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Association of annexin V with mitochondria

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Annexin V is an intracellular protein recently shown to be localized to nucleoli and cytosol. In this study we show that cytosolic annexin V is associated with mitochondria. To assess the nature of the annexin V–mitochondrial interaction, an annexin V binding activity was partially purified from placental cytosol by annexin V-affinity chromatography. Five polypeptides in the eluate appeared to be associated with annexin V, with a predominant species of 27 kDa. Antibodies to the 27 kDa polypeptide recognised mitochondria but not nucleoli. We conclude that annexin V interacts with a 27 kDa mitochondrial polypeptide that is possibly part of a larger complex.

Annexin V; Mitochondrion

1. INTRODUCTION

The annexin superfamily consists of a growing number of proteins that can be classified into sub-groups on the basis of structural similarities and the nature of the calcium-dependent phospholipid binding they exhibit [1]. Like most other annexins, annexin V has a fourfold internal repeat of a hydrophobic 70 amino acid domain flanked by short amino and carboxy terminal regions [2]. It is a symmetrical molecule that binds 5 atoms of calcium [3], resembles membrane-channel forming proteins [4], and is a potent anti-phospholipase and anticoagulant. Although extensively studied, its true physiological function is unknown.

The intracellular and apparently ubiquitous occurrence of annexin V [5,6] suggests that it has a crucial role to play at the cellular level. To attempt to elucidate the possible function of annexin V we are investigating its subcellular distribution. In a previous study we showed that it is located in the nucleoli and cytosol of a variety of cell lines, and suggested that this distribution is consistent with a role in ribosomal assembly and transport [6]. In this study we have examined the cytosolic distribution of annexin V in more detail and find that it is associated with mitochondria. Partial purification of interacting proteins by affinity chromatography suggests that annexin V binds to a 27 kDa mitochondrial polypeptide that is probably part of a larger protein complex.

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2. MATERIALS AND METHODS

2.1. *Cells and antibodies*

A549 cells were purchased from the ATCC and cultured as described previously [6]. Anti-annexin V antibody was prepared and affinity purified as before [6]. A hybridoma culture supernatant containing a monoclonal antibody recognising human mitochondria (MAB1273) was purchased from Chemicon International Inc., USA.

2.2. *Preparation of mitochondria*

Approximately 10^7 A549 cells were washed and resuspended in 500 μl 10 mM Tris pH 8.0, 1 mM $MgCl_2$. After 15 min on ice the suspension was subjected to 10–12 strokes of a Dounce homogenizer and added to 500 μl 40 mM Tris pH 8.0, 0.3 M sucrose, 2 mM $MgCl_2$. The homogenate was fractionated by differential centrifugation according to Nigam and Blobel [7]. The post-nuclear supernatant from this procedure was subjected to $10{,}000 \times g$ for 10 min to pellet the mitochondria. The pellet was resuspended in the same buffer, re-centrifuged, and assayed for the mitochondrial marker enzyme, cytochrome c oxidase [8], and a cytosolic enzyme, lactate dehydrogenase [9]. Mitochondria were lysed by the addition of 1% (v/v) Triton X-100.

2.3. *Immunoassays*

Immunoblotting and indirect immunofluorescence were performed as described [6], except that in the latter technique cells were fixed and permeabilized for 3 min in ice-cold acetone/methanol (1:1, v/v). Fluorescein isothioscyanate (FITC) labeled annexin V was prepared and used as previously described [6]. Fluorescence microscopy was performed using an Olympus BH2 microscope. Samples labeled with both FITC and rhodamine isothiocyanate (RITC) were visualized with a supplementary barrier filter in place. This filter reduces the intensity of the FITC signal but prevents the RITC fluorescence overlapping with the FITC fluorescence. Immunoprecipitations (200 μl total volume) were performed by the addition of 10 μg/ml of affinity purified annexin V antibodies to a sample (0.5 μg/ml) of iodinated eluate, in the presence or absence of 1 μg/ml annexin V. Following 4 h at 4°C, 2.5% (w/v) protein A sepharose (Pharmacia) was added and the incubation continued for 2 h. Complexes were collected by centrifugation and washed three times in 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, 0.05% (v/v) Nonidet P40 prior to analysis by SDS-PAGE.

2.4. *Annexin V gel shift assay*

Purified annexin V [6,10] was labeled with Na-^{125}Iodine (NEN Du-

Pont) using Iodogen (Pierce Chemical Co.). The iodinated protein, with or without the addition of other compounds, was then subjected to 7.5% non-denaturing (non-dissociating continuous buffer system) polyacrylamide gel electrophoresis [11] and autoradiography.

