% SDS-PAGE.

3. RESULTS

3.1. *Isolation and characterization of two novel calcium-dependent proteins from bovine lung*

The two proteins can be rapidly extracted from bovine lung using the method described. Fig.1a shows SDS-PAGE of the final supernatant of the preparation of calcium-dependent proteins. This supernatant was further fractionated by anion-exchange chromatography on DEAE-cellulose (fig.2). Samples of the unbound fraction of the column and the two peak fractions were analyzed by SDS-PAGE (fig.1b–d). The unbound fraction contained polypeptides of apparent molecular mass 34–38 kDa (fig.1b). The protein peak eluting at 0.08 M NaCl contained predominantly a polypeptide of apparent molecular mass 32 kDa (fig.1c) which cross-reacted with an antiserum to

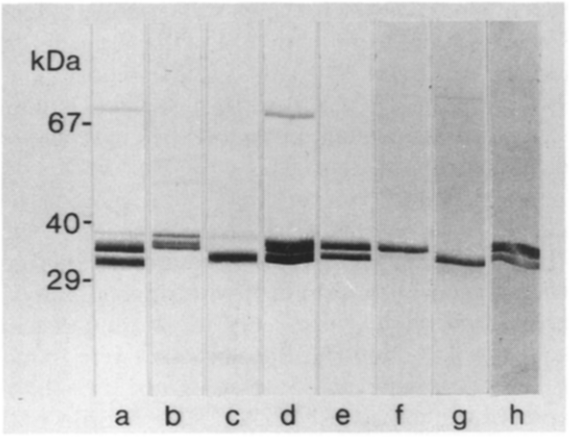

Fig.1. Isolation of the 32 and 34 kDa proteins from bovine lung. Coomassie blue-stained 10% SDS-PAGE of: (a) final supernatant of the lung preparation; (b) unbound fraction of the DEAE-cellulose column; (c) peak eluting at 0.08 M NaCl from DEAE-cellulose; (d) peak eluting at 0.23 M NaCl from DEAE-cellulose; (e) 32 and 34 kDa proteins after gel filtration; (f) purified 34 kDa protein; (g) purified 32 kDa protein. (h) Immunoblot of (e) using antiserum to *Torpedo* calelectrin, followed by peroxidase-labelled second antibody and staining with diaminobenzidine.

endonexin [9] (not shown). The majority of the protein eluted at 0.23 M NaCl, and showed two major polypeptides of apparent molecular mass 32 and 34 kDa, together with a minor component of apparent molecular mass 70 kDa (fig.1d). Approx. 70 mg/kg of each of the 32 and 34 kDa proteins was obtained. The 32 and 34 kDa polypeptides could be separated from the 70 kDa polypeptide by gel filtration (fig.1e). All three proteins eluted

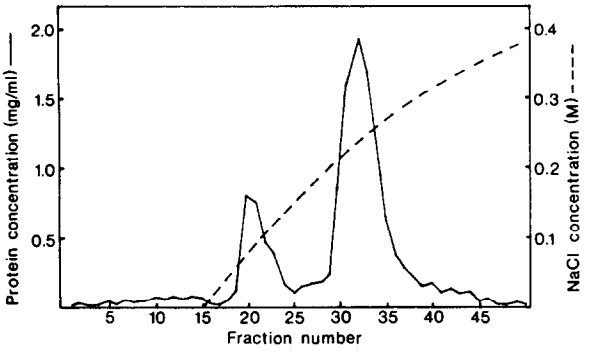

Fig.2. Anion-exchange chromatography on DEAE-cellulose at pH 7.4 of the final supernatant of the lung preparation. 1-ml fractions were collected.

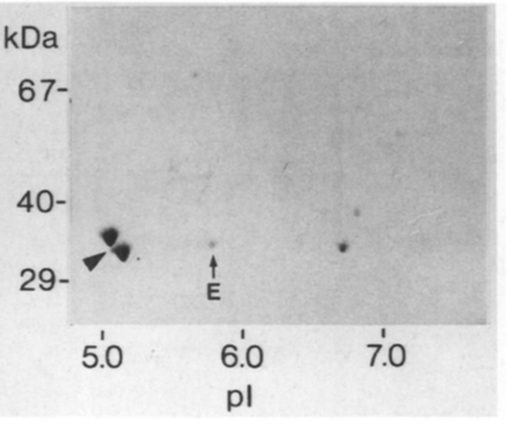

Fig.3. Two-dimensional gel electrophoresis of a fraction similar to that in fig.1a. The positions of the 32 and 34 kDa proteins are marked (►). E, position of endonexin.

as monomers. Partial separation of the 32 and 34 kDa proteins could be achieved by using a shallow NaCl gradient applied to the DEAE-cellulose column. By taking the initial and final fractions of the overlapping peaks, each protein could be obtained as over 95% pure as judged by densitometric tracings of polyacrylamide gels. Fig.1f and g shows the purified proteins.

