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A novel, specific pro-urokinase complex on monocyte-like cells, detected by transglutaminase-catalyzed cross-linking

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Radiolabeled pro-urokinase plasminogen activator (pro-uPA) was cross-linked to a specific protein on the surface of human monocyte-like U937 cells in a reaction catalyzed by tissue transglutaminase. The conjugate formed with this unknown component had a much higher molecular weight (apparent M_r 250,000–300,000) than the complex of pro-uPA and the urokinase plasminogen activator receptor (uPAR). There was a strong preference for the pro-form of uPA. The conjugate was recognized by antibodies against uPA but not by anti-uPAR antibodies. Nevertheless, the blocking of uPAR with a monoclonal antibody abolished the formation of the conjugate, thus showing a role of uPAR in this process.

Prourokinase; Urokinase receptor; Plasminogen activation; Transglutaminase; Cross-linking

1. INTRODUCTION

The urokinase plasminogen activator (uPA) is a serine protease which plays a trigger role in the proteolytic cascade of plasminogen activation. This cascade system has critical functions in degradation processes in the extracellular matrix which occur under a number of normal as well as pathological conditions, including cancer invasion [1,2]. uPA is synthesized and secreted in the form of a proenzyme (pro-uPA) which is activated by plasmin or certain other proteases [2] into the active two-chain enzyme.

A number of cell types possess a specific uPA receptor (uPAR) [3–5], a glycoplipid-anchored cell surface protein which has been thoroughly characterized [6–8] and which plays a critical role in the localization and regulation of plasminogen activation. Thus, in some cell types receptor-binding confines uPA to cell–cell and focal cell–substratum contact sites [9,10], and the concomitant binding of pro-uPA to uPAR and of plasminogen to unknown components on the cell leads to a strong enhancement of plasmin generation [11]. These and other processes dependent on receptor binding of uPA are assumed to involve interactions with other, as yet unidentified cell surface molecules [11]. In order to study such interactions we have in the present work utilized tissue transglutaminase, an abundant enzyme capable of introducing a covalent cross-link between certain proteins [12–14].

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Abbreviations: u-PA, urokinase-type plasminogen activator; pro-uPA, single-chain proenzyme form of uPA; u-PAR, u-PA receptor; DFP, diisopropylfluorophosphate; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DSS, *N,N'*-disuccinimidyl-suberate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Proteins*

The following reagents were purchased from the sources indicated: tissue transglutaminase from guinea pig liver (Sigma, Saint Louis, MO), active (two-chain) human uPA (Serono, Aubonne, Switzerland) and laminin from mouse EHS sarcoma (Boehringer-Mannheim, Germany). Purified, recombinant human pro-uPA was a kind gift from Dr. P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy. Diisopropylfluorophosphate-(DFP)-treated two-chain uPA was prepared as described [15]. The monoclonal and polyclonal antibodies used have been described previously [16–18]. Proteins were labeled with ^{125}I as published [6], except that an incubation period of only 10 min was used.

2.2. *Ligand binding and cross-linking with transglutaminase*

Human histiocytic lymphoma cells of the U937 line were grown as described [4] and were seeded in fresh medium the day before the experiment. Immediately before the assay, the cells were washed in 50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (binding buffer) and resuspended in binding buffer with 0.1% BSA at a density of 5×10^6 cells per ml. Each sample was made up of 200 μl of this cell suspension. The cells were preincubated in the presence or absence of various competitor proteins as indicated, for 15 min at 4°C on a shaking table. ^{125}I-labeled pro-uPA (or other labeled ligands, as specified) was then added to the samples at approx. 1.5 nM final concentration. The samples were incubated as above at 4°C for 1 h, after which the volume was made up to 1 ml with binding buffer. The cells were collected by centrifugation, and 800 μl of the supernatant was removed and discarded. The cells were resuspended in the remaining 200 μl of buffer, after which tissue transglutaminase (final concentration 20 μg/ml) was added to the samples. Control samples received no enzyme. The cells were transferred to 37°C and incubated for 30 min, after which the reaction was stopped by the addition of 800 μl of phosphate-buffered saline with 10 mM EDTA (PBS-EDTA). The cells were washed twice in PBS-EDTA and recovered in a volume of

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100 μl. The samples were then placed on ice, and the cells were lysed by the addition of CHAPS (1% final concentration). The lysates were clarified by centrifugation at 20,000 × *g* for 10 min at 4°C. All cell lysates were analyzed by SDS-PAGE followed by autoradiography.