2.5. *Partial purification of annexin V binding proteins*

An affinity column consisting of 20 mg of pure annexin V coupled to 10 ml Affigel-15 (Bio-Rad) was prepared according to the manufacturer's instructions. 500 ml of human placental cytosolic extract [12] (5 mg/ml total protein, in a buffer consisting of 10 mM imidazole pH 7.2, 0.25 M sucrose, 5 mM β-mercaptoethanol, 3 mM $MgCl_2$, 1 mM EDTA, 1% (v/v) Triton X-100) was applied to the column. The column was washed extensively with 20 mM Tris pH 7.0, 0.5 M NaCl and protein remaining on the column eluted with 20 mM Tris pH 7.0, 2 M NaCl, 1% (v/v) Triton X-100. Fractions were assayed for annexin V binding activity using the gel shift assay, and those showing peak activity were pooled, subjected to SDS-PAGE, and stained with 0.1% (w/v) Coomassie Blue in 10% (v/v) methanol, 0.5% (v/v) acetic acid. A band corresponding to a 27 kDa polypeptide was excised from the gel, emulsified in marcol/montanide oil (9:1, v/v) and used to immunize rabbits [13].

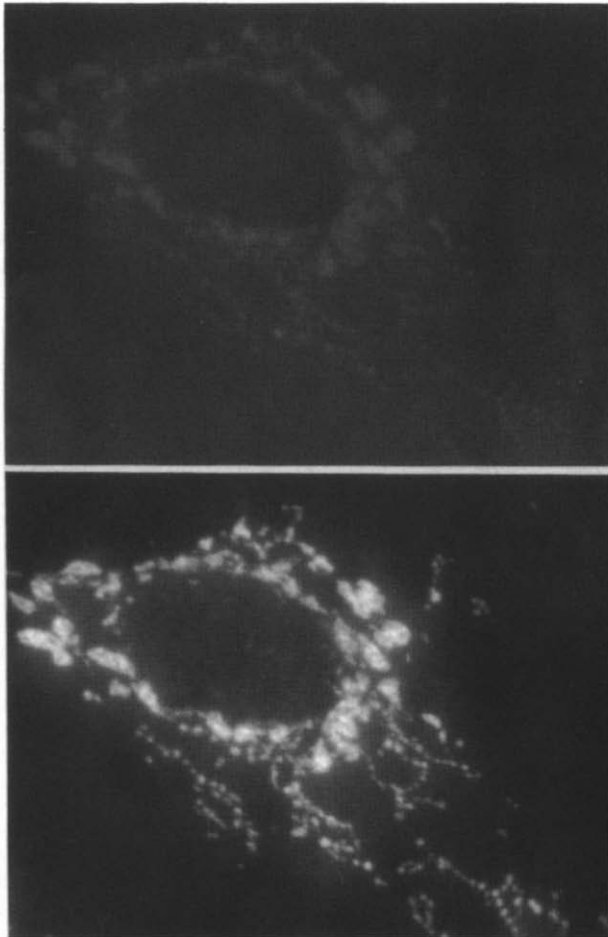

Fig. 1. Association of annexin V with mitochondria. Solvent-fixed and permeabilized A549 cells were simultaneously stained with 1 μg/ml FITC-conjugated annexin V (upper panel) and a 1:200 dilution of an anti-mitochondrial monoclonal antibody (lower panel). The monoclonal was visualized following incubation with a 1:200 dilution of a RITC-conjugated anti-mouse IgG second antibody (Silenus, Australia). Note the nucleolar staining on incubation with annexin V.

3. RESULTS

3.1. *Localization of annexin V to mitochondria by fluorescence microscopy*

We have previously used indirect immunofluorescence to show that annexin V is located in the nucleolus and cytosol of many cell lines. Strong staining was observed in the cytoplasm, which obscured many of the intracellular structures and rendered the nucleolar staining almost invisable [6]. Given the phospholipid binding properties of annexin V, we wondered whether part of this strong cytoplasmic staining represents annexin V bound to phospholipid. To test this we altered our fixing procedure from treatment with formaldehyde (which cross-links phospholipid) to treatment with acetone/methanol (which solubilizes and removes phospholipid). Indirect fluorescent staining of solvent-fixed A549 cells with affinity-purified anti-annexin V antibodies revealed a greatly diminished intensity of cytosolic staining and a clear, intracellular, punctate pattern reminiscent of mitochondria (data not shown).

