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Purification and internal amino acid sequence of the 80 kDa protein kinase C substrate from Swiss 3T3 fibroblasts

Homology with substrates from brain

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The acidic 80 kDa protein kinase C (PKC) substrate was purified from 2.3×10^{10} Swiss 3T3 fibroblasts. Partial amino acid sequence data were obtained from five peptides generated by *S. aureus* V8 cleavage of the protein, enabling a total of 91 amino acid residues to be assigned. The sequences of these five peptides were compared to the deduced amino acid sequences of acidic 80–87 kDa PKC substrates from both actively proliferating A431 epidermal carcinoma cells, and fully differentiated neural tissue. Despite their similar physical properties, there was no homology between the peptides derived from the fibroblast 80 kDa protein and the PKC substrate from A431 cells. However, there was 66% homology with the 87 kDa bovine brain protein within the regions covered by the peptides (about 30% of the total protein). Furthermore, comparison of the peptides from the fibroblast 80 kDa protein with proteolytic peptides derived from the acidic 80 kDa rat brain protein revealed an overall homology of 89%. These data provide the first direct evidence that the 80 kDa PKC substrate from Swiss 3T3 fibroblasts is closely related to the 80–87 kDa PKC substrates detected in fully differentiated neural tissue.

Protein kinase C substrate; Amino acid sequence; Cellular signalling

1. INTRODUCTION

The non-tumorigenic murine Swiss 3T3 fibroblast has proved to be an extremely useful model system for identifying both extracellular factors that modulate cell growth and for elucidating the early signals that lead to mitogenesis. One of the major signal transduction pathways activated by a variety of extracellular stimuli involves the enzyme protein kinase C (PKC) [1]. Since the mechanism(s) through which PKC-mediated signals elicit mitogenesis remains largely unknown, it is important to characterize the physiological substrates of this kinase.

An acidic protein that migrates with an apparent molecular mass of 80 kDa in SDS polyacrylamide gels has been identified as a major and specific substrate for PKC in quiescent mouse 3T3 fibroblasts [2–6]. The phosphorylation of this protein is stimulated by phorbol esters [2,4,7], diacylglycerols [7–9], platelet derived growth factor [2,7,10], fibroblast growth factor [3,7,11], bombesin [6,10,12], vasopressin [13,14], bradykinin [15] and *Pasteurella multocida* toxin [16], all of which stimulate DNA synthesis in quiescent Swiss 3T3 cells [1]. In addition, removal of either phorbol esters or mitogenic peptides results in rapid dephosphorylation of this protein [17]. These findings raise the possibility that the 80 kDa protein is involved in PKC-mediated mitogenic signal transduction.

Acidic 80–87 kDa PKC substrates have also been identified in the brain of several species [18,19,21,22]. In rodents, the protein migrates slightly faster by SDS-PAGE, is more acidic and shows different immunological cross-reactivity than in other species [18,19]. Chromatographic heterogeneity has also been reported within the same species [20,21]. Recently, the isolation of a cDNA encoding the 87 kDa PKC substrate from bovine brain was reported [23]. The purification and sequencing of proteolytic peptides of the rat brain 80 kDa PKC substrate enabled a comparison to be made of the rat and bovine proteins. This revealed an overall homology of 54% for the regions covered by the peptides [24]. Hence, these acidic PKC substrates are similar but not identical.

Acidic 65–87 kDa PKC substrates that appear to be related to the Swiss 3T3 fibroblast 80 kDa protein have also been detected in a variety of mammalian cells in culture [2,4,7,18,22,25,26]. However, no direct evidence is yet available to suggest that these proteins are related to each other or to the neuronal 80–87 kDa PKC substrates. Indeed, there has been a recent report on the cloning and sequencing of an acidic 80 kDa PKC substrate from the human epidermal carcinoma cell line A431 [27]. Comparison of the deduced amino acid se-

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quence of this protein with peptides generated from the rat brain 80 kDa PKC substrate [24] or the deduced amino acid sequence of the 87 kDa bovine brain protein [23], indicates that the protein from A431 cells is unrelated to the acidic 80–87 kDa PKC substrates isolated from brain. Consequently, it was important to determine whether the 80 kDa protein detected in non-excitable Swiss 3T3 fibroblasts, which are a model cell line for elucidating mitogenic signalling pathways, expresses an 80 kDa PKC substrate related to any of those identified in brain or cultured epidermal carcinoma cells.

