FEBS Letters 475 (2000) 52–56 FEBS 23751

Shedding and cleavage of the urokinase receptor (uPAR): identification and characterisation of uPAR fragments in vitro and in vivo

Nicolai Sidenius*, Cornelis F.M. Sier, Francesco Blasi

Department of Molecular Pathology and Medicine, Molecular Genetics Unit, DIBIT, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy

Received 9 May 2000

Edited by Veli-Pekka Lehto

Abstract Applying a novel, highly specific and sensitive immunoabsorption/Western blotting technique we have identified in vitro in conditioned cell culture medium and in vivo in human urine different soluble forms of the urokinase-type plasminogen activator receptor (uPAR/CD87). These include the uPAR fragment D2D3 and the never before identified domain 1 (D1) fragment. These forms correspond to fragments previously characterised as biologically active as inducers of chemotaxis and cell adhesion. We find that stimulation of U937 cells is associated with increased uPAR expression, cleavage of surface uPAR, and release of soluble fragments to the culture medium suggesting that monocytes are a source of the circulating and urinary soluble uPAR fragments found in vivo. Our study demonstrates that potentially biologically active uPAR fragments are produced in the human body, indicating a possible function in the regulation of not only proteolysis but also signal transduction related processes.

Key words: Urokinase-type plasminogen activator receptor; Fragment; Cleavage; Shedding; Plasma; Urine

1. Introduction

The interaction between urokinase-type plasminogen activator (uPA) and its receptor (uPAR/CD87) plays a key role in physiological and pathological processes like cell migration, tissue remodelling, cancer invasion and metastasis [1,2]. uPAR is composed of three homologous domains (Fig. 1) and is linked to the cell surface at the carboxy terminus by a glycosylphosphatidylinositol (GPI) anchor [3]. The amino terminal domain of uPAR (D1) contains the main uPA-binding site [4], whereas the next two domains of uPAR (D2D3) bind the extracellular matrix protein vitronectin [5]. However, full-size uPAR is required to reach high affinity in both interactions [6–8]. The region linking D1 and D2D3 in uPAR is prone to cleavage by several proteases including physiologically relevant enzymes such as neutrophil elastase, plasmin, and uPA itself [4,9,10]. Chymotrypsin-cleaved or recombinant soluble uPAR (suPAR) fragments have strong and specific chemotactic activities on several different cell types in vitro [11–14], but the existence and possible biological function of these fragments in vivo has never been addressed. A variant of uPAR lacking domain 1 has been identified on several cultured tumour cell lines, in experimental tumours, and similar fragments are found in the fluid from human ovarian cysts [9,15–17]. This D2D3 form of uPAR is formed by the cleavage of the full-size molecule [18], however the released soluble D1 has never been identified in any cell culture medium or biological fluid.

We have searched for the presence and possible origin of suPAR fragments in vivo. Our data demonstrate that uPAR fragments, including the D2D3 fragment and the never before identified D1 fragment, are released from cells and can be detected in vivo. The data suggest that potentially biologically active suPAR fragments might actually play a role in processes like cell migration and tissue remodelling, not only locally but also at a distance. The data are thus in accordance with the growing body of evidence that the uPA/uPAR interaction has another function in addition to the regulation of extracellular proteolysis.

2. Materials and methods

2.1. Materials

Chymotrypsin, PNGaseF, and immobilised streptavidin were from Roche; 12-*O*-tetradecanoylphorbol-13-acetate (PMA) was from Sigma; cell culture reagents (RPMI, glutamine, penicillin, streptomycin, foetal calf serum (FCS), trypsin) were purchased from Gibco-BRL; anti-uPAR antibodies were kindly provided by Dr. Gunilla Høyer-Hansen (Finsen Laboratory, Copenhagen, Denmark); biotinylated anti-uPA antibody c6 was from Monozyme (Denmark); uPA was a kind gift from Dr. J. Henkin (Abbott Laboratories, Chicago, IL, USA).