Since the two proteins could be isolated using a procedure similar to that used for *Torpedo* calelectrin [5], we investigated whether they were related immunologically. Fig.1h shows that both bovine proteins cross-react strongly on an immunoblot with the anti-*Torpedo* calelectrin antiserum.

As a further characterization of the proteins, we analyzed the final supernatant of the lung preparation by two-dimensional gel electrophoresis (fig.3). The new proteins migrate with apparent isoelectric points of 5.1 (32 kDa) and 5.0 (34 kDa). Their position on the gel was confirmed by two-dimensional gel electrophoresis of the purified proteins (not shown). The acidic isoelectric points of the proteins show them to be distinct from the previously characterized annexins of similar molecular mass, calpactin I heavy chain (p*I* 7.2 and 7.4) [6], lipocortin 1 (p*I* 6.8) [33], endonexin (p*I* 5.6) [9] and protein II (p*I* 6.5) [11].

3.2. *Amino acid composition and partial sequence*

Amino acid analysis showed that the two proteins have a very similar amino acid composition to

Table 1

Amino acid composition of the 32 and 34 kDa proteins and comparison with human placental anticoagulant protein (IBC) [38]

Amino acid	32 kDa	34 kDa	IBC[a]
Asx	10.3	9.7	9.7
Thr	6.4	6.4	7.2
Ser	5.7	5.5	6.6
Glx	14.0	13.9	12.9
Pro	2.2	2.2	1.6
Gly	7.8	7.6	6.9
Ala	8.2	8.2	8.2
Cys	0.0	0.0	0.3
Val	5.2	5.1	5.0
Met	1.7	1.7	2.2
Ile	5.1	5.2	5.6
Leu	11.5	11.9	11.9
Tyr	3.7	3.9	3.8
Phe	3.9	4.1	4.1
His	1.1	1.1	0.9
Lys	6.7	6.5	6.9
Arg	6.6	6.8	6.0
Trp	ND	ND	0.3

[a] Predicted from nucleotide sequence

Tryptophan was not determined (ND). Values are expressed as mol%

each other and to the previously described proteins *Torpedo* calelectrin [4], p70 [9,14], endonexin [9] and protein II [12]. The amino acid composition is also very similar to a recently described human placental anticoagulant protein related in sequence to lipocortin [38] (table 1).

The individual proteins were both found to be blocked to Edman degradation at the NH_2-terminal. Peptides were therefore produced by cleavage with trypsin or lysyl endopeptidase-C, separated by reverse-phase HPLC and amino acid sequence data obtained. Both proteins were found to contain sequences homologous to those reported for calpactin I [20,24,25], lipocortin I [8] and protein II [12]. The new proteins showed the closest homology to sequences reported for endonexin II [13] and the sequence of human placental anticoagulant protein [37,38] (table 2).

3.3. *Characterization of Ca^{2+}-dependent phospholipid-binding properties*

Since the two new proteins were related immunologically and by amino acid sequence to other annexins, we used a centrifugation assay to

Table 2

Sequence data for the 32 and 34 kDa proteins

```
32                                  (32) GTVADFPGFD
IBC                                MAQVLRGTVTDFPGFD
LIPO I    MAMVSEFLKQAWFIENEEQEYVQTVKSSKGGPGSAVSPYPTFN

32/34     ER      (32) GLGTDEESILTLLT                    (34) RELTGLIEK (34)MKPX
IBC       ERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISAAFKTLFGRDLLDDLKSELTGKFEKLIVALMKPS
ENDO II                                                                    KPS
LIPO I    PSSDVAALHKAIMVKGVDEATIIDILTKRNNAQRQQIKAAYLQETGKPLDETLKKALTGHLEEVVLALLKTP

32/34     RLYDAYELK       (32) VLTEIIASRXLEDD AIEQVTEEEYGSSL (M)
IBC       RLYDAYELKHALKGAGTNEKVLTEIIASRTPEELRAIKQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAG-IDEAQV
ENDO II   RLYDAYELKHALKGAGTNEKVLTEIIASRTPEELRAIKQVYEEW
LIPO I    AQFDADELRAAMKGLGTDEDTLIEILASRTNKEIRDINRVYREELKRDLAKDITSDTSGDFRNALLSLAKGDRSEDFGVN-EDLA

IBC       EQDAQALFQAGELKWGTDEEKFITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSI
LIPO I    DSDARALYEAGERRKGTDVNVFNTILTTRSYPQLRRVFQKYTKYSKHDMNKVLDLELKGDIEKCLTAIVKCATSK

IBC       PAYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKKALLLLCGEDD
ENDO II                KGAGTDDHTLIRV VSRSEIDLFNIRKEFRKNFATSLY  IKGDTSGDYKKALLLLXGEDD
LIPO I    PAFFAEKLHQAMKGVLTRHKALIRIMVSRSEIDMNDIKAFYQKMYGISLCQAILDETKGDYEKILVALCGG-N
```