In the present study, we report the purification and partial amino acid sequence of the acidic 80 kDa PKC substrate from Swiss 3T3 fibroblasts. These results provide direct evidence that this protein is related to the acidic 80–87 kDa PKC substrate proteins isolated from neural tissue, but not from epidermal carcinoma cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Sephadex G-150 (fine), phenylsepharose and liquid chromatography columns were purchased from Pharmacia Biotechnology; [γ-^{32}P]ATP (3.0 Ci/mmol) was from Amersham International UK; DEAE-cellulose was from Whatman; phorbol dibutyrate (PDBu), histone IIIS and phosphatidylserine (PS) were from Sigma (St Louis, MO); protein assay reagent was from Pierce, Aquacide II from Calbiochem and silver staining kits were from Koch-Light Ltd.

Protein kinase C was partially purified from rat brain by the method of Walsh et al. [28] and assayed using the phosphocellulose spot technique [29] as described [13].

2.2. *Methods*

2.2.1. Assay of the 80 kDa protein

The 80 kDa protein was assayed by phosphorylation. Protein samples from chromatographic fractions were incubated with 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 500 ng/ml phorbol dibutyrate, 500 μM $CaCl_2$, 100 μg/ml phosphatidylserine, 50 μM [γ-^{32}P]ATP (5×10^5 cpm/nmol) and 5 μl PKC (spec. act. 22 nmol ^{32}P/mg/min) in a final volume of 100 μl. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 20 μl five-times concentrated SDS-sample buffer (50% glycerol, 12.5% SDS, 50 mM Tris/HCl pH 8.0, 10 mM EDTA, 12.5% 2-mercaptoethanol). Samples were boiled for 5 min, and then subjected to SDS-PAGE. The bands corresponding to the 80 kDa protein on autoradiographs of dried gels were excised and quantitated by Cerenkov counting using a liquid scintillation counter (Beckman Instruments).

2.2.2. Cell culture

Cultures of Swiss 3T3 cells [30] were maintained in 90 mm Nunc Petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in humidified air/CO_2 (9:1) at 37°C. For the preparation of the cytosolic fraction, 3×10^6 cells were subcultured into 1850 cm^2 Falcon roller bottles and were grown to confluence without a change of medium for 6–7 days. The final density was $3–5 \times 10^7$ cells/flask.

2.2.3. Preparation of the cytosolic fraction

600 roller bottles, containing 2.3×10^{10} cells, were washed twice with 150 ml phosphate-buffered saline (PBS; 0.14 M NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) at room temperature. All subsequent steps were carried out at 4°C. The cells were harvested by gentle scraping into PBS containing 5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bacitracin, 10 μg/ml aprotinin, 1 mg/ml soybean trypsin inhibitor and 50 μM phenylmethanylsulphonyl fluoride. The cells were then pelleted by centrifugation at $750 \times g$ for 10 min and resuspended at 5×10^6 cells/ml in 50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA and the above protease inhibitors. Cells were then disrupted using a dounce homogenizer (A pestle, 75 strokes). The homogenate was centrifuged at $500 \times g$ for 10 min to remove nuclear material and intact cells, and the supernatant was centrifuged again at $30000 \times g$ for 30 min. The resulting supernatant, containing the 80 kDa protein was collected.

