FEBS 18129

FEBS Letters 402 (1997) 181–184

Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin

A possible cell surface activator

Yuushi Okumura[a], Hiroshi Sato[b], Motoharu Seiki[b], Hiroshi Kido[a,*]

[a]*Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan*
[b]*Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920, Japan*

Received 29 October 1996; revised version received 17 December 1996

Abstract Membrane type 1 matrix metalloproteinase (MT1-MMP) was suggested to play a critical role in the regulation of tissue invasion by normal and neoplastic cells by directly mediating the activation of pro-gelatinase A. Recently, the proteolytic activation of a pro-MT1-MMP by an intracellular proprotein convertase, furin, was reported. In this study, we found that plasmin efficiently activates the pro-MT1-MMP by cleaving immediately downstream of Arg^{108} and Arg^{111} in the multi-basic motif between its pro- and catalytic domains that participates in the activation of pro-gelatinase A. Our present data suggest that pro-MT1-MMP transported to the plasma membrane is activated by plasmin extracellularly and thus it may play an important role in the matrix degradation process.

Key words: Membrane type 1 matrix metalloproteinase; Plasmin; Zymogen convertase; Pro-gelatinase A

1. Introduction

Membrane type 1 matrix metalloproteinase (MT1-MMP) is believed to play a critical role in tumor cell invasion and degradation of the extracellular matrix by directly mediating the activation of pro-gelatinase A [1,2]. Like all other MMPs, MT-MMP is synthesized as an inactive precursor, and the zymogen is activated on proteolytic cleavage at a site in the multi-basic motif (RRK/RR) between its pro- and catalytic domains [3,4]. It was recently reported that furin, a proprotein convertase present in the Golgi apparatus, activates a transmembrane deletion mutant of pro-MT1-MMP [5] and GST-pro-MT1-MMP [6], resembling the action observed previously for stromelysin-3 [7]. In addition to the intracellular proteolytic activation of pro-MT1-MMP by furin, a number of lines of evidence suggest that MT1-MMP is transported to the plasma membrane in a latent precursor form. The MT1-MMP expressed in transfected cells, human fibroblasts and HT-1080 human fibrosarcoma cells is a 63 kDa latent precursor enzyme with a propeptide domain [1,8], or a partially processed 60 kDa latent protease [9], respectively. Based on these data, we proposed a mechanism of pro-MT1-MMP activation on the cell surface, besides the intracellular mechanism of activation mediated by furin, and searched for a potential extracellular protease activator of pro-MT1-MMP. Among extracellular trypsin-like proteases, only plasmin was found to activate pro-MT1-MMP. N-Terminal amino acid sequence analyses of the products showed that plasmin has two processing sites, i.E. the R^{111}-Y^{112}, and R^{108}-R^{109} bonds in the multi-basic cleavage motif of the propeptide. In gelatin zymography experiments, pro-gelatinase A was activated by plasmin-activated MT1-MMP, and this activation was inhibited by the plasmin inhibitor, aprotinin, and the substrate analogue, dec-RVKR-cmk. Based on these results, it appears that pro-MT1-MMP is activated not only by furin in the Golgi apparatus but also by plasmin on the cell surface.

2. Materials and methods

2.1. Materials

Human plasmin was purchased from Boehringer Mannheim GmbH (Germany). Urokinase and protease inhibitors, aprotinin and α1-antitrypsin, were from Sigma (Tokyo, Japan). Leupeptin, Boc-LKR-MCA, Boc-RVRR-MCA and Pyr-RTKR-MCA were from the Peptide Institute (Osaka, Japan). Inhibitors of furin, dec-RVKR-cmk and its derivative, dec-FAKR-cmk, were gifts from Dr. H.D. Klenk, University of Marburg. Tryptases from human lung were purified to homogeneity by the method described by Smith et al. [10]. Thrombin and factor Xa from bovine plasma were purified by the methods described by Hashimoto et al. [11]. A recombinant GST-pro-MT1-MMP fusion protein containing the pro-, catalytic and hinge domains of MT1-MMP was obtained by the method described by Kinoshita et al. [12]. Pro-gelatinase A was obtained by the method described by Sato and Seiki [13].

