Autoactivation of Avian Urokinase-type Plasminogen Activator (uPA)

A NOVEL MODE OF INITIATION OF THE uPA/PLASMIN CASCADE*

(Received for publication, December 2, 1997)

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In contrast to mammalian urokinase-type plasminogen activator (uPA), which is produced and maintained inzymogen form, avian uPA is found in the active two-chain form in cultures of normal and transformed chicken cells in the absence of plasmin, the putative natural activator of pro-uPA. Recombinant chicken uPA (ch-uPAwt) synthesized in two distinct expression systems also presents in the active two-chain form. In addition, conversion to the active uPA in both natural and recombinant expression systems could be prevented by uPA-specific inhibitors including a monoclonal antibody that uniquely inhibits the catalytic activity of ch-uPA. Most significantly, an active site mutant of avian uPA (ch-uPAS353A) that lacks catalytic activity is produced and maintained in single-chain form. Furthermore, the single-chain ch-uPAS353A mutant can be converted to the two-chain form by purified active ch-uPAwt. These results strongly indicate an autocatalytic mechanism of activation of ch-uPA. Autoactivation appears to be an intrinsic property of ch-uPA and may be the initiating molecular event in uPA-mediated proteolytic cascades.

With few exceptions, serine proteases are secreted as single-chainzymogens and require limited proteolysis for conversion to a two-chain form that possesses full enzymatic activity. Accordingly, mammalian urokinase-type plasminogen activator (uPA) is secreted and maintained as a zymogen pro-uPA that possesses very low intrinsic activity (1–7). The precipitating event in the initiation of the uPA/plasmin proteolytic cascade remains elusive, as the natural mechanism of activation of uPA is not well understood. Whereas the activation of pro-uPA is presumed to be mediated primarily by plasmin in a feedback mechanism (2, 8, 9), plasmin itself must be generated from plasminogen by active uPA and thus cannot be envisioned as the single initiating factor in the proteolytic process. In addition, in plasminogen-deficient mice, uPA is found in its active two-chain form (10), suggesting that mechanisms other than activation by plasmin are responsible for uPA activation. There is accumulating evidence that the single-chain human uPA through interactions with substrates, inhibitors, cell surface receptors, or fibrin clots (7, 8, 11–13) is capable of acquiring transient conformations that are similar to its active two-chain configuration, and the resulting enhanced catalytic activity of thezymogen may serve as the initiating event of this proteolytic cascade. Definitive proof for this mechanism of pro-uPA activation, however, is lacking.

In striking contrast to mammalian cells, which generally produce, secrete, and maintain uPA in the single-chain,zymogen form, transformed chicken embryo fibroblasts (RSVCEF) consistently produce active two-chain uPA in the complete absence of plasminogen and plasmin (14). It was suggested that malignant transformed cells possess a unique structural or enzymatic process that brings about uPA activation (14). We investigated this plasmin-independent activation of avian uPA in a number of cell culture systems, including recombinant expression systems, and present evidence that the activation of chicken uPA is not dependent on malignant transformation but rather is an autocatalytic event that appears to reflect an intrinsic property of avian uPA.

EXPERIMENTAL PROCEDURES

Cell Cultures—Primary cultures of chick embryo fibroblasts (CEF) were prepared from 11-day old embryos as described (14). Secondary cultures of CEF were infected with the BH strain of Rous sarcoma virus (RSV) generating highly transformed cultures of RSVCEF that were propagated, maintained, and characterized as described previously (14). Cultures of the chicken macrophage cell line, HD11, obtained from M. Hayman (SUNY, Stony Brook, NY) and the duck embryo cell line, CCL 141, obtained from ATCC (Rockville, MD) were maintained under identical culture conditions as CEF and RSVCEF. The human epidermoid carcinoma cell line, HeP3, and the mouse myeloma cell line, NS0, were cultured and maintained as described previously (15).