2.2.4. Purification of the 80 kDa protein from Swiss 3T3 fibroblasts

The supernatant fractions (2.60 liters) were heated at 100°C in 200 ml aliquots for 3 min. Heat-labile proteins were pelleted by centrifugation at $30000 \times g$ for 30 min. The supernatant, containing the 80 kDa protein was collected. All subsequent steps were carried out at 4°C. 2.56 liters of the heat-stable extract were incubated with 600 ml of settled DEAE-cellulose previously equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE buffer), with occasional stirring for 1 h. After washing with 2 liters of the TE buffer, the slurry was poured into a column (5 cm × 30 cm), and bound proteins were eluted with a 2 liter linear gradient of 0 to 1.0 M NaCl in TE buffer at 1.25 ml/min. 15 ml fractions were collected. The 80 kDa protein eluted between 135 and 265 mM NaCl. Active fractions were pooled and dialysed against 3 changes of 4 liters TE buffer. The dialysate was then applied to four DEAE-cellulose minicolumns (2 ml) equilibrated in TE buffer. The 80 kDa protein was eluted with 10×1 ml aliquots of 2 M NaCl, 20 mM HCl. Active fractions were pooled and concentrated to 8.0 ml with Aquacide II. The concentrated sample containing the 80 kDa protein was applied to a Sephadex G-150 gel-filtration column (82 cm × 2.5 cm) equilibrated with 1 M NaCl, 20 mM HCl. The 80 kDa protein eluted between 200 and 230 ml. Active fractions were pooled and dialyzed against 3 changes of 4 liter TE buffer before concentration to 1.9 ml with Aquacide II. The 80 kDa protein was phosphorylated with PKC and then electrophoresed on a 1.5 mm thick 7.5% SDS-polyacrylamide gel. After electrophoresis, protein was visualized by brief staining with Coomassie blue (0.1%). The band corresponding to the 80 kDa

Table I

The purification of the 80 kDa protein from Swiss 3T3 fibroblasts

Stage	Volume (ml)	Total protein (mg)	Total activity (10^4 cpm)	Specific activity (10^4 cpm/mg)	Fold purification	Yield (%)
Cytosolic fraction	2600	936	402	0.43	1.0	100
Boiled extract	2560	163.8	221	1.35	3.1	54.9
DEAE-cellulose	581	28.2	124	4.4	10.2	30.8
G-150 gel filtration	31.5	0.267	17.7	66	153.5	4.4
Electro-elution	0.25	0.0098	15.7	1604	3730	3.9

All procedures described in section 2

protein was excised and electroeluted from the gel slice as previously described [24]. A summary of the purification of the 80 kDa protein from 2.3×10^{10} Swiss 3T3 fibroblasts is shown in Table I.

2.2.5. Proteolytic cleavage of the 80 kDa protein and peptide separation

The purified 80 kDa protein (10 μg) was digested with *S. aureus* V8 protease at 25°C for 24 h, with an enzyme/substrate ratio of 1:30. The resulting peptides were resolved on a reverse-phase HPLC system (Waters), using a Brownlee Aquapore RP-300 column (2.1 × 100 mm). The following buffer system was used: Buffer A (0.08% trifluoroacetic acid in acetonitrile/water 1:99 v/v); Buffer B (0.06% trifluoroacetic acid in acetonitrile/water 90:10 v/v). The column was equilibrated in Buffer A and developed at a flow rate of 0.4 ml/min with a gradient of 0–30% Buffer B in 35 min, 30–45% Buffer B in 70 min followed by 45–100% Buffer B in 90 min. Peptides were detected by monitoring absorbance at 220 nm.

2.2.6. Peptide sequence analysis

HPLC-purified peptides generated from the *S. aureus* V8 digest of the 80 kDa protein were subjected to gas/liquid phase microsequencing on an Applied Biosystems model 477A peptide sequencer, equipped with a miniaturised reaction cartridge and utilising rapid cycle chemistry and on-line analysis programs (Totty and Mattaliano, submitted for publication).

3. RESULTS AND DISCUSSION

To determine whether the acidic 80 kDa protein of Swiss 3T3 fibroblasts is related to any of the 80–87 kDa PKC substrates from brain [18–21] or cultured epidermal carcinoma cells [27], we have undertaken to characterize the fibroblast protein by purification, proteolysis and subsequent amino acid sequencing of the peptide fragments.

Previous studies have shown that the 80 kDa PKC substrate from Swiss 3T3 fibroblasts is heat stable [18,19]. Therefore, a purification scheme involving heat treatment, DEAE-cellulose chromatography, G-150 gel filtration and electroelution from preparative SDS polyacrylamide gels was followed as previously described for the purification of the rat brain protein [19,24]. Approximately 10 μg of homogeneous 80 kDa protein was isolated from the cytosolic fraction of 2.3×10^{10} confluent, quiescent Swiss 3T3 fibroblasts (Table I).