To confirm that annexin V localizes to mitochondria, we incubated solvent-fixed cells with purified annexin V conjugated to FITC in a double-labeling experiment that also included a monoclonal antibody recognising mitochondria (and subsequently marked with an anti-mouse RITC-conjugated antibody). As shown in Fig. 1, with the exception of the nucleolar staining we have previously reported, the pattern of annexin V staining was clearly coincident with that observed for the monoclonal antibody, suggesting strongly that annexin V also binds to mitochondria. Staining of mitochondria with FITC-labeled annexin V was not calcium dependent, and could be abolished by the addition of excess unlabeled annexin V (data not shown).

3.2. *Demonstration of annexin V and annexin V-binding activity in mitochondrial lysates*

To investigate the association of annexin V with mitochondria, we purified mitochondria from cultured A549 cells [7]. The purity of the mitochondrial preparations was assessed by testing for the presence of cytochrome *c* oxidase (mitochondrial marker) and lactate dehydrogenase (cytosolic marker). The mitochondrial pellet routinely contained 80–100 U cytochrome *c* oxidase/mg protein and 15–20 U lactate dehydrogenase/mg protein. By contrast, the supernatant contained 5–10 U cytochrome *c* oxidase/mg protein and 900–1,000 U lactate dehydrogenase/mg protein.

Purified mitochondria were lysed, the mitochondrial proteins were separated by SDS-PAGE, and then tested by immunoblotting for the presence of annexin V using affinity-purified anti-annexin V antibodies. As shown in Fig.2A, a unique band of 35 kDa was observed on the immunoblot, confirming that annexin V is associated with mitochondria.

To show that mitochondria contain an annexin V

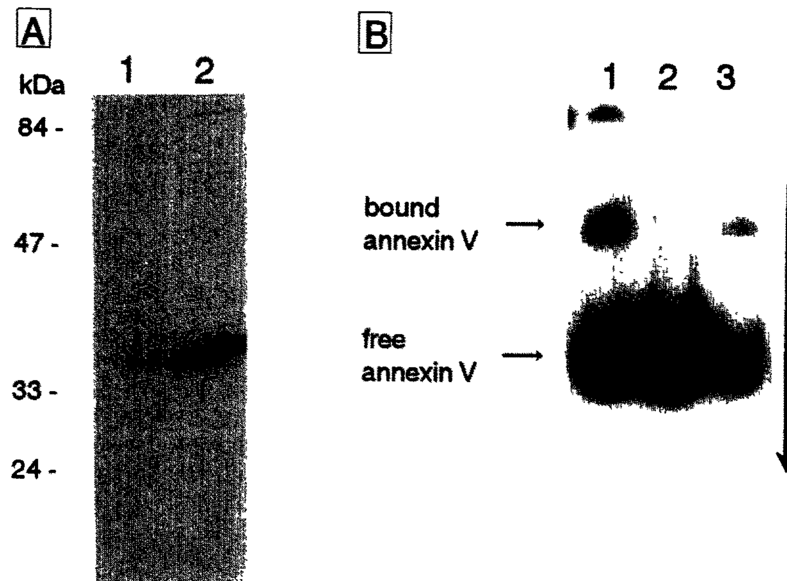

Fig. 2. (A) Demonstration of annexin V in mitochondrial lysates. 5 μg mitochondrial protein (lane 1) or 100 ng purified annexin V (lane 2) were separated on 12% SDS-PAGE gels and transferred to nitrocellulose. The membrane was probed with 2 μg/ml affinity purified anti-annexin V antibodies, and visualized using a horse-radish peroxidase-conjugated second antibody and 4-chloro-1-naphthol development system (Bio-Rad). (B) Annexin binding activity in mitochondria. 3 nM ^{125}I-annexin V was incubated without (lane 2) or with 2 μg mitochondrial protein (lanes 1,3) for 1 h at 37°C, in the presence of 5 mM EDTA (lane 1) or 2.5 mM calcium (lane 3). Samples were subjected to non-dissociating electrophoresis on 7.5% PAGE gels followed by autoradiography. The vertical arrow shows the direction of migration of samples.

binding activity, and to determine whether it is likely to be a phospholipid or protein, mitochondrial lysates were incubated with ^{125}I-labeled annexin V and subjected to non-dissociating (native) gel electrophoresis. In this system, annexin V in the presence of calcium and phospholipid does not enter the gel, whereas free annexin V does (unpublished data). As shown in Fig. 2B, annexin V incubated with mitochondrial protein entered the gel but migrated more slowly than annexin V alone. This alteration in mobility was observed in the absence and presence of calcium, suggesting that it is probably due to a calcium-independent protein-protein interaction, rather than a calcium-dependent annexin V-phospholipid interaction.