Expression of Recombinant Chicken uPA (ch-uPA)—The cDNA encoding ch-uPAwt was cloned into Bluescript KS+ (Stratagene, La Jolla, CA) as described previously (16). The ch-uPAwt insert was ligated into the EcoRI and BclI restriction sites of the pEE12 expression vector, a cytomegalovirus promoter-driven expression vector obtained from C. Bebington (Celltech, Slough, UK). The ch-uPAwt,pEE12 vector was transfected by electroporation into mouse myeloma NS0 cells. The transfected cultures were grown in the absence of glutamine under limiting dilution to select for cells expressing glutamine synthetase encoded by the pEE12 expression vector, a cytomegalovirus promoter-driven expression vector obtained from C. Bebington (Celltech, Slough, UK). The ch-uPAwt-containing colonies were picked, subcloned, expanded in culture, and harvested in serum-free medium. A subclone, 2F7NS0/ch-uPAwt, produced 0.1 μg/ml of ch-uPAwt in serum-free conditioned medium.

The ch-uPAwt cDNA also was transferred from Bluescript into pVL1392 a baculovirus-based transfer vector that contains the complete polyhedron gene locus of the Autographa californica nuclear polyhedrosis virus (PharMingen, San Diego, CA). The ch-uPAcontaining vector was co-transfected with BaculoGold viral DNA into Sf9 insect cells. Recombinant baculovirus containing the ch-uPA insert was amplified through sequential infections of Sf9 cells and then used to infect Hi5 insect cells, a high level protein producer insect cell line.

* This work was supported in part by Grant CA76039 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: uPA, urokinase-type plasminogenactivator; ch-uPA, chicken uPA; ch-uPAS353A, wild-type ch-uPA; CEF, chicken embryo fibroblasts; RSV, Rous sarcoma virus; DFP, diisopropyl fluorophosphate; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; CM, conditioned medium.

This paper is available on line at http://www.jbc.org
(Invitrogen, San Diego, CA). Infected cells expressing and secreting ch-uPAwt protein were harvested and analyzed for the production and zymogen status of ch-uPA.

**Disopropyl Fluorophosphate (DFP) Treatment and Casein-Plasminogen Zymography**—Treatment with the irreversible serine protease inhibitor DFP leads to the generation of single-chain zymogen uPA, which is resistant to DFP and two-chain active uPA, which is completely inhibited by DFP. Samples of conditioned media are incubated at 20 °C with 5 mM DFP for 1 h followed by a second 1 h treatment with 5 mM DFP. The samples are then electrophoresed on SDS-polyacrylamide gels (12%) under nonreducing conditions alongside untreated samples or solvent (isopropyl alcohol) only treated samples. The gels are then washed with 50 mM Tris-Cl, followed by a 1 h wash in H2O. The washed gel is placed over an underlay consisting of 1% agarose, 5% nonfat dry milk (casein) and 50 μg/ml chicken plasminogen incubated at 37 °C until pronounced zones of lysis appear at the electrophoretic position of uPA.

**Reducing SDS-PAGE, Western Blotting, and Immunodetection—**Samples of conditioned media are electrophoresed under nonreducing (no βME) or reducing (5% v/v βME) conditions on SDS-polyacrylamide gels (12%) gels. The gels are transferred to nitrocellulose membranes. The membranes are blocked for 2 h in 5% casein-0.1% Tween 20 then incubated overnight at 4 °C with 1 μg/ml rabbit polyclonal antibody raised against ch-uPA. After washing, the membranes are incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and following extensive washing are developed by ECL chemiluminescence (Amersham Pharmacia Biotech). The active site serine residue (Ser353) of ch-uPA was mutated to alanine that converts rapidly and efficiently to the active two-chain form, indicating that recombinant ch-uPA is found in the two-chain form identically to the native ch-uPA expressed by RSVCEF (lanes 1 and 2).