The N-terminus of the 80 kDa PKC substrate from Swiss 3T3 fibroblasts has been reported to be blocked by myristoylation [25]. Therefore, to obtain amino acid sequence information, purified samples of the 80 kDa protein were digested with *S. aureus* V8 protease and the resulting peptides separated by reverse-phase HPLC (Fig. 1). Selected peaks were applied to a gas/liquid phase sequencer, and amino acid sequence obtained at the 30–100 pmol level. A total of 9 peaks yielded homogeneous peptides. The amino acid sequences of these peptides are presented in Fig. 1. In all, 91 amino acid residues were assigned from the analysis. Peptides C_1 and E_1 are extended versions of peptides C and E respectively, and were presumably generated through incomplete proteolytic cleavage of the protein by *S. aureus* V8 protease.

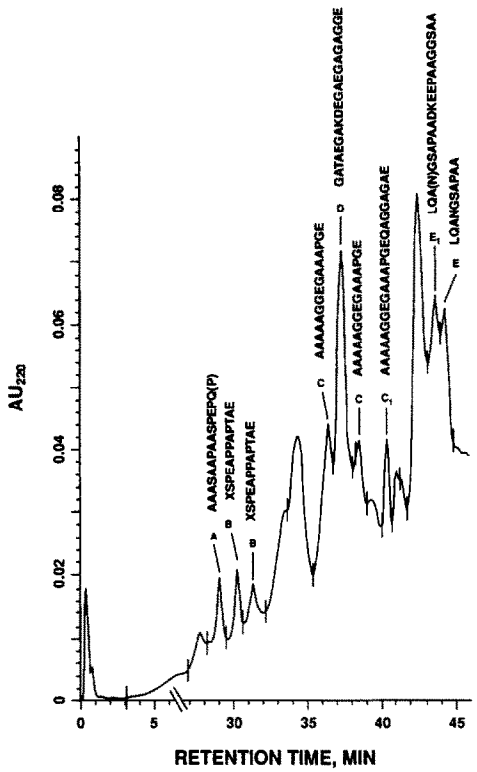

Fig. 1. Purification of *S. aureus* V8 proteolytic peptides from the Swiss 3T3 80 kDa protein by reverse phase HPLC. 10 μg of 80 kDa protein purified from preparative polyacrylamide gels was digested with *S. aureus* V8 protease and separated by HPLC as described in section 2. Amino acid sequence information was obtained from peptides labelled A–E_1, and is shown in single amino acid code above each peak. X indicates that no residue could be defined. Residues that could not be unequivocally identified are enclosed in parentheses. Sequences of peptide B were observed in two peaks of the digest. In both cases, the signal of the PTH-derivative dropped sharply in the 12th cycle of sequencing, and after this cycle, no further PTH-derivatives were detected. Therefore, it is likely that peptide B was sequenced to the end. Sequences of peptide C were observed in four separate peaks. Similarly, sequences of peptide E were detected in three separate peaks of the digest. Peptides C_1 and E_1 are extended versions of peptides C and E respectively, and are likely to originate from partial proteolytic cleavage by *S. aureus* V8 protease.

The sequence data we have obtained of the 80 kDa protein from Swiss 3T3 cells provide an excellent opportunity to define the relationship between the fibroblastic protein and other acidic PKC substrates. A comparison of the partial amino acid sequence of the fibroblast 80 kDa protein with the predicted amino acid sequence of the acidic 80 kDa protein from the human epidermal carcinoma cell line A431 [27] revealed no regions of homology, despite their similar apparent molecular weight on SDS-PAGE, ability to be phosphorylated by PKC, heat stability and acidic p*I*. In addition, no significant homology was found between the fibroblast 80 kDa protein and the PKC substrates GAP-43 [31] and P47 [32].