2.3. *Other methods*

Chemical cross-linking with *N,N'*-disuccinimidylsuberate (DSS) and termination of this reaction with ammonium acetate were performed as described [15], except that the samples were handled in the buffers specified above for transglutaminase-catalyzed cross-linking. SDS-PAGE was performed according to [19] and was done under non-reducing conditions unless otherwise stated, on a 6–16% gradient slab gel.

3. RESULTS

3.1. *Tissue transglutaminase cross-links a specific, cell surface pro-uPA complex*

U937 cells were incubated with ^{125}I-labeled pro-uPA, followed by tissue transglutaminase, in a Ca^{2+}-containing buffer. Clarified detergent lysates of the cells were analyzed by SDS-PAGE and autoradiography (Fig. 1A).

The presence of transglutaminase led to the formation of an SDS-resistant, radiolabeled conjugate which migrated with an apparent M_r of 250–300,000 (lane 2; see also Fig. 2), irrespective of whether reducing (result not shown) or non-reducing sample treatment was used before electrophoresis. The reaction was strikingly specific; this sharp protein band was the only labeled product of the transglutaminase treatment (compare lanes 2 and 3 of Fig. 1A).

When labeled, active uPA or labeled, DFP-inactivated two-chain uPA was used as the ligand instead of pro-uPA, the transglutaminase failed to form the conjugate (Fig. 1B, lane 2; result shown for labeled DFP-uPA).

In a variant of the assay, we subjected the cells to acid treatment for removal of endogenous, receptor-bound uPA [15] and used unlabeled instead of labeled ligands for incubation, followed by detection by Western blotting (results not shown). After incubation of the cells with 120 nM of pro-uPA followed by 50 μg/ml of tissue transglutaminase, the M_r 250–300,000 conjugate was detected as a strong band, using monoclonal anti-uPA antibodies (clones 5 and 6 [16,17]) or polyclonal antibodies against uPA [17]. However, a very weak band was also detected at the conjugate position in samples incubated with active uPA or DFP-treated two-chain uPA in the same, high concentration, indicating that the prefernce for the pro-form of uPA was not absolute.

3.2. *The transglutaminase-catalyzed cross-linking is dependent on the binding of pro-uPA to the uPA receptor*

The present conjugate was clearly different from the complex formed with the previously characterized uPAR of M_r 55,000 [6] which could be demonstrated in the same samples by chemical cross-linking with DSS, using radiolabeled ligands (Fig. 1A and B, lane 1; labeled conjugate of apparent M_r 90,000).

Nevertheless, we found that preincubation of the cells with the monoclonal anti-uPAR antibody, R3, which has previously been shown to inhibit uPA-binding to uPAR [18], completely prevented the formation of the conjugate, whereas there was no effect of the antibodies R2 and R4, which bind to uPAR without preventing ligand binding [18] (Fig. 2). Preincubation with an excess of unlabeled uPA (Fig. 2) or pro-uPA (result not shown), both of which are known to block uPAR [4], also efficiently prevented the transglutaminase cross-linking reaction. However, while pointing to a role of uPAR in complex formation, these findings do not implicate that uPAR itself is part of the covalent adduct. In Western blotting experiments, performed as above, the M_r 250–300,000 conjugate failed to react with monoclonal (clones R2, R3 or R4 [18]) or polyclonal anti-uPAR antibodies, even under conditions where a clear reaction was found with the unconjugated uPAR. Furthermore, using tissue transglutaminase, we have been unable to cross-link pro-uPA to purified, recombinant uPAR [20], or to polymerize pro-uPA:uPAR complexes (results not shown).