**RESULTS AND DISCUSSION**

To investigate the mechanism of activation of chicken uPA, the cDNA was cloned into a baculovirus expression vector and in a mammalian expression vector for expression in two distinct expression systems: Hi5 insect cells and NS0 mouse myeloma cells, respectively. Cells that synthesized and secreted ch-uPA were selected, expanded in culture, and incubated to release ch-uPA into the conditioned medium. All conditioned media were obtained under serum-free conditions in the absence of plasminogen and in the presence of aprotinin, a potent plasmin inhibitor. The recombinant ch-uPA from the two expression systems was tested for its zymogen status by two independent methods. In the first method, which tests the conformational status of the active site, conditioned media from cultures of RSVCEF, Hi5-ch-uPAriv and NS0-ch-uPAriv are left untreated or treated with DFP, an irreversible active site inhibitor of serine proteases that targets only the active form of the enzymes. Subsequently, the samples are subjected to SDS-PAGE and underlay zymography. Active two-chain uPA would be sensitive to DFP, resulting in the diminution of zones of lysis in the zymograph. In contrast, single-chain zymogen uPA would be resistant to inhibition by DFP, displaying a zone of lysis comparable to that created by the untreated sample. As shown in Fig. 1A, recombinant ch-uPA produced in both expression systems (lanes 3–6) is as sensitive to DFP as the native RSVCEF-produced ch-uPA (lanes 1–2), indicating that ch-uPA presents entirely in its active form.

The expressed recombinant ch-uPA is in the two-chain active protease form was determined by an alternative method based on protein structure, namely electrophoresis on SDS-polyacrylamide gels under nonreducing or reducing conditions, followed by Western blotting and probing with a polyclonal anti-ch-uPA antibody (Fig. 1B, lanes 1–6). Upon reduction, zymogen ch-uPA would be maintained as a 48-kDa single-chain form, whereas two-chain uPA would dissociate into a 35-kDa B-chain and a 12–14-kDa A-chain. The recombinant ch-uPA secreted in both the insect and the mammalian expression systems (lanes 3 and 4, and 5 and 6, respectively) exhibits only the 35-kDa B-chain upon reduction (A-chain is not immunoreactive with this antibody), with little or no evidence of the 48-kDa single-chain form, indicating that recombinant ch-uPA is found in the two-chain form identically to the native ch-uPA expressed by RSVCEF (lanes 1 and 2).

In contrast to avian uPA, human uPA obtained from conditioned medium of human tumor cells is maintained in the single-chain zymogen form, as seen by resistance to DFP treatment (Fig. 1A, lanes 7 and 8). Furthermore, recombinant human uPA expressed in the same insect cell expression system was found to be expressed and maintained in single-chain zymogen form (Fig. 1B, lanes 7 and 8), under identical conditions to those that allowed complete conversion of ch-uPA to the active two-chain form, indicating that there is no ubiquitous single-chain uPA-converting enzyme in the recombinant expression system that had activated the chicken pro-uPA.

Thus, the recombinant ch-uPA produced by two heterologous expression systems is found to be in the active two-chain form, indicating that ch-uPA is an intrinsically unstable zymogen that converts rapidly and efficiently to the active two-chain uPA form independent of the culture system in which it is expressed. Interestingly, this intrinsic conversion process generates the same two-chain uPA molecule as plasmin-mediated activation of uPA. Recombinant ch-uPA harvested from NS0-ch-uPAwt cultures was purified and subjected to SDS-PAGE under reducing conditions. The B-chain was blotted onto polyvinylidene difluoride membrane and subjected to amino-terminal sequencing. The first 8 residues obtained were Ile, Val, Gly, Gly, Ser, Gln, Ala, Glu, corresponding to ch-uPA sequence 150VYGGSQAE160 indicating that the cleavage site is at lysine 152. The homologous position in all mammalian uPA expressed by RSVCEF (lanes 1 and 2).

**Construction and Expression of the Active Site Mutant of ch-uPA—**

The active site serine residue (Ser353) of ch-uPA was mutated to alanine utilizing the method of splicing by overlap extension and polymerase chain reaction (21). The internal 5′ primer, 5′-GGGAATTCGGC-3′, was used to incorporate the nucleotide change (TCT→GCT) that mutates Ser353 to alanine. The resulting cloned DNA was sequenced to verify the mutation and subcloned into both the pEE 12 and pAAEC vectors for expression in NS0 and Hi5 cells, respectively, as described previously (14, 20). Human plasminogen, plasmin, uPA, and antibodies to human uPA were from American Diagnostica (Greenwich, CT).