2.2. Cell culture

U937 cells were cultured in RPMI medium supplemented with 5% FCS, glutamine (5 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). For shedding experiments cells were seeded at 0.2×10^6/ml in the presence or absence of 100 ng/ml PMA. At the indicated times the conditioned medium was collected, centrifuged for 10 min at 14 000 rpm to remove debris and stored at −80°C. Cells were washed twice with cold phosphate buffered saline (PBS), lysed in PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (Complete™, Roche), kept on ice for 1 h, centrifuged for 10 min at 14 000 rpm, and the supernatants were stored at −80°C until analysis.

2.3. uPAR enzyme-linked immunosorbent assay (ELISA)

The ELISA used to quantify uPAR antigen in biological samples has been described previously [19]. The ELISA uses a polyclonal rabbit anti-human uPAR as catching antibody and a mixture of three monoclonal antibodies (mAb R2, R3 and R5, see Fig. 1) as detecting antibodies. It detects full-size uPAR, D1 and D2D3 fragments, as well as complexes with uPA.

*Corresponding author. Fax: (39)-2-2643-4844.
E-mail: sidenius.nicolai@hsr.it

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GPI, glycosylphosphatidylinositol; PMA, 12-*O*-tetradecanoylphorbol-13-acetate; (pro)uPA, (pro-)urokinase-type plasminogen activator; (s)uPAR, (soluble) uPA receptor

0014-5793/00/$20.00
PII: S0014-5793(00)01624-0

2.4. Immunoprecipitation, deglycosylation and immunoblotting

Samples were diluted to 1 ml using RIPA buffer (0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholate, 0.15 M NaCl and 0.05 M Tris–HCl pH 7.6) containing a cocktail of protease inhibitors and immunoprecipitated for 16 h at 4°C using biotinylated monoclonal antibodies (1 μg), previously immobilised on streptavidin coated beads (5 μl). After washing with RIPA buffer the absorbed material was eluted by boiling in non-reducing sample buffer and the proteins size fractionated in 12% SDS–PAGE under non-reducing conditions. Proteins were transferred to Immobilon-P™ membranes (Millipore) and probed with a rabbit polyclonal anti-uPAR antibody. The immune complexes were visualised by incubation with a peroxidase conjugated donkey anti-rabbit antibody (Amersham) and chemoluminescent detection (SuperSignal West Dura Extended, Pierce). For Western blot analysis of deglycosylated uPAR, the washed beads were added 10 μl PBS containing 0.5% SDS and 2 mM DTT, and incubated at 95°C for 3 min. Proteins were deglycosylated by addition of 20 μl of deglycosylation buffer (PBS containing 0.5% Triton X-100 and 15 mM EDTA), 1 unit of peptide-*N*-glycosidase F (PNGase-F) and incubated at 37°C for 2 h. Control samples were treated in the same manner but did not receive PNGase-F. For Western blotting the samples were analysed by 12% SDS–PAGE gels under reducing conditions. Antibodies were biotinylated using Sulfo-NHS-Biotin (Pierce) according to the manufacturers instructions and using a 100-fold molar excess of biotinylation reagent over antibody.

2.5. Purification and iodination of recombinant suPAR

Recombinant soluble uPAR was purified by affinity chromatography from conditioned medium of Chinese hamster ovary cells transfected with a mutant uPAR cDNA encoding soluble uPAR (amino acid 1–276) lacking the GPI anchor [20]. Purified suPAR was labelled with ^{125}I using the Iodogen procedure (Pierce Chemicals), resulting in a specific activity of 80×10^6 cpm/μg. For the analysis of suPAR degradation in urine, aliquots were supplemented with ^{125}I-suPAR to a concentration of 2 ng/ml and incubated for 24 h at 37°C. Non-reducing sample buffer was added, and the samples boiled and separated by 12% SDS–PAGE. Dried gels were analysed by autoradiography at −80°C using two amplifying screens.