In contrast, a striking relationship was observed between the 3T3 80 kDa protein and acidic PKC substrates

Alignment of experimentally determined amino acid sequences from the Swiss 3T3 fibroblast and rat brain 80kDa PKC substrates with the predicted sequence of the bovine 87kDa PKC substrate

PEPTIDE			SEQUENCE	% HOMOLOGY
A	BOV	308	RASSACSAPSQEAQP	53
	3T3		AAASAAPAASPEPQP	
B	BOV	324	CSPEAPPAEAAE	82
	3T3		XSPEAPPAPTAE	
	RAT		XSPEAPPAPVAE	91
C_1	BOV	202	AAAAAG-EAGAAPGEPTAAPGEEAAAG E E GA	58
	3T3		AAAAAGGEGAAAPGEQAGGAGAEGAE	
	RAT		AAAAAGGDAAAAPGEQAGGAGAEGAEG(G)E(SR)	92
D	BOV	179	EGGEAEGAAGASAEGGK DEASGGAAA	47
	3T3		GATAEGAK DEGAGAGGE	
	RAT		EAGEGAEAEGATADGAX(DE)	80
E_1	BOV	59	LQANGSAPAAD K EEPAAAGS GA	91
	3T3		LQANGSAPAAD K EEPAAGGS AA	
	RAT		LQAXGSAPAAD(K)EEPASGGX(A)TP	94

Fig. 2. The amino acid sequences of peptides A–E_1 of the Swiss 3T3 fibroblast 80 kDa protein were aligned with the deduced amino acid sequence of the bovine brain 87 kDa PKC substrate [23] and with proteolytic peptides from the rat brain 80 kDa PKC substrate [24] (unpublished data). Vertical lines between amino acids denote identical residues. The numbers to the left of the bovine brain peptides indicate the position of the sequence within the entire protein. The numbers to the right of the bovine and rat brain sequences represent the % homology between these peptides and those of the fibroblast protein. A gap after amino acid 207 of the bovine brain protein was introduced to allow optimum alignment.

identified in brain tissue. Fig. 2 shows the experimentally determined peptide seqeunces of the fibroblast and rat brain PKC substrates aligned with the predicted sequence of the bovine brain protein. Within the regions covered by the fibroblast peptides, which represent 30% of the bovine brain 87 kDa protein, there was 66% homology between the two PKC substrates. The overall homology between the peptides of the rat brain and fibroblast 80 kDa proteins was 89%. Furthermore, all of the non-matching residues of the mouse fibroblast and rat brain peptides are replaced by conservative substitutions, and are therefore unlikely to have a profound effect on the tertiary structure of the protein. Analysis of the homology between individual peptides identified certain regions within these proteins that display a high degree of conservation between species (Fig. 2). Peptides B and E, which can be aligned with regions at the N-terminus and extreme C-terminus of the bovine brain protein, respectively, are highly conserved in both bovine and rodent species. This may suggest that these domains have functional significance. Regions of greatest divergence can be identified in the central portion of the protein, where the mouse fibroblast peptides A, C and D show only 53, 58 and 47% homology with the bovine brain protein, respectively.

In conclusion, the data presented here provide the first direct evidence that the acidic 80 kDa protein identified as a major substrate of PKC in Swiss 3T3 fibroblasts is closely related, but not identical to the 80–87 kDa PKC substrates isolated from the brain of several species. Recently, the bovine brain 87 kDa PKC substrate was reported to be a calmodulin-binding protein in vitro. The affinity for calmodulin was shown to be decreased by PKC-mediated phosphorylation of the protein [33]. Thus, in neurones, this PKC substrate may regulate ionic conductance through alterations in free Ca^{2+}. In non-excitable cells, similar molecular interactions could play a role in Ca^{2+} signalling of long-term responses such as gene expression and cellular proliferation, a proposition that warrants further experimental work.