3.3. *Partial purification of annexin V binding proteins from human placenta*

To investigate the nature of this annexin V binding activity, it was partially purified from the supernatant (cytosolic fraction) of a 48,000 × *g* centrifugation of post-nuclear placental extracts prepared as described [12]. The cytosol was passed over an annexin V affinity column, the column was washed extensively in the presence of 0.5 M NaCl, then eluted using 2 M NaCl. Fractions were collected and assayed for the ability to alter the mobility of ^{125}I-labeled annexin V in native gels. Fractions showing peak annexin V binding activity were analysed by SDS-PAGE. On Coomassie stained gels, up to five major polypeptide species were observed in these fractions (data not shown).

To determine which of these polypeptides interacted with annexin V, a sample of the peak fraction was iodinated and used in a simple immunoprecipitation experiment. The labeled eluate was mixed with unlabeled annexin V, immunoprecipitated with anti-annexin V antibodies, washed extensively, then analysed by SDS-PAGE and autoradiography. As shown in Fig. 3 (lane

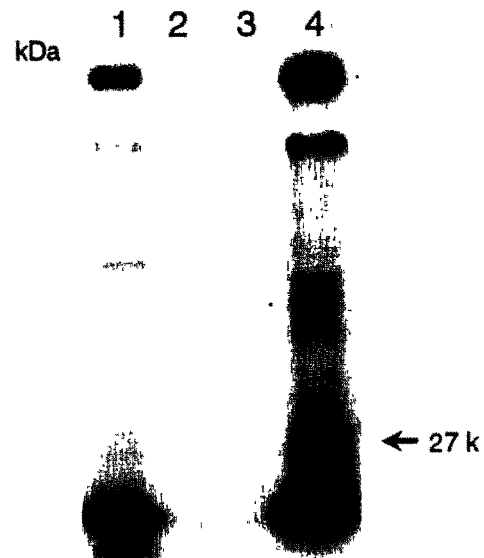

Fig. 3. Immunoprecipitation of annexin V-associating proteins from affinity purification eluate. Iodinated eluate (lane 1) was mixed with unlabeled annexin V and protein A (lane 2), or affinity-purified anti-annexin V antibodies and protein A (lane 3), or unlabeled annexin V, antibodies and protein A (lane 4). Samples were analyzed by 10% SDS-PAGE followed by autoradiography.