3. Results

3.1. Assay development

To determine the nature of suPAR in biological fluids we developed a sensitive immunoprecipitation/Western blotting assay. The assay is based on the capture of the suPAR antigen(s) in the biological sample with biotinylated monoclonal antibodies recognising epitopes on D1 (mAb R3, Fig. 1) and D3 (mAb R2), followed by analysis of the absorbed material by Western blotting using a polyclonal anti-uPAR antibody. The specificity of the assay was verified using human recombinant suPAR partially digested by chymotrypsin (Fig. 2). Under mild conditions, chymotrypsin cleaves suPAR specifically in the linker region between D1 and D2 [4] generating a mixture of full-size suPAR, a fragment consisting of domains D2 and D3 (D2D3), and the free domain D1. From this mixture the mAb R2, specific for an epitope in domain 3, precipitated two peptides with approximate molecular weight of 55 kDa and 35 kDa, i.e. the size of the full-length suPAR and of the D2D3 fragment. The mAb R3, which recognises an epitope in domain 1, in addition to the 55 kDa peptide also precipitated a 16 kDa peptide, the predicted size of the D1 fragment. Inclusion of both R2 and R3 antibodies in the immunoprecipitation precipitated all three peptides. Another set of monoclonal antibodies having similar recognition specificity (mAb R4 and mAb R5, see Fig. 1) displayed identical patterns, confirming the identity of these fragments (data not shown). Although the D1 and D2D3 fragments are present in equimolar amounts in chymotrypsin digested recombinant suPAR, the overall efficiency of detection of the D1 fragment was several-fold lower than that of the D2D3 fragment. Compared to R2, the R3 antibody was less efficient to immunoprecipitate full-size suPAR, but indispensable for the detection of D1.

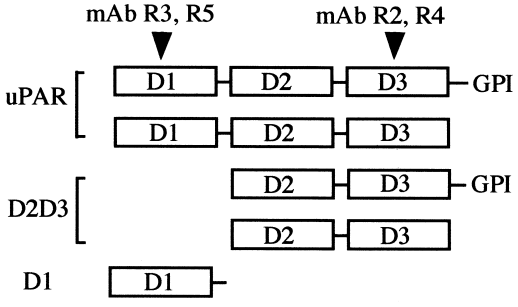

Fig. 1. Diagram illustrating the domain structure of full-size uPAR and its proteolytic fragments. uPAR is composed of three domains (D1, D2, and D3) and is linked to the cell surface by a GPI anchor attached to D3. Proteolytic cleavage in the linker region between D1 and D2 results in release of the D1 fragment, leaving behind the D2D3 fragment on the cell surface. Soluble forms, lacking the GPI anchor, for both uPAR and D2D3. Monoclonal antibodies specific for D1 (mAb R3 and R5) and D3 (mAb R2 and R4) are used in this study to identify the different uPAR fragments.

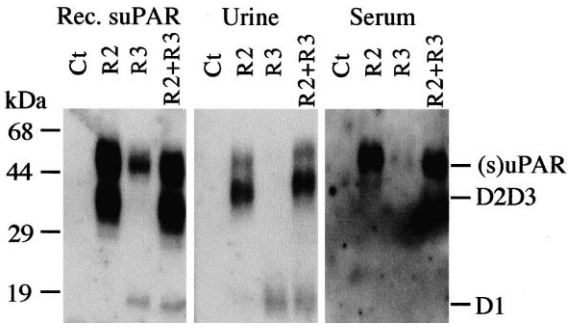

Fig. 2. Immunoblot demonstrating the domain structure of suPAR fragments present in human serum and urine. Serum (0.1 ml) and urine (0.4 ml) from a healthy male, or partially chymotrypsin digested recombinant human suPAR (Rec. suPAR, 1 ng) was immunoprecipitated with mouse monoclonal antibodies R2, R3, R2 plus R3, or with a control mAb (Ct). The precipitated material was separated by 12% SDS–PAGE and analysed by Western blotting using a rabbit polyclonal antibody directed against human uPAR. The migration of suPAR and the suPAR fragments D2D3 and D1 are indicated on the right side of the panel and the position of molecular weight markers on the left side of the panel.