4. DISCUSSION

The present work demonstrates a novel pro-uPA complex on the surface of U937 cells. The complex was

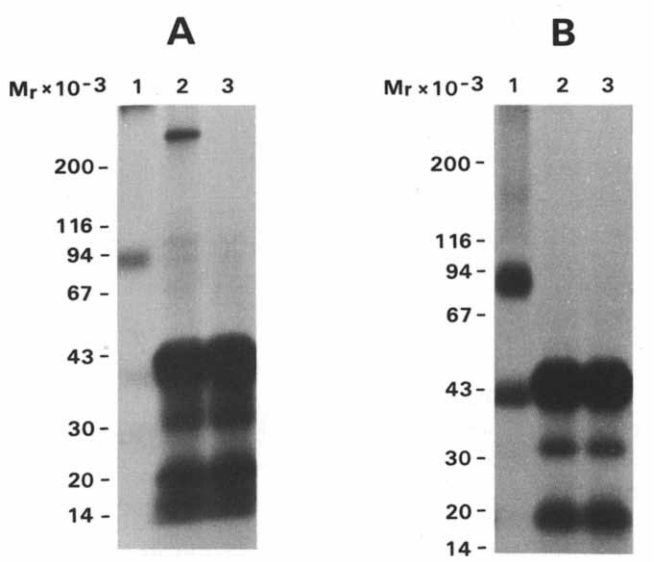

Fig. 1. Tissue transglutaminase incorporates pro-uPA into a specific, high molecular weight complex on U937 cells. Cells were incubated with ^{125}I-labeled pro-uPA (Panel A) or DFP-uPA (Panel B), followed by either of the following treatments: chemical cross-linking with DSS (lane 1), incubation with tissue transglutaminase (lane 2) or incubation with buffer alone (lane 3). Detergent cell lysates were analyzed by SDS-PAGE and autoradiography. The M_r of molecular weight marker proteins are indicated; the top of the figure aligns with the upper edge of the separation gel. In this gel system with non-reduced samples, the unconjugated ligands migrate with an apparent M_r of 42,000 (pro-uPA) and 46,000 (DFP-uPA), respectively.

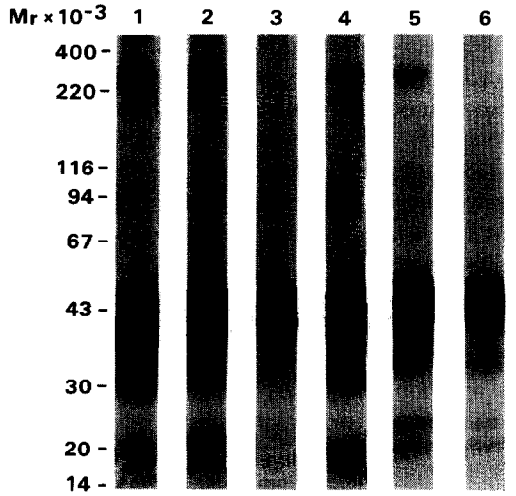

Fig. 2. Formation of the complex is dependent on uPAR. Binding of radiolabeled pro-uPA to U937 cells followed by incubation with transglutaminase were performed as in Fig. 1A, lane 2, except that the cells had been pre-incubated with the following monoclonal antibodies or other reagents: buffer (lane 1), anti-trinitrophenol (irrelevant antibody control; 20 μg/ml) (lane 2), anti-uPAR R3 (20 μg/ml) (lane 3), anti-uPAR R2 (20 μg/ml) (lane 4), anti uPAR R4 (20 μg/ml) (lane 5), or uPA (100 nM) (lane 6). The molecular weight marker proteins include a sample of reduced and alkylated laminin (A-chain: M_r 400,000; B_1 and B_2 chains: M_r 220,000 [14]).

found after transglutaminase-catalyzed cross-linking, but the detection of just a single binding product, as well as the requirement for an unblocked uPAR, makes it clear that the complex formation was the result of specific recognition events and/or steric arrangements of the reactants. Transglutaminase-catalyzed fixation of non-covalent protein complexes is a well established phenomenon [12]. In the present reaction, there was a strong preference for the pro-form of uPA.

The demonstration of a new, uPAR-dependent, pro-uPA binding product is of considerable interest, because the glycolipid-anchored uPAR:ligand complex is involved in several processes which appear to require interactions with other cell surface molecules. These processes include the potentiation of the plasminogen activation cascade and the directioning of surface-bound pro-uPA to contact sites, discussed above, and also internalization of uPA:inhibitor complexes [21–23] and a proposed role of uPAR in cellular signal transduction [24].

It is a relevant possibility that, also in vivo, covalent fixation of the present complex may occur in addition to the non-covalent interactions that form the background for the specificity. Tissue transglutaminase and closely related transglutaminases are widespread in the organism, e.g. occurring in erythrocytes, endothelial cells and hepatocytes [12]. In the latter cell type, tissue transglutaminase participates in covalent reactions on the extracellular surface [13], and the enzyme has also been shown to bind to extracellular matrix components [25].