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REFERENCES

[1] Rozengurt, E. (1986) Science 234, 161–166.
[2] Rozengurt, E., Rodriguez-Pena, A. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[3] Tsuda, T., Kaibuchi, K., Kawahara, Y., Fukazaki, H. and Takai, Y. (1985) FEBS Lett. 191, 205–210.
[4] Rodriguez-Pena, A. and Rozengurt, E. (1985) EMBO J. 4, 71–76.
[5] Rodriguez-Pena, A. and Rozengurt, E. (1986) EMBO J. 5, 77–83.
[6] Zachary, I., Sinnett-Smith, J. and Rozengurt, E. (1986) J. Cell Biol. 102, 2211–2222.
[7] Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F. and Quamo, S.N. (1985) J. Biol. Chem. 260, 13304–13315.
[8] Rozengurt, E., Rodriguez-Pena, A., Coombs, M. and Sinnett-Smith, J. (1984) Proc. Natl. Acad. Sci. USA 81, 5748–5752.
[9] Issandou, M. and Rozengurt, E. (1989) Biochem. Biophys. Res. Commun. 163, 201–208.
[10] Isacke, C.M., Meisenheider, J., Brown, K.D., Gould, K.L., Gould, S.J. and Hunter, T. (1986) EMBO J. 5, 2889–2898.
[11] Nånberg, E., Morris, C., Higgins, T., Vara, F. and Rozengurt, E. (1990) J. Cell. Physiol. 143, 232–242.
[12] Erusalimsky, J.D., Friedberg, I. and Rozengurt, E. (1988) J. Biol. Chem. 263, 19188–19194.
[13] Rodriguez-Pena, A. and Rozengurt, E. (1986) J. Cell Physiol. 129, 124–130.
[14] Erusalimsky, J.D. and Rozengurt, E. (1989) J. Cell. Physiol. 141, 253–261.
[15] Issandou, M. and Rozengurt, E. (1990) J. Biol. Chem. (in press).
[16] Staddon, J.M., Chanter, N., Lax, A.J., Higgins, T.E. and Rozengurt, E. (1990) J. Biol. Chem. (in press).
[17] Rodriguez-Pena, A., Zachary, I. and Rozengurt, E. (1986) Biochem. Biophys. Res. Commun. 140, 379–385.
[18] Blackshear, P.J., Wen, L., Glynn, B.P. and Witters, L.A. (1986) J. Biol. Chem. 261, 1459–1469.
[19] Morris, C. and Rozengurt, E. (1988) FEBS Lett. 231, 311–316.

[20] Albert, K.A., Nairn, A.C. and Greengard, P. (1987) Proc. Natl. Acad. Sci. USA 84, 7046–7050.
[21] Patel, J. and Kligman, D. (1987) J. Biol. Chem. 262, 16686–16691.
[22] Albert, K.A., Walaas, S.I., Wang, J.K.T. and Greengard, P. (1986) Proc. Natl. Acad. Sci. USA 83, 2822–2826.
[23] Stumpo, D.J., Graff, J.M., Albert, K.A., Greengard, P. and Blackshear, P.J. (1989) Proc. Natl. Acad. Sci. USA 86, 4012–4016.
[24] Erusalimsky, J.D., Morris, C., Perks, K., Brown, R., Brooks, S. and Rozengurt, E. (1989) FEBS Lett. 255, 149–153.
[25] James, G. and Olsen, E.N. (1989) J. Biol. Chem. 264, 20928–20933.
[26] Aderem, A.A., Albert, K.A., Keum, M.M., Wang, J.K.T., Greengard, P. and Cohn, Z.A. (1988) Nature 332, 362–364.
[27] Sakai, K., Hirai, M., Minoshima, S., Kudoh, J., Fukuyama, R. and Shimizu, N. (1989) Genomics 5, 309–315.
[27] Sakai, K., Hirai, M., Minoshima, S., Kudoh, J., Fukuyama, R. and Shimizu, N. (1989) Genomics 5, 309–315.
[28] Walsh, M.P., Valentine, K.A., Ngai, P.K., Caruthers, C.A. and Hollenburg, M.D. (1984) Biochem. J. 224, 117–127.
[29] Roskoski Jr., R. (1983) Methods Enzymol. 99, 3–6.
[30] Todaro, G.J. and Green, H. (1963) J. Cell Biol. 17, 299–312.
[31] Karns, L.R., Ng, S.-C., Freeman, J.A. and Fishman, M.C. (1987) Science 236, 597–600.
[32] Tyres, M., Rachubinski, R.A., Stewart, M.I., Varrichio, A.M., Shorr, R.G.L., Haslam, R.J. and Harley, C.B. (1988) Nature 333, 470–473.
[33] Graff, J.M., Young, T.N., Johnson, J.D. and Blackshear, P.J. (1989) J. Biol. Chem. 264, 21818–21823.