3.2. suPAR and suPAR fragments in urine and blood

The developed assay identified three different uPAR related polypeptides in urine from healthy volunteers (Fig. 2). The antibody specificity identifies the three peptides as full-size suPAR, D2D3 and D1. In serum, only one peptide corresponding to full-size uPAR was observed. The intensity ratio between the D1 and the D2D3 fragments in urine was comparable to that of chymotrypsin digested suPAR suggesting that the amounts of these two fragments present in urine are close to an equimolar ratio. The slight difference in apparent molecular weight between the fragments present in urine and those present in the chymotrypsin digestion of recombinant

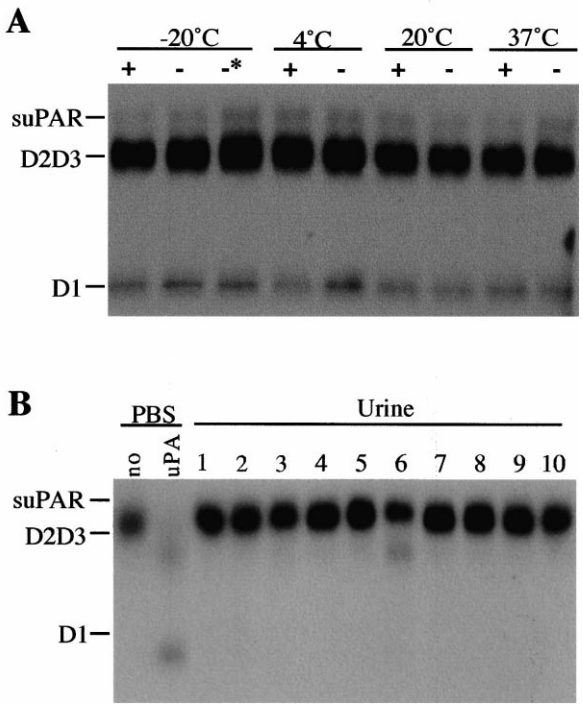

Fig. 3. Stability of suPAR and suPAR fragments in human urine. A: Aliquots (0.2 ml) of freshly obtained urine were incubated for 24 h at the indicated temperatures in the presence (+) or absence (−) of protease inhibitors (1 mM PMSF and 5 mM EDTA). One sample (*) was subjected to five freeze/thaw cycles. The samples were analysed by immunoprecipitation/Western blotting as described in the legend to Fig. 4. B: Urine samples from 10 different individuals were added ^{125}I-suPAR to 2 ng/ml and incubated for 24 h at 37°C after which the samples were analysed by 12% SDS–PAGE and autoradiography. As a control ^{125}I-suPAR was incubated in buffer (PBS) in the absence (no) or presence of 1 μM uPA (uPA).

suPAR is due to the difference in molecular size and glycosylation of the recombinant protein.

3.3. suPAR and suPAR fragments are stable in urine

The abundant presence of suPAR fragments in urine could reflect the generation of the fragments *in* the urine by proteolytic degradation of full-length suPAR. We therefore analysed whether the pattern of suPAR fragments in urine samples was stable when stored for 24 h at different temperatures (−20°C, 4°C, 20°C and 37°C), or when subjected to repeated freeze/thaw cycles in the presence and absence of protease inhibitors (Fig. 3A). The pattern of suPAR fragments was found to be minimally affected by the storage temperature, thawing and by the presence or absence of protease inhibitors. Finally, when radiolabelled recombinant suPAR was added to 10 independent urine samples, minimal degradation after incubation for 24 h at 37°C was observed (Fig. 3B). We conclude that the presence of D2D3 and D1 fragments in the urinary compartment is unlikely to be due to urinary degradation of full-size suPAR.

3.4. U937 cells shed soluble uPAR and uPAR fragments into the growth medium

The finding that suPAR and suPAR fragment are stable in urine suggests that the presence of these fragments in this compartment reflects proteolytic cleavage and shedding of uPAR occurring in distant parts of the organism. This possibility prompted us to investigate whether uPAR fragments are produced and released by cell types known to express uPAR. For this purpose we analysed the cleavage and shedding of uPAR in cultured U937 cells in the presence or absence of the phorbol ester PMA (Fig. 4). Measured by ELISA the amount of cellular uPAR in untreated cells was relatively constant during the experiment, while PMA induced a strong and persistent increase, as expected, visible already between 24 and 72 h (Fig. 4A, top). Throughout culture suPAR antigen accumulated in the medium indicating a continuous shedding of uPAR from the cells to the medium (Fig. 4B, top). The amount of uPAR antigen released from non-treated cells during the first 24 h of culture (0.4 ng/10^6 cells) corresponded approximately to a complete turnover of cellular uPAR in 24 h. The release rate was much higher in PMA stimulated cells (6.1 ng/10^6 cells) corresponding to a turnover rate of cellular uPAR of approximately 3.5 h.