2.2. Cleavage of the GST-pro-MT1-MMP fusion protein

Cleavage of pro-MT1-MMP by various extracellular proteases was analyzed with the GST-pro-MT1-MMP fusion protein as a substrate without removing the GST domain, for elimination of protease contamination of the assay system, and for elimination of denaturation of pro-MT1-MMP in the process of purification. The GST-pro-MT1-MMP fusion protein (0.8 μg) was incubated with various proteases (2.7 pmol), and then incubated further for 15–60 min at 37°C in 20 μl of a reaction buffer (plasmin and urokinase, 0.1 M Tris-HCl buffer, pH 7.0; human lung tryptase, 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 2.5 μg/ml heparin; thrombin, 50 mM Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 20 mM EDTA; and factor Xa, 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$). The reaction was terminated by cooling in ice and the addition of 3×SDS-PAGE sample loading buffer [14], followed by analysis by SDS-PAGE under reducing conditions.

2.3. Enzyme and inhibitor assays

The amidolytic activities of proteases toward various synthetic peptides were analyzed as follows: 25 μl of a test sample or diluted

*Corresponding author.

Abbreviations: pro-MT1-MMP, precursor of membrane type 1 matrix metalloproteinase; dec, decanoyl; cmk, chloromethyl ketone; mAb, monoclonal antibody; Boc, *N*-tert-butyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide; Pyr, L-pyroglutamyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; pro-gelatinase A, precursor of gelatinase A; uPA, urokinase-type plasminogen activator; PBS, phosphate-buffered saline

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(96)01523-2

sample was mixed with 2.5 μl (20 mM) of a substrate in 472.5 μl of the reaction buffer in a quartz cuvette controlled thermostatically at 37°C. The reaction was started by the addition of the substrate, and then the amount of 7-amino-4-methylcoumarin liberated from the substrate was determined fluorimetrically, with excitation and emission wavelengths of 380 and 460 nm, respectively, using a Hitachi fluorescence spectrophotometer, 650-10MS model. One unit of enzyme activity was defined as the amount degrading 1 μmol of substrate per min. The K_m values were determined from the hydrolysis rates at four separate substrate concentrations by Lineweaver-Burk plotting. To determine the effects of inhibitors, enzyme preparations were preincubated for 5 min with various inhibitors at 37°C and then the residual activity was measured. The inhibition constant (K_i) was determined from a Dixon plot.

2.4. Electrophoresis and Western immunoblotting

SDS-PAGE (10–20% gradient gel; Daiichi Pure Chemicals, Tokyo) was performed by the method of Laemmli [14] at room temperature. The gel was stained with silver. SDS-PAGE low range standards (APRO Science, Inc., Japan) were used as molecular weight markers (phosphorylase b (97.2 kDa), BSA (66.4 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa)). For Western blot analysis, the reaction samples were subjected to SDS-PAGE, and then transferred electrophoretically to an Immobilon transfer membrane (Millipore): excess sites were blocked with 3.5% skim milk in PBS. Each membrane was then probed with a mAb against MT1-MMP (114-2F2) [1] overnight at 4°C. The final concentration of the mAb was 1 μg/ml in 3.5% skim milk in PBS. After extensive washing with TBS (20 mM Tris-HCl buffer, pH 7.5/0.5M NaCl), the membrane was reprobed with anti-mouse IgG conjugated with horseradish peroxidase (Amersham, Amersham, UK), 1:1000, in 3.5% skim milk in PBS for 1 h at room temperature. The bound antibodies were detected with ECL Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

2.5. Amino acid sequences

The N-terminal amino acid sequences of the proteolytic products were determined by the microsequencing method with an Applied Biosystems 492 model gas-phase sequencer/140C HPLC system after the reaction samples had been electrophoretically transferred to a ProBrot membrane (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

2.6. Activation of pro-gelatinase A and inhibition of its activation

Pro-gelatinase A (0.7 μg) was incubated with GST-pro-MT1-MMP (0.8 μg) and plasmin (2.7 pmol) in a reaction buffer (50 mM Tris-HCl buffer, pH 7.5/0.15 M NaCl/50 mM $CaCl_2$/0.25 μM $ZnCl_2$) in the presence or absence of a protease inhibitor (10 μM), for 1 h at 37°C, and then analyzed by gelatin zymography.