Amino acid sequences were obtained for peptides derived either from purified 32 kDa (32) and 34 kDa (34) proteins, or from a mixture of the two (M). For comparison the partial sequence of endonexin II (ENDO II) and the complete sequences of lipocortin I (LIPO I) and human placental anticoagulant protein (IBC) are given [8,13,37,38]

investigate whether they also showed calcium-dependent binding to phospholipids. When the two proteins were mixed with pure PC vesicles, no binding was detectable even at 1 mM calcium. However, both proteins bound in a calcium-dependent manner to liposomes made from equimolar mixtures of PC and PA, PS, PE, or PI (fig.4). Binding to PI-containing liposomes was only detected at 1 mM Ca^{2+}, whereas binding to PA-, PS- and PE-containing liposomes occurred at 100 μM free Ca^{2+}. The same result was obtained when each protein was incubated independently with the liposomes (not shown).

4. DISCUSSION

We describe here the isolation and preliminary characterization of two new calcium-dependent proteins which are major constituents of bovine lung. By the criteria of calcium-dependent binding to acidic phospholipids, immunological similarity to *Torpedo* calelectrin, amino acid composition and possession of the highly conserved sequence, the proteins represent new members of the annexin family. The two proteins are very similar to each other in molecular mass, p*I*, amino acid composition and phospholipid-binding properties, and the present study cannot rule out the possibility that the 32 kDa protein is a proteolysis product of the 34 kDa protein. However, both proteins are NH_2-terminally blocked and we note that calpactin I, lipocortin I and protein II are all sensitive to proteolysis at the NH_2-terminal tail [12,19,34,35].

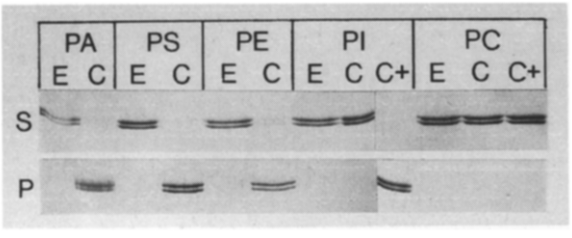

Fig.4. Calcium-dependent binding of the 32 and 34 kDa proteins to phospholipids. Coomassie blue-stained 10% SDS-PAGE of equal proportions of supernatants (S) and pellets (P) following incubation of the proteins with liposomes and centrifugation as described in section 2. Liposomes were either pure PC (PC) or equimolar mixtures of PC and PA (PA), PS (PS), PE (PE) or PI (PI). The experiment was performed in the presence of 1 mM EGTA (E), 100 μM free Ca^{2+} (C), or 1 mM free Ca^{2+} (C+).

Two proteins with a similar migration pattern on two-dimensional gels have been observed in preparations of the chromobindins, proteins from bovine adrenal medulla which bind to chromaffin granule membranes in a calcium-dependent manner [17,26]. These two proteins, chromobindins 5 and 7, have very similar peptide maps to each other and are distinct from clathrin light chains [36], but have not been further characterized. However, their molecular masses, isoelectric points and calcium-dependent binding to membranes suggest that they are probably homologous to the bovine lung proteins reported here.

A comparison of amino acid sequences indicates that the new proteins are most closely related to human placental endonexin II [13] and to the very recently described human placental anticoagulant protein; 34 kDa, p*I* 4.9 [37,38]. The bovine sequences we report can be aligned to the sequence of human placental anticoagulant protein with 86% identity.

Saris et al. [24] have divided the annexins into four groups of homologous proteins (calpactin I-, lipocortin I-, endonexin- and p70-like proteins). We suggest that endonexin II [13], the human placental anticoagulant protein [37,38], chromobindins 5 and 7 [17,26] and the two new proteins reported here represent a fifth group.

Although there have been a number of studies on the in vitro properties of the annexins, their physiological role remains undefined. Sequence similarity suggests that the new proteins would be expected to inhibit phospholipase A_2 and our preliminary studies indicate that this is the case. Further structural and functional studies will be needed to elucidate the role of the two new proteins and to compare this with the other members of the annexin family.

Acknowledgement: C.M.B. was supported by a project grant from the Medical Research Council.

REFERENCES

[1] Wassermann, R.H., Corradino, R.A., Carafoli, E., Kretsinger, R.H., MacLennan, D.H. and Siegel, F.L. (1977) Calcium-Binding Proteins and Calcium Function, North-Holland, New York.
[2] Geisow, M.J. and Walker, J.H. (1986) Trends Biochem. Sci. 11, 420–423.