To determine the molecular structure of the uPAR antigen present in the cell lysates and in the conditioned medium we performed immunoprecipitation experiments using a combi-

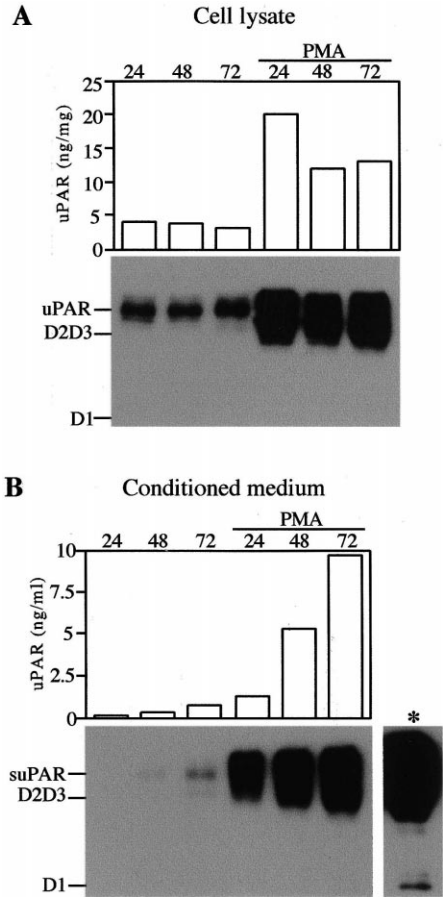

Fig. 4. uPAR antigen in U937 cell lysates (A) and in conditioned media (B). U937 cells were plated in the absence or presence of 100 nM PMA and after 24, 48 and 72 h the cells and conditioned media were harvested. The total level of uPAR antigen in the cell lysates and conditioned medium was measured by ELISA, and the molecular structure determined by immunoprecipitation using a combination of the R2 and R3 antibodies as described in the legend to Fig. 2. The positions of molecular weight markers, full-length uPAR, cleaved uPAR (D2D3) and the released domain 1 (D1) are indicated. The panel marked with an asterisk (*) is a long exposure of the lane containing conditioned medium from U937 cells treated with PMA for 72 h.

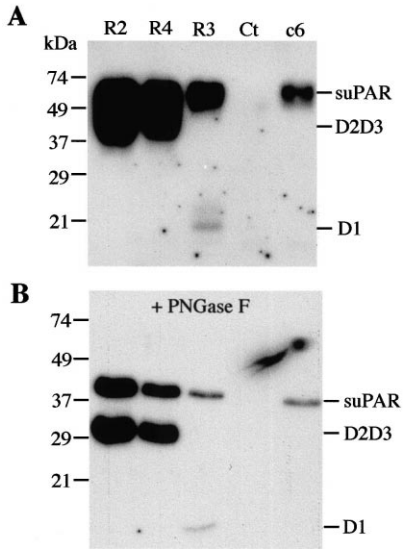

Fig. 5. Molecular structure of uPAR fragments in conditioned medium from U937 cells. Conditioned medium obtained from PMA stimulated U937 cells was either immunoprecipitated with antibodies recognising uPAR domain 3 (R2 and R4), or uPAR domain 1 (R3), or an anti-uPA antibody (c6), or an irrelevant antibody (Ct). After washing, the precipitates were denatured, reduced and treated for 2 h without (A) of with PNGase-F (B) and analysed by Western blotting.

nation of R2 and R3 monoclonal antibodies (Fig. 4A and B, bottom). In non-stimulated cells a prominent band with a molecular weight around 50–55 kDa representing full-length uPAR could be observed both in cell lysates and in conditioned media. Stimulation with PMA resulted in a strong increase in the intensity of this band and the appearance of shorter polypeptides. The intense band with a molecular weight around 35 kDa suggests a cleaved receptor composed of domains 2 and 3 (D2D3), and could be observed in both the cell lysates and in the conditioned media. Long exposure of the blots revealed the presence of a fast migrating band in conditioned medium from PMA stimulated cells with a molecular weight around 15 kDa possibly representing the domain 1 fragment (D1). Despite long exposures, the D1 band was never observed in cell lysates (not shown) in accordance with the model that D1 is released from the cell surface upon receptor cleavage.