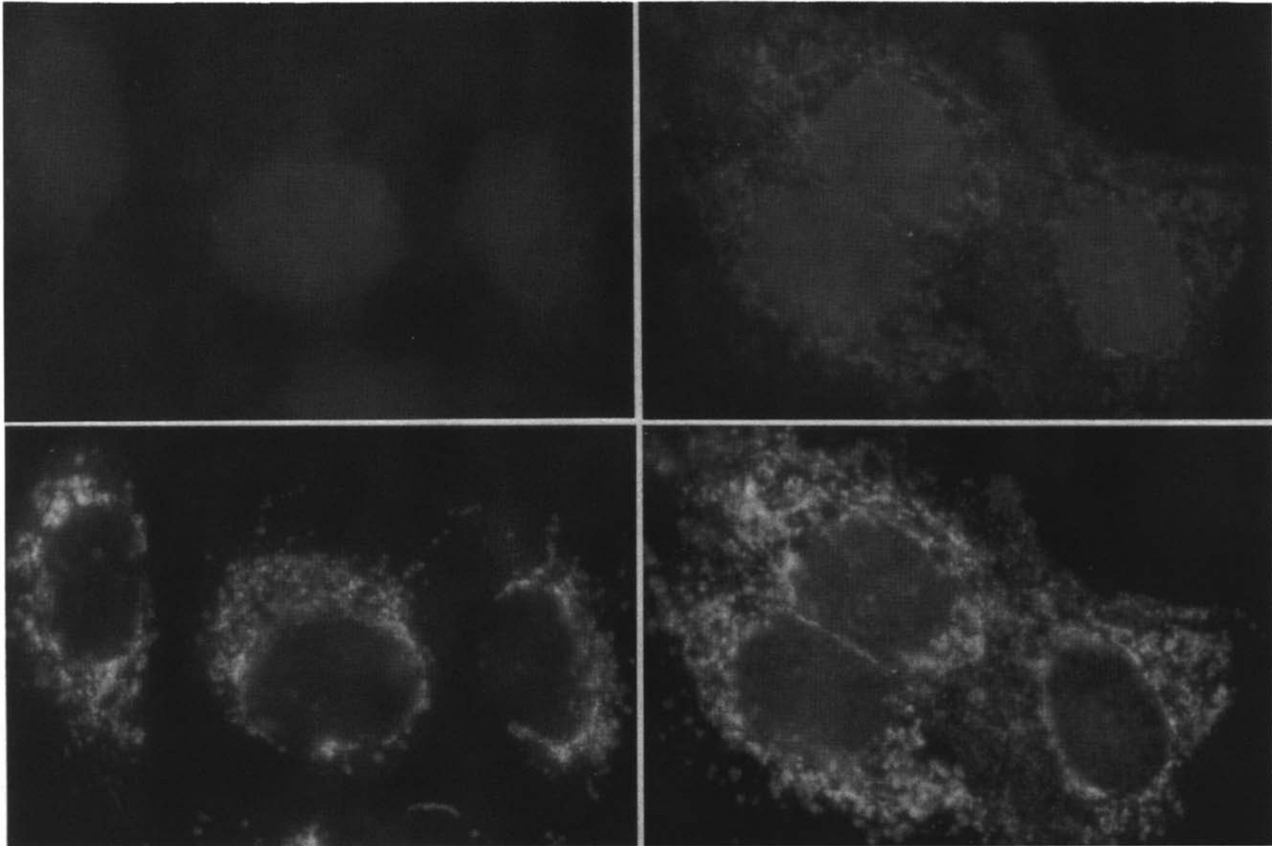

Fig. 4. Intracellular localization of the 27 kDa polypeptide. Fixed and permeabilized A549 cells were incubated with a 1:200 dilution of rabbit pre-immune serum (upper left) or a 1:200 dilution of immune serum raised against the 27 kDa polypeptide (upper right). The same cells were also incubated with a 1:200 dilution of the monoclonal antibody (lower left and right). Rabbit antibodies were marked with a FITC-conjugated second antibody, while the monoclonal was marked with a RITC-conjugated second antibody.

1), besides material that did not enter the gel or ran at the dye front, the predominant band in the labeled eluate prior to immunoprecipitation was at 70 kDa, which did not correspond to any of the major polypeptide species previously observed in the peak fraction. However, eluate samples incubated with unlabeled annexin V and protein A (lane 2) or annexin V antibodies and protein A (lane 3) showed no bands on the gel. By contrast, eluate incubated with unlabeled annexin V, anti-annexin V antibodies and protein A (lane 4) resulted in a reduction in the intensity of the 70 kDa band and the appearance of bands at 62 kDa, a doublet at 55 kDa, 45 kDa, and 27 kDa, with the major species at 27 kDa. In addition there was enhancement of the amount of material that did not enter the gel or migrated at the dye front.

One interpretation of this result is that annexin V binds to a 27 kDa component of a protein complex. This explains the simultaneous appearance of several bands apparently associated with annexin V, the selective enhancement of the 27 kDa band, and the appearance of high MW material (undissociated complex or aggregates) that does not enter the gel.

If the 27 kDa band does bind annexin V, we predicted that it should be associated with mitochondria. To test this prediction we separated the polypeptides in the peak fraction from the affinity purification by SDS-PAGE, excised the 27 kDa band from the gel and used it to immunize a rabbit. The resulting antiserum was then used in indirect immunofluorescence experiments on A549 cells. As shown in Fig. 4, cells incubated with both the anti-27 kDa antiserum and the anti-mitochondrial monoclonal antibody showed a coincident pattern of staining. Neither the 27 kDa antiserum nor the monoclonal antibody stained nucleoli. This is consistent with a mitochondrial localization of the 27 kDa polypeptide, and supports the view that it is associated with annexin V in mitochondria but not in nucleoli.

4. DISCUSSION

Although all annexins share the property of calcium-dependent phospholipid binding, physiological roles have been suggested for only three of the more than 13 members of the family. These are annexin II which participates in calcium-dependent exocytosis [14]; annexin III, which is an inositol 1,2-cyclic phosphate 2-phosphohydrolase [15]; and annexin VI, which is pro-

posed to be involved in budding of clathrin-coated endocytic pits [16].