2.7. Gelatin zymography

Zymography was performed as described [15]. Samples were mixed with the SDS-PAGE sample buffer in the absence of a reducing agent and then incubated for 20 min at room temperature to denature the MMPs. Electrophoresis was performed on 12.5% polyacrylamide gels containing 0.1% SDS and gelatin at a final concentration of 0.1% (w/v). Thereafter, the gels were washed in 2.5% Triton X-100 for 2 h at room temperature to remove the SDS. The gels were then incubated for 24 h at 37°C in a reaction buffer (50 mM Tris-HCl buffer, pH 7.6/0.15 M NaCl/10 mM $CaCl_2$), and then stained with 0.1% Coomassie brilliant blue R-250. The location of gelatinolytic activity was detected as a clear band in the background of uniform staining.

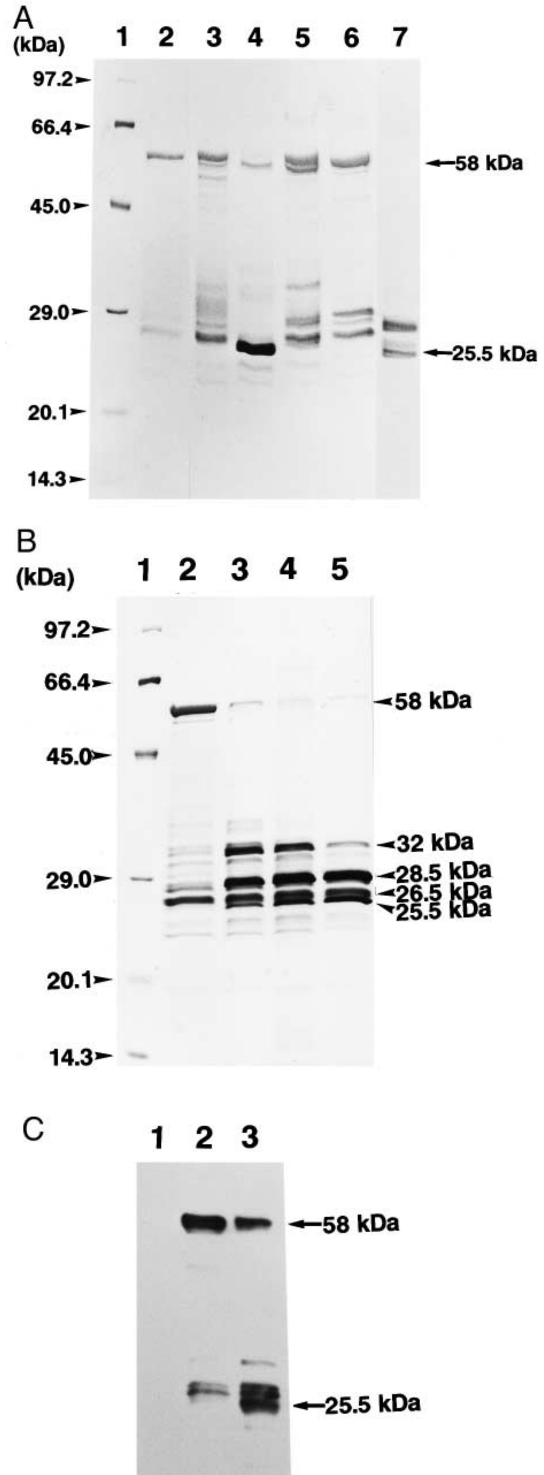

Fig. 1. A: Cleavage of GST-pro-MT1-MMP by various trypsin-type proteases. GST-pro-MT1-MMP (0.8 μg) (lane 2) was incubated for 60 min with various extracellular proteases (2.7 pmol), such as human lung tryptase (lane 3), thrombin (lane 4), factor Xa (lane 5), urokinase (lane 6), and plasmin (lane 7), and the reaction products were separated by SDS-PAGE. Molecular weight markers (lane 1). B: Time course of GST-pro-MT1-MMP cleavage by human plasmin. GST-pro-MT1-MMP (0.8 μg) was incubated with plasmin (2.7 pmol) for 0 (lane 2), 15 (lane 3), 30 (lane 4), and 60 min (lane 5), followed by SDS-PAGE. Molecular weight markers (lane 1). C: Western immunoblotting analysis. The proteolytic products in B were also analyzed by Western immunoblotting with a mAb against the catalytic domain of MT-MMP-1. GST-pro-MT-MMP-1 (lane 2) was incubated with plasmin for 15 min (lane 3). GST as a control (lane 1).