[3] Geisow, M.J., Walker, J.H., Boustead, C.M. and Taylor, W. (1987) Biosci. Rep. 7, 289–298.
[4] Walker, J.H. (1982) J. Neurochem. 39, 815–823.
[5] Sudhof, T.C., Walker, J.H. and Fritsche, U. (1985) J. Neurochem. 44, 1302–1307.
[6] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[7] Glenney, J.R. (1987) Bioessays 7, 173–175.
[8] Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L.K., Foeller, C., Chow, E.P., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Nature 320, 77–81.
[9] Sudhof, T.C., Ebbecke, M., Walker, J.H., Fritsche, U. and Boustead, C. (1984) Biochemistry 23, 1103–1109.
[10] Geisow, M.J., Fritsche, U., Hexham, J.M., Dash, B. and Johnson, T. (1986) Nature 320, 636–638.
[11] Shadle, P.J., Gerke, V. and Weber, K. (1985) J. Biol. Chem. 260, 16354–16360.
[12] Weber, K., Johnsson, N., Plessman, U., Van, P.N., Soeling, H.-D., Ampe, C. and Vandekerckhove, J. (1987) EMBO J. 6, 1599–1604.
[13] Schlaepfer, D.D., Mehlman, T., Burgess, W.H. and Haigler, H.T. (1987) Proc. Natl. Acad. Sci. USA 84, 6078–6082.
[14] Owens, R.J. and Crumpton, M.J. (1984) Biochem. J. 219, 309–316.
[15] Glenney, J.R. (1985) FEBS Lett. 192, 79–82.
[16] Schlaepfer, D.D. and Haigler, H.T. (1987) J. Biol. Chem. 262, 6931–6937.
[17] Creutz, C.E., Dowling, L.G., Sando, J.J., Villar-Palasi, C., Whipple, J.H. and Zaks, W.J. (1983) J. Biol. Chem. 258, 14664–14674.
[18] Shadle, P.J. and Weber, K. (1987) Biochim. Biophys. Acta 897, 502–506.
[19] Haigler, H.T., Schlaepfer, D.D. and Burgess, W.H. (1987) J. Biol. Chem. 262, 6921–6930.
[20] Huang, K.-S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E. and Pepinsky, R.B. (1986) Cell 46, 191–199.
[21] Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R. (1987) J. Biol. Chem. 262, 1698–1705.
[22] Geisow, M.J., Childs, J., Dash, B., Harris, A., Panayotou, G., Sudhof, T.C. and Walker, J.H. (1984) EMBO J. 3, 2969–2974.
[23] Martin, F., Derancourt, J., Capony, J.-P., Colote, S. and Cavadore, J.-C. (1987) Biochem. Biophys. Res. Commun. 145, 961–968.
[24] Saris, C.J.M., Tack, B.F., Kristensen, T., Glenney, J.R. and Hunter, T. (1986) Cell 46, 201–212.
[25] Kristensen, T., Saris, C.J.M., Hunter, T., Hicks, L.J., Noonan, D.J., Glenney, J.R. and Tack, B.F. (1986) Biochemistry 25, 4497–4503.
[26] Creutz, C.E., Zaks, W.J., Hamman, H.C., Crane, S., Martin, W.H., Gould, K.L., Oddie, K.M. and Parsons, S.J. (1987) J. Biol. Chem. 262, 1860–1868.
[27] Hudson, L. and Hay, F.C. (1976) Practical Immunology, Blackwell, London.
[28] Laemmli, U.K. (1970) Nature 227, 680–685.
[29] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[30] Anderson, L. and Anderson, N.G. (1977) Proc. Natl. Acad. Sci. USA 74, 5421–5425.
[31] Walker, J.H., Kristjansson, G.I. and Stadler, H. (1986) J. Neurochem. 46, 875–881.
[32] Reeves, J.P. and Dowben, R.M. (1969) J. Cell Physiol. 73, 49–60.
[33] Fava, R.A. and Cohen, S. (1984) J. Biol. Chem. 259, 2636–2645.
[34] Glenney, J.R. and Tack, B.F. (1985) Proc. Natl. Acad. Sci. USA 82, 7884–7888.
[35] Johnsson, N., Vandekerckhove, J., VanDamme, J. and Weber, K. (1986) FEBS Lett. 198, 361–364.
[36] Creutz, C.E. and Harrison, J.R. (1984) Nature 308, 208–210.
[37] Funakoshi, T., Hendrickson, L.E., McMullem, B.A. and Fujikawa, K. (1987) Biochemistry 26, 8087–8092.
[38] Iwasaki, A., Suda, M., Nakao, H., Nagoya, T., Saino, Y., Arai, K., Mizoguchi, T., Sato, F., Yoshizaki, H., Hirata, M., Miyata, T., Shidara, Y., Murata, M. and Maki, M. (1987) J. Biochem. (Tokyo) 102, 1261–1273.