The role of annexin V is presently unknown, although on the basis of its association of the nucleolus, we have suggested that it may function in either the establishment and maintenance of nucleolar structure, or in ribosome assembly and transport [6]. Our present finding that annexin V is also associated with mitochondria prompts a re-evaluation of this proposal. Because mitochondria and the nucleolus are structurally distinct it is unlikely that annexin V contributes to either organelle structurally. Likewise, since mitochondria encode their own ribosomal RNAs, assemble their own ribosomes, and do not transport them, it is unlikely that annexin V is involved directly in ribosomal assembly or in the transport of completed ribosomes.

However, one obvious similarity remains between the processes of ribosome biosynthesis in both organelles. The proteins that are associated with ribosomes bind to ribosomal RNA during or very soon after transcription and so must be imported from their site of synthesis to the site of ribosome assembly. As most of the mitochondrial ribosomal proteins are encoded by nuclear genes, a common requirement of both mitochondrial and nucleolar ribosome assembly is the import of ribosomal proteins. A model can therefore be envisaged in which annexin V promotes the transport of these proteins to the mitochondria and the nucleolus. This model can be developed further to take into account the known calcium-binding properties of annexin V [3,4], to propose that it binds or promotes binding of ribosomal proteins in the low Ca^{2+} environment of the cytoplasm. On encountering higher Ca^{2+} levels in the mitochondria or nucleolus, annexin V binds extra Ca^{2+} ions, undergoes a conformational change, and promotes the release of the ribosomal proteins.

In this model the polypeptides that are apparently associated with annexin V could be either components of the transport apparatus, or passengers. Purification and identification of these proteins may enable this issue to be addressed. Comparison of these polypeptides with nuclear/nucleolar proteins associated with annexin V may also be illuminating since the isolation and identification of the latter proteins was excluded in the present studies by our use of post-nuclear cytosolic preparations. At present we can suggest that the 27 kDa polypeptide is specific to mitochondria since antibodies recognising it failed to stain nucleoli.

Finally, this model implies the existence of protein transport systems from the cytoplasm to the nucleolus and mitochondria. The role of annexin V in such a system is consistent with the view that many annexins are involved in aspects of protein or vesicular trafficking [14,16]. It remains to be seen whether the structural similarities of distinct annexins translate into related physiological roles.

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REFERENCES

[1] Schlaepfer, D.D., Mehlman, T., Burgess, W.H. and Haigler, H.T. (1987) Proc. Natl. Acad. Sci. USA 88, 6078–6082.
[2] Funakoshi, T., Hendrickson, L.E., McMullen, B.A. and Fujikawa, K. (1987) Biochemistry 26, 8087–8092.
[3] Huber, R., Romisch, J. and Paques, E.-P. (1990) EMBO J. 9, 3867–3874.
[4] Brisson, A., Mosser, G. and Huber, R. (1991) J. Mol. Biol. 220, 199–203.
[5] Bohn, H., Kraus, W. and Winkler, W. (1985) Arch. Gynaekol. 236, 225–233.
[6] Sun, J., Salem, H.H. and Bird, P. (1992) FEBS Lett. 314, 425–429.
[7] Nigam, S.K. and Blobel, G. (1989) J. Biol. Chem. 264, 16927–16932.
[8] Storrie, B. and Madden, E.A. (1990) Methods Enzymol. 182, 203–223.
[9] Gay, R.J., McComb, R.B. and Bowers, G.H. (1968) Clin. Chem. 14, 740.
[10] Funakoshi, T., Heimark, R.L., Hendrickson, L.E., McMullen, B.A. and Fujikawa, K. (1987) Biochemistry 26, 5572–5578.
[11] Hames, B.D. (1981) in: Gel Electrophoresis of Proteins. A Practical Approach (Hames, B.D. and Rickwood, D. Eds.) IRL Press, Oxford.
[12] Laxminarayan, K.M., Matzaris, M., Speed, C.J. and Mitchell, C.A. (1993) J. Biol. Chem. 268, 4968–4974.
[13] Lane, D. and Harlow, E. (1988) Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, New York.
[14] Sarafian, T., Pradel, L.A., Henry, J.P., Aunis, D. and Bader, F. (1991) J. Cell. Biol. 114, 1135–1147.
[15] Ross, T.S., Tait, J.F. and Majerus, P.W. (1990) Science 248, 605–607.
[16] Lin, H.C., Sudhof, T.C. and Anderson, R.G.W. (1992) Cell 70, 283–291.