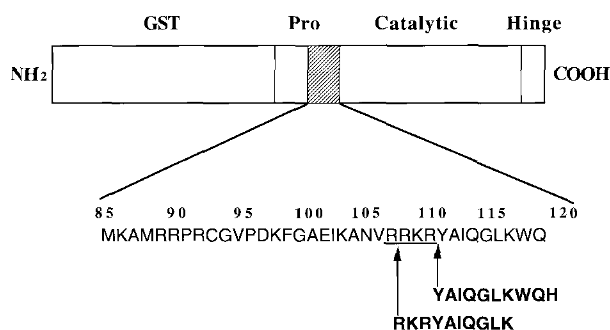

Fig. 2. Identification of the plasmin cleavage sites in GST-pro-MT1-MMP during activation. The N-terminal sequence of the 25.5 kDa fragment in Fig. 1 generated by plasmin was analyzed as described in Section 2, and is indicated by bold letters. The aligned amino acid sequence is the sequence of MT1-MMP deduced on cDNA sequencing [1,12]. The multi-basic cleavage motif of pro-MT1-MMP is underlined.

3. Results and discussion

3.1. Proteolytic activation of the GST-pro-MT1-MMP fusion protein

We examined the effects of various extracellular trypsin-type proteases on proteolytic activation of the GST-pro-MT1-MMP fusion protein, which comprises GST, and the pro-, catalytic and hinge domains of MT1-MMP, and retains the latency of MT-MMP (Fig. 1A). Among the proteases tested, plasmin (lane 7) specifically converted the 58 kDa GST-pro-MT1-MMP fusion protein to a 25.5 kDa fragment, which roughly corresponds to the molecular mass of the catalytic domain of MT1-MMP. On the other hand, thrombin cleaved GST-pro-MT1-MMP at the thrombin cleavage site at the junction between GST and the pro-domain of MT1-MMP, producing a 26.5 kDa fragment (lane 4). The N-terminal amino acid sequence of the 26.5 kDa fragment produced by thrombin supported this interpretation (data not shown). However, other extracellular or secretory proteases did not liberate a 25.5 kDa fragment, though some of them produced fragments with molecular masses higher than 27 kDa. Then, we examined the time-course of GST-pro-MT1-MMP cleavage by plasmin, the products being analyzed by SDS-PAGE (Fig. 1B) and by Western immunoblotting with 114-1F2 mAb [1] against the catalytic domain of MT1-MMP (Fig. 1C). As was expected, the 58-kDa GST-pro-MT1-MMP band material was degraded in a time-dependent manner with the successive formation of 32, 28.5 and 25.5 kDa fragments. Degradation of the intermediate 32 kDa fragment was also observed on incubation for 15 min. Of these fragments, the 25.5 kDa one, but not the 32 and 28.5 kDa one, was stained with the mAb (Fig. 1C), suggesting that the 25.5 kDa fragment contains the catalytic domain of MT1-MMP. The N-terminal amino acid sequences of the 32, 28.5 and 26.5 kDa fragments revealed the N-terminal sequence of GST (data not shown), indicating that plasmin cleaved not only pro-MT1-MMP but also at sites in the GST domain. The contaminating protein with a molecular mass of about 26.5 kDa in the original sample of GST-pro-MT1-MMP (Fig. 1A,B, lane 2; and C, lanes 2 and 3), which had the N-terminal sequence of GST and was slightly cross-reactive with the mAb, was produced during the process of purification of the fusion protein by glutathione-sepharose affinity chromatography. The protein by itself, however, exhibited no ability of pro-gelatinase A activation, as suggested below in Fig. 3, lane 3. Taken together, the results indicate that the 25.5 kDa fragment is the only major one of MT1-MMP, the others being N-terminal side fragments of GST.