To unequivocally identify the different uPAR polypeptides present in the U937 conditioned media we selectively immunoprecipitated conditioned medium from PMA stimulated cells with the above characterised panel of different monoclonal antibodies (Fig. 5). Antibodies recognising domain 3 (R2 and R4) precipitated the two larger fragments in accordance with the identity of these bands being full-length suPAR and D2D3. The antibody against D1 (R3) precipitated full-length uPAR and the small 15 kDa fragment but not the D2D3 band, confirming the identity of these peptides to be full-length uPAR and the D1 fragment. Full-length uPAR could furthermore be precipitated with a monoclonal antibody against uPA (mAb c6) demonstrating that the receptor in the conditioned medium, at least partially, is associated with uPA. A control antibody did not precipitate any of these proteins. The highly heterogeneous glycosylation of uPAR causes a 'smeary' appearance of uPAR and uPAR fragments in Western blotting preventing a good separation of full-length uPAR and the D2D3 fragment. To eliminate this problem we performed Western blotting on immunoprecipitates that had been reduced, denatured, and deglycosylated. Indeed, the migration pattern of uPAR, D2D3 and D1 after deglycosylation was in complete accordance with the molecular weights based on the cDNA derived amino acid sequence of uPAR and a cleavage in the region between domains 1 and 2. Also, the separation of full-length uPAR and D2D3 was excellent under these conditions allowing an accurate identification of the different uPAR fragments.

4. Discussion

uPA and uPAR are known to be involved in proteolytic processes during wound healing and cell migration by activating plasminogen activation. Recently however, a number of studies have indicated that there might be additional important functions for this system, with uPAR as the key component [2,21].

uPAR is able to interact with several soluble and membrane proteins other than uPA, e.g. vitronectin, integrins, and thrombospondin. Even though major binding sites for one or more of these ligands reside in one of the three domains of uPAR, the full-size structure appears to be essential for high affinity [7,8,22]. Thus, the finding that uPAR is cleaved under physiological conditions implies interesting consequences for the regulation of extracellular proteolysis and cell adhesion. First, the abolished binding of uPA is expected to result in a reduced potential of the cell to promote plasminogen activation [9]. Second, abolished vitronectin binding reduces uPAR dependent cell adhesion to this substrate [8,23]. Cleavage of uPAR however, will not only bear negative influence on its function. The part of uPAR that has been found to be susceptible to cleavage is the linker region between domains 1 and 2 [4,9,10]. The chemotactic properties transduced by the binding of uPA to uPAR are in fact mediated by an epitope within this linker region of uPAR, which is uncovered by uPA binding or by cleavage [11,13]. Although soluble D1 or D2D3 have not previously been found in conditioned media, or in blood, our data demonstrates for the first time that these fragments are indeed released from cells and can be found in vivo.

The question why in serum the domains D1 and D2D3 were not found, still remains to be answered. Their half-life in the circulation may be too short, possibly due to the reduced molecular size or by impaired ability to bind stabilising molecules like for instance uPA and/or vitronectin. Indeed, the possibility that full-length suPAR is associated with other molecules in serum and urine is strongly supported by our data. The R3 antibody is known not to bind suPAR associated with uPA or vitronectin [7,24] and fails to efficiently precipitate full-length suPAR from urine and serum (Fig. 2).

Our data suggest that the type of uPAR fragments present in urine might reflect the cleavage state of uPAR in tissues distant from the urinary compartment. It has been suggested that uPAR cleavage occurs predominantly in areas of tissue remodelling where both uPAR and uPA are expressed at high levels [18]. The detection and molecular characterisation of these fragments in urine and blood might therefore reflect remodelling phenomena in physiological or pathological processes in which the uPA/uPAR system is involved, including cancer.