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REFERENCES

[1] Danø, K., Andreasen, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266.
[2] Pöllänen, J., Stephens, R.W. and Vaheri, A. (1991) Adv. Cancer Res. 57, 273–328.
[3] Vassalli, J.D., Baccino, D. and Belin, D. (1985) J. Cell. Biol. 100, 86–92.
[4] Nielsen, L.S., Kellerman, G.M., Behrendt, N., Picone, R., Danø, K. and Blasi, F. (1988) J. Biol. Chem. 263, 2358–2363.
[5] Møller, L.B. (1993) Blood Coagulation and Fibrinolysis 4, 293–303.
[6] Behrendt, N., Rønne, E., Ploug, M., Petri, T., Løber, D., Nielsen, L.S., Schleuning, W.D., Blasi, F., Appella, E. and Danø, K. (1990) J. Biol. Chem. 265, 6453–6460.
[7] Ploug, M., Rønne, E., Behrendt, N., Jensen, A.L., Blasi, F. and Danø, K. (1991) J. Biol. Chem. 266, 1926–1933.
[8] Behrendt, N., Ploug, M., Patthy, L., Houen, G., Blasi, F. and Danø, K. (1991) J. Biol. Chem. 266, 7842–7847.
[9] Pöllänen, J., Hedman, K., Nielsen, L.S., Danø, K. and Vaheri, A. (1988) J. Cell Biol. 106, 87–95.
[10] Hebert, C.A. and Baker, J.B. (1988) J. Cell Biol. 106, 1241–1247.
[11] Ellis, V., Pyke, C., Eriksen, J., Solberg, H. and Danø, K. (1992) N.Y. Acad. Sci. 667, 13–31.
[12] Greenberg, C.S., Birckbichler, P.J. and Rice, R.H. (1991) FASEB J. 5, 3071–3077.
[13] Barsigian, C., Stern, A.M. and Martinez, J. (1991) J. Biol. Chem. 266 22501–22509.
[14] Aeschlimann, D. and Paulsson, M. (1991) J. Biol. Chem. 266, 15308–15317.
[15] Behrendt, N., Ploug, M., Rønne, E., Høyer-Hansen, G. and Danø, K. (1993) Methods Enzymol. 223, 207–222.
[16] Nielsen, L.S., Grøndahl-Hansen, J., Andreasen, P.A., Skriver, L., Zeuthen, J. and Danø, K. (1986) J. Immunoassay 7, 209–228.
[17] Grøndahl-Hansen, J., Agerlin, N., Munkholm-Larsen, P., Bach, F., Nielsen, L.S., Dombernovsky, P. and Danø, K. (1988) J. Lab. Clin. Med. 111, 42–52.
[18] Rønne, E., Behrendt, N., Ellis, V., Ploug, M., Danø, K. and Høyer-Hansen, G. (1991) FEBS Lett. 288, 233–236.
[19] Laemmli, U.K. (1970) Nature 277, 680–685.
[20] Ploug, M., Kjalke, M., Rønne, E., Weidle, U., Høyer-Hansen, G. and Danø, K. (1993) J. Biol. Chem. 268, 17539–17546.
[21] Olson, D., Pöllänen, J., Rønne, E., Høyer-Hansen, G., Wun, T.-C., Sakaguchi, K., Appella, E., Danø, K. and Blasi, F. (1992) J. Biol. Chem. 267, 9129–9133.
[22] Herz, J., Clouthier, D.E. and Hammer, R.E. (1992) Cell 71, 411–421.
[23] Nykjær, A., Petersen, C.M., Møller, B., Jensen, P.H., Moestrup, S.K., Holtet, T.L., Etzerodt, M., Thøgersen, H.C., Munch, M., Andreasen, P.A. and Gliemann, J. (1992) J. Biol. Chem. 267, 14543–14546.
[24] Nusrat, A.R. and Chapman, H. (1991) J. Clin. Invest. 87, 1091–1097.
[25] Upchurch, H.F., Conway, E., Patterson, M.K. and Maxwell, M.D. (1991) J. Cell. Physiol. 149, 375–382.