3.2. Cleavage site determination

To determine the site of cleavage of GST-pro-MT1-MMP by plasmin, the N-terminal amino acid sequence of the 25.5 kDa fragment was analyzed (Fig. 2). Sequence analysis of the 25.5 kDa fragment revealed two sequences, Y(1.2)-A(1.2)-I(2.2)-Q(1.7)-G(2.1)-L(1.4)-K(1.1)-W(1.0)-Q(1.3)-H(0.6), and R(1.4)-K(1.4)-R(1.2)-Y(1.3)-A(1.4)-I(2.5)-Q(1.8)-G(2.0)-L(1.1)-K(1.2) (the values in parentheses are the yields in pmol). This indicates that the R^{108}-R^{109} and R^{111}-Y^{112} bonds of the multi-basic cleavage motif between the pro- and catalytic domains of MT1-MMP are cleaved with almost the same efficiency during the activation by plasmin. In the case of furin, the R^{111}-Y^{112} bond is specifically processed [5,6], and the same site of cleavage by pancreatic trypsin of the proform of the catalytic domain of MT1-MMP was previously reported [2]. Analysis of the substrate specificity of plasmin revealed that it exhibits similar K_m values for a synthetic plasmin substrate (Boc-LKR-MCA) ($K_m = 4.2 \times 10^{-4}$ M) and a synthetic furin substrate (Boc-RVRR-MCA) ($K_m = 4.8 \times 10^{-4}$ M). Taken together, these results suggest that the consensus cleavage motif, RXK/RR, of pro-MT1-, 2-, 3- and 4-MMP, and stromelysin-3 zymogen is recognized by plasmin as well as furin.

3.3. Activation of pro-gelatinase A by plasmin-activated MT1-MMP and its inhibition by plasmin inhibitors

In order to confirm the proteolytic activity of MT1-MMP produced from the zymogen by plasmin, we analyzed the activation of pro-gelatinase A. Current evidence indicates that MT1-MMP initiates 68-kDa pro-gelatinase A activation via a two-step process comprising initial cleavage of the N^{37}-L^{38} bond, followed by autocatalytic conversion of the 64 kDa L^{38} intermediate to a 62 kDa active enzyme with an N-terminal Y^{81} residue [16,17]. As shown in Fig. 3, neither plasmin (lane 2) nor thrombin (lane 3) activated pro-gelatinase A directly, and only pro-MT1-MMP incubated with plasmin (lane

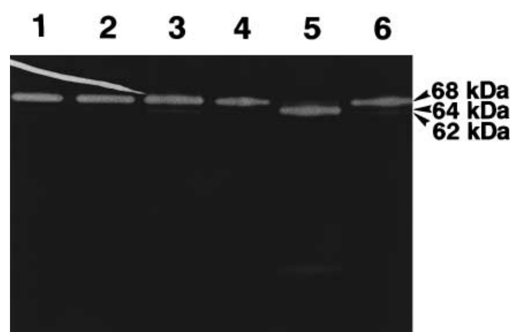

Fig. 3. Cleavage of pro-gelatinase A by plasmin-activated pro-MT1-MMP. Gelatin zymography of pro-gelatinase A (0.7 μg) (lane 1), pro-gelatinase A treated with various proteases (2.7 pmol), such as plasmin (lane 2) and thrombin (lane 3), GST-pro-MT1-MMP (0.8 μg) (lane 4), GST-pro-MT1-MMP treated with plasmin (lane 5), and GST-pro-MT1-MMP treated with thrombin (lane 6) was performed. The experimental conditions for gelatin zymography are given in Section 2.

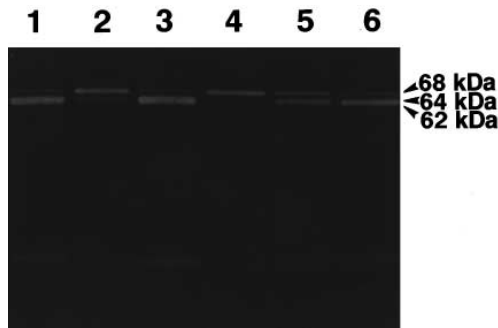

Fig. 4. Inhibition of pro-gelatinase A activation by various protease inhibitors. Pro-gelatinase A (0.7 μg) was incubated with GST-pro-MT1-MMP (0.8 μg) and plasmin (2.7 pmol) in the absence (lane 1) or presence of various inhibitors (10 μM), such as dec-RVKR-cmk (lane 2), dec-FAKR-cmk (lane 3), aprotinin (lane 4), leupeptin (lane 5), and α_1-antitrypsin (lane 6), followed by gelatin zymography.