Acknowledgements: This study was supported by the Biomed-2 program of the European Union and the Italian Ministry of University and Technological Research (MURST). N.S. and C.F.M.S. were fellows of the Danish Research Academy and the Italian Association for Cancer Research (AIRC), respectively.

References

[1] Andreasen, P.A., Kjøller, L., Christensen, L. and Duffy, M.J. (1997) Int. J. Cancer 72, 1–22.
[2] Blasi, F. (1997) Immunol. Today 18, 415–417.
[3] Ploug, M., Rønne, E., Behrendt, N., Jensen, A.L., Blasi, F. and Danø, K. (1991) J. Biol. Chem. 266, 1926–1933.
[4] Behrendt, N., Ploug, M., Patthy, L., Houen, G., Blasi, F. and Danø, K. (1991) J. Biol. Chem. 266, 7842–7847.
[5] Wei, Y., Waltz, D.A., Rao, N., Drummond, R.J., Rosenberg, S. and Chapman, H.A. (1994) J. Biol. Chem. 269, 32380–32388.
[6] Ploug, M., Østergaard, S., Hansen, L.B., Holm, A. and Danø, K. (1998) Biochemistry 37, 3612–3622.
[7] Høyer-Hansen, G., Behrendt, N., Ploug, M., Danø, K. and Preissner, K.T. (1997) FEBS Lett. 420, 79–85.
[8] Sidenius, N. and Blasi, F. (2000) FEBS Lett. 470, 40–46.
[9] Høyer-Hansen, G., Rønne, E., Solberg, H., Behrendt, N., Ploug, M., Lund, L.R., Ellis, V. and Danø, K. (1992) J. Biol. Chem. 267, 18224–18229.
[10] Plesner, T., Behrendt, N. and Ploug, M. (1997) Stem. Cells 15, 398–408.
[11] Resnati, M., Guttinger, M., Valcamonica, S., Sidenius, N., Blasi, F. and Fazioli, F. (1996) EMBO J. 15, 1572–1582.
[12] Wei, Y., Lukashev, M., Simon, D.I., Bodary, S.C., Rosenberg, S., Doyle, M.V. and Chapman, H.A. (1996) Science 273, 1551–1555.
[13] Fazioli, F., Resnati, M., Sidenius, N., Higashimoto, Y., Appella, E. and Blasi, F. (1997) EMBO J. 16, 7279–7286.
[14] Degryse, B., Resnati, M., Rabbani, S.A., Villa, A., Fazioli, F. and Blasi, F. (1999) Blood 94, 1–15.
[15] Solberg, H., Rømer, J., Brünner, N., Holm, A., Sidenius, N., Danø, K. and Høyer-Hansen, G. (1994) Int. J. Cancer 58, 877–881.
[16] Ragno, P., Montuori, N., Covelli, B., Høyer-Hansen, G. and Rossi, G. (1998) Cancer Res. 58, 1315–1319.
[17] Wahlberg, K., Høyer-Hansen, G. and Casslèn, B. (1998) Cancer Res. 58, 3294–3298.
[18] Høyer-Hansen, G., Ploug, M., Behrendt, N., Rønne, E. and Danø, K. (1997) Eur. J. Biochem. 243, 21–26.
[19] Stephens, R.W. et al. (1997) Clin. Chem. 43, 1868–1876.
[20] Masucci, M.T., Pedersen, N. and Blasi, F. (1991) J. Biol. Chem. 266, 8655–8658.
[21] Chapman, H.A. (1997) Curr. Opin. Cell Biol. 9, 714–724.
[22] Ploug, M., Ellis, V. and Danø, K. (1994) Biochemistry 33, 8991–8997.
[23] Waltz, D.A. and Chapman, H.A. (1994) J. Biol. Chem. 269, 14746–14750.
[24] List, K., Høyer Hansen, G., Rønne, E., Danø, K. and Behrendt, N. (1999) J. Immunol. Methods 222, 125–133.