**Protein Purification and Analysis—**Chicken plasminogen was purified from chicken plasma by lysine-Sepharose affinity chromatography as described (19). The ch-uPAriv was purified by benzamidine-Sepharose affinity chromatography and gel filtration chromatography as described (19). Polyclonal antibodies to ch-uPA and a neutralizing monoclonal antibody to ch-uPA were purified by affinity chromatography as described previously (19, 20). Human plasminogen, plasmin, uPA, and antibodies to human uPA were from American Diagnostica (Greenwich, CT).

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Inhibition of the conversion process using highly specific reagents indicates that the conversion of ch-uPA requires its own catalytic activity. A monoclonal antibody to ch-uPA added to cultures expressing ch-uPAriv (Fig. 2) prevents the conversion reaction, yielding a DFP-insensitive chicken uPA zymogen (lanes 3 and 4). This monoclonal antibody has been shown to be...
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highly specific in blocking the catalytic activity of ch-uPA (20). Low molecular weight inhibitors of uPA activity such as benzamidine and nitrophenyl guanidinobenzoate also prevent single-chain or two-chain status by Western blotting, it presents in the 48-kDa single-chain form under reducing conditions (Fig. 3, lane 2), whereas the wild-type ch-uPAwt expressed in parallel cultures exhibits only the two-chain form (Fig. 3, lane 1), dissociating under reducing conditions into a 35-kDa B-chain and a 14-kDa A-chain. Therefore, abrogating the catalytic activity of chicken uPA by mutagenesis of the active site serine residue completely ablates conversion to the two-chain uPA under conditions identical to those permissive for the generation of the active two-chain form of the wild-type enzyme. This result is consistent with and confirmatory to the preventive effects of the exogenously added uPA-specific inhibitors (Fig. 2) and strongly supports an autocatalytic mechanism of conversion.

To ensure that the serine to alanine mutation of chicken uPA did not alter its conformation so as to make the activation site (Lys152-Ile153) inaccessible, or the entire molecule incapable of conversion, the mutant ch-uPAS353A was treated with plasmin, the putative natural activator of uPA. Indeed, the mutant chicken uPA is converted by plasmin in a dose-dependent manner (Fig. 3, lanes 3 and 4) to yield a B-chain indistinguishable from that of ch-uPAtot. These data attest that the mutant is conformationally capable of being converted but remains in the single-chain form under the identical conditions that allowed for complete conversion of the wild-type chicken uPA to the two-chain form. These results also further support the absence of any distinct converting enzyme in these expression systems. If such a convertase existed, it should be, like plasmin, equally capable of converting ch-uPAS353A as it would ch-uPAtot, yet no two-chain ch-uPAS353A has ever been observed.

To demonstrate that ch-uPA autoactivation could be recapitulated in vitro, the inactive ch-uPAS353A mutant was radiolabeled and utilized as a single-chain substrate for conversion by purified active ch-uPAtot. Fig. 4 illustrates that ch-uPAtot−containing conditioned medium (lane 2) as well as 50–150 ng of purified ch-uPAtot (lanes 3 and 4) can process 1 μg of single-
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Fig. 3. Mutation of the active site serine to alanine in chicken uPA (ch-uPA<sub>SS353A</sub>) results in abrogation of catalytic activity and in the stabilization and maintenance of single-chain ch-uPA. Wild-type chicken uPA (ch-uPA<sub>wt</sub>, lane 1) and a catalytically inactive mutant (ch-uPA<sub>SS353A</sub>, lanes 2-4) expressed in the insect cell/baculoviral system under identical culture conditions are compared by electrophoresis on 12% SDS-polyacrylamide gels under reducing conditions, followed by Western blotting with anti-chicken uPA antibody. Lanes 3 and 4 illustrate the dose-dependent conversion of the single-chain catalytically inactive mutant of chicken uPA, ch-uPA<sub>SS353A</sub>, by plasmin (5 and 25 ng, respectively) with the progressive appearance of B-chain, indicative of two-chain formation. Molecular weight markers are shown on the left, and uPA forms are indicated on the right. Identical results were obtained with ch-uPA<sub>wt</sub> and ch-uPA<sub>SS353A</sub> expressed in the NS0 cell culture system.