5), i.e. not that incubated with thrombin (lane 6), induced pro-gelatinase A activation. Moreover, this pro-gelatinase A activation was markedly inhibited by a plasmin inhibitor, aprotinin, and a substrate analogue and furin inhibitor, dec-RVKR-cmk. But dec-FAKR-cmk and other trypsin inhibitors had little effect on the plasmin-mediated pro-gelatinase A activation (Fig. 4). The K_i values of aprotinin and dec-RVKR-cmk as to plasmin were 5.3×10^{-9} M and 1.3×10^{-6} M, respectively.

These data suggest that plasmin plays an important role in the activation of pro-MT-MMPs on the cell surface as furin does intracellularly. The cell surface plasmin formation is controlled by uPA. In most malignant tumors, increases in the expression of the uPA and uPA dependent pathways of malignant tumor dissemination are supported by a large amount of evidence [18–23]. Inhibition of uPA by serine protease inhibitors and by antibodies blocking the activity results in a drastic reduction of tumor metastasis [19,20,22,23]. The increase in the expression of cell surface-bound uPA in malignant tumors greatly facilitates plasmin formation, which in turn, activates pro-MT-MMPs following activation of pro-gelatinase A in the plasma membrane. In view of the invasive and metastatic properties of malignant tumors, cell surface activation of pro-MT-MMPs by plasmin may be of specific advantage to malignant cells.

References

[1] Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. (1994) Nature 370, 61–65.
[2] Will, H., Atkinson, S.J., Butler, G.S., Smith, B. and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123.
[3] Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B. L., Grants, G.A. and Goldberg, G.I. (1995) J. Biol. Chem. 270, 5331–5338.
[4] Imai, K., Ohuchi, E., Aoki, T., Nomura, H., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1996) Cancer Res. 56, 2707–2710.
[5] Pei, D. and Weiss, S.J. (1996) J. Biol. Chem. 271, 9135–9140.
[6] Sato, H., Kinoshita, T., Takino, T., Nakayama, K. and Seiki, M. (1996) FEBS Lett. 393, 101–104.
[7] Pei, D. and Weiss, S.J. (1995) Nature 375, 244–247.
[8] Cao, J., Sato, H., Takino, T. and Seiki, M. (1995) J. Biol. Chem. 270, 801–805.
[9] Lohi, J., Lehti, K., Westermarck, J., Kähäri, V.M. and Keski-oja, J. (1996) Eur. J. Biochem. 239, 239–247.
[10] Smith, T.J., Hougland, M.W. and Johnson, D.A. (1984) J. Biol. Chem. 259, 11046–11051.
[11] Hashimoto, N., Morita, T. and Iwanaga, S. (1985) J. Biochem. 97, 1347–1355.
[12] Kinoshita, T., Sato, H., Takino, T., Itoh, M., Akizawa, T. and Seiki, M. (1996) Cancer Res. 56, 2535–2538.
[13] Sato, H. and Seiki, M. (1993) Oncogene 8, 395–405.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Hibbs, M.S., Hasty, K.A., Seyer, J.M., Kang, A.H. and Marinardi, C.L. (1985) J. Biol. Chem. 260, 2493–2500.
[16] Atkinson, S.J., Crabbe, T., Cowell, S., Ward, R.V., Butler, M. J., Sato, H., Seiki, M., Raynolds, J.J. and Murphy, G. (1995) J. Biol. Chem. 270, 30479–30485.
[17] Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stevenson, W.G.S. and Seiki, M. (1996) FEBS Lett. 385, 238–240.
[18] Stoppelli, M.P., Tacchetti, C., Cubellis, M.V., Corti, A., Hearing, V.J., Cassani, G., Appella, E. and Blasi, F. (1986) Cell 45, 675–684.
[19] Stephens, R.W., Pöllänen, J., Tapiovaara, H., Leung, K.-C., Sim, P.-S., Salonen, E.-M., Rønne, E., Behrendt, N., Danø, K. and Vaheri, A. (1989) J. Cell Biol. 108, 1987–1995.
[20] Ossowski, L. (1988) Cell 52, 321–328.
[21] Hearing, V.J., Law, L.W., Corti, A., Appella, E. and Blasi, F. (1988) Cancer Res. 264, 1180–1189.
[22] Ossowski, L. (1988) J. Cell Biol. 107, 2437–2445.
[23] Axelrod, J.H., Reich, R. and Miskin, R. (1989) Mol. Cell. Biol. 9, 2133–2141.