Fig. 4. Conversion of ch-uPA<sub>SS353A</sub> by purified ch-uPA<sub>wt</sub> in vitro. Conditioned medium (1 ml) from radiolabeled NS0 ch-uPA<sub>SS353A</sub> cultures containing 1 μg/ml <sup>35</sup>S-labeled ch-uPA<sub>SS353A</sub> was incubated in the absence (lane 1) or presence of either 1 ml of conditioned medium from NS0 ch-uPA<sub>wt</sub> cultures (containing 0.1 μg of active ch-uPA<sub>wt</sub>, lane 2) or purified ch-uPA<sub>wt</sub> (50 and 150 ng, in lanes 3 and 4, respectively). After overnight incubation, the samples were immunoprecipitated utilizing a polyclonal rabbit anti-ch-uPA antibody, electrophoresed on 12% SDS gels under reducing conditions, and subjected to autoradiography. Molecular weight markers in kDa are indicated on the left, and the electrophoretic mobility of chicken and duck uPA are shown on the right.

Finally, it appears that this plasmin-independent generation of active two-chain avian uPA is not unique to the transformed chicken fibroblast culture system. Normal avian macrophages stimulated with phorbol ester also produce active two-chain uPA in the absence of plasmin and in the presence of aprotinin (Fig. 5A, lane 2), similar to RSVEC (lane 1). Furthermore, a normal duck cell line, cultured as well in the absence of plasmin and in the presence of the plasmin inhibitor, also produces the active (DFP-sensitive) form of avian uPA (Fig. 5B, lanes 3 and 4). Thus, the unusual occurrence of active two-chain uPA appears to be a distinct feature of the avian fibrinolytic system.

This is the first description of autoactivation of a protease of the fibrinolytic cascade. There are, however, other studies that document a mechanism of autoactivation for factor VII (22, 23) and factor XII (24–26), the two serine proteases that initiate the intrinsic and extrinsic coagulation cascade, respectively. Furthermore, the initiation of the classical pathway of the complement cascade, namely activation of C1, also is an autocatalytic event (27, 28). It is interesting to note that activation of PA is believed to be the initiating molecular event in the fibrinolytic cascade, possibly indicating a pattern that the primary initiation of biologically significant proteolytic cascades may be an autocatalytic event.

Mammalian uPA is controlled post-translationally at multiple levels, including synthesis and secretion in a stable zymogen form and tight regulation of its catalytic activity and turnover by PA-specific serpins PAI-1 and PAI-2 (29–31). This study demonstrates that avian uPA is capable of autoactivation, thus precluding the regulatory requirement of another proenzyme to initiate pro-uPA activation. In addition, previous studies in our laboratory have shown that chicken uPA is resistant to inhibition by the PA-inhibiting serpins PAI-1 and PAI-2 (16). Thus, it appears that chicken uPA lacks the two major post-translational regulatory mechanisms that control mammalian uPA. The physiological significance of this apparent lack of regulation in the avian fibrinolytic/proteolytic system is not known, but it appears from studies on both avian embryo development and the promoter region of the uPA gene...
that chicken uPA is regulated spatially and temporally at the level of expression rather than post-translationally (32–35). Furthermore, since the existence and location of specific avian PAIs have never been reported, it is difficult to assess how the intrinsic autoactivation of avian uPA is controlled catalytically and spatially at the tissue level.

In conclusion, chicken uPA constitutes an interesting natural variant of uPA that can evade most regulatory mechanisms that constrain the behavior of mammalian uPA. Identification of the structural motif(s) that dictate the autoactivating propensity of chicken uPA will not only yield mechanistic insight into the process but also will substantiate the corresponding structural motifs in human uPA that apparently stabilize the zymogen and limit pro-uPA autoactivation. This unusual uPA variant has already shed light on the activation modality of proteolytic cascades in the avian system and may continue to confer insights into the structural/functional features of serine proteases as well as on the exquisite control systems that have developed in the course of mammalian evolution.

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