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INTRACELLULAR PROCESSING OF COMPLEMENT PRO-C3 AND PROALBUMIN IS INHIBITED BY RAT α_1-PROTEASE INHIBITOR VARIANT(Met^{352}→Arg) IN TRANSFECTED CELLS

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SUMMARY: Complement C3, when its cDNA was transfected into COS-1 cells, was synthesized as a precursor, pro-C3, which was intracellularly processed into the α and β subunits, although not completely. A cDNA for rat α_1-protease inhibitor (α_1-PI) was mutated *in vitro* to encode its variant with the modified active site (Met^{352}→Arg). In cells co-transfected with the mutant α_1-PI cDNA and the C3 cDNA, pro-C3 expressed was secreted without being processed into the subunits. Co-transfection of the mutant α_1-PI cDNA and the albumin cDNA also resulted in the inhibition of intracellular conversion of proalbumin into serum-type albumin. No inhibition of the processing of each proform was observed in cells co-transfected with the normal α_1-PI cDNA. Taken together, the results indicate that the α_1-PI variant (Met^{352}→Arg) expressed inhibits specifically an intracellular enzyme which is involved in the proteolytic processing of both pro-C3 and proalbumin.

α_1-Protease inhibitor (α_1-PI) is a serum glycoprotein produced by hepatocytes and serves as the major inhibitor of elastase released by neutrophils, although it also inhibits other serine proteases (1). Of many human α_1-PI variants so far reported, α_1-PI Pittsburgh associated with a fatal bleeding disorder (2, 3) is of particular interest. The mutant inhibitor has a single substitution of the reactive site Met^{358}→Arg, resulting in its acquisition of antithrombin III activity (3, 4). In addition, the mutant α_1-PI has been shown to inhibit the yeast Kex2 protease (5) and a Kex2-like protease in rat

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Abbreviations used: α_1-PI, α_1-protease inhibitor; C3, the third component of complement; pro-C3, precursor of C3; RSA, rat serum albumin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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liver Golgi membranes (6), both of which can convert proalbumin to serum-type albumin by cleavage at paired basic amino acid residues.

The third component of complement (C3), mainly produced by hepatocytes, is synthesized as a single polypeptide precursor (pro-C3) and processed into the α and β subunits with disulfide linkage before its secretion (7-9). The cloning and sequencing of cDNAs for mouse (10), human (11) and rat (12) C3 demonstrated that pro-C3 has the structure NH_2-(β subunit)-Arg-Arg-Arg-Arg-(α subunit)-COOH. Thus, it is evident that the dimeric structure of the mature molecule is formed by proteolytic removal of the four intervening arginine residues from pro-C3. This processing is suggested to occur during the intracellular transport of pro-C3, possibly at the trans Golgi region (9, 13).

In the present study we prepared a mutant cDNA encoding an α_1-PI variant (Met^{352}→Arg) by site-directed mutagenesis of rat α_1-PI cDNA, and examined the effect of the mutant inhibitor on the intracellular processing of pro-C3 in COS-1 cells which had been co-transfected with the mutant α_1-PI cDNA and C3 cDNA.

MATERIALS AND METHODS

cDNAs. Full length cDNAs encoding rat α_1-PI (14) and C3 (12) were cloned and characterized as described previously. The rat albumin cDNA was kindly provided by Dr. Y. Kaneda (Osaka University).

Oligonucleotide site-directed mutagenesis. Site-directed mutagenesis of the α_1-PI cDNA was designed for Met-352(ATG) → Arg(AGG) and carried out by the method of Kunkel (15). The α_1-PI cDNA was subcloned into the EcoRI site of pUC118 plasmid vector, and a sense strand of the cDNA was used as a template for in vitro synthesis of the complementary strand. A 17-mer synthetic oligonucleotide (5'-pCAGAGACCTGGGGACGG-3') was used as a primer for the second strand. The mutant clone obtained was designated as pcα_1PIM/R. To destroy two EcoRI sites in rat C3 cDNA, oligo-directed silent mutations were introduced at Glu-96(GAA) → Glu(GAG) and Ile-970(ATT) → Ile(ATC). The C3 cDNA was cloned into pBluescriptSK$^-$ by auto-excision process (16). The single strand cDNA of the plasmid was used as a template for site-directed mutagenesis (15). 18-mer synthetic oligonucleotides (5'-pGTAACTTGAGGAATGACC-3' and 5'-pCGTCCTCCTAAGAC-CAGA-3') were used as primers for the second strand. The rat C3 cDNA clone with no EcoRI sites thus prepared was named as pcRCM. The nucleotide sequences of these mutant clones were verified by the dideoxynucleotide chain termination method (17).

Construction of expression plasmids. Expression vector (pSVM) was constructed from pSV2 by destroying its EcoRI site and by introducing multicloning sites (HindIII, EcoRI, SmaI and BglII). pSV2 was digested with EcoRI, filled in with the Klenow enzyme, and self-ligated. pSV2 that lost the EcoRI site was digested with HindIII and BglII. Two 18-mer synthetic oligonucleotides

(5'-pAGCTTCGAATTCCCGGGA-3' and 5'-pGATCTCCCGGGAATTCGA-3') were inserted into the HindIII-BglII site of the vector (pSVM).

cDNA inserts of α_1-PI, α_1-PIM/R (Met^{352}→Arg), C3 and albumin were prepared by EcoRI digestion of each plasmid, and inserted into the EcoRI site of pSVM expression vector. The insert orientation was confirmed by restriction endonuclease mapping. The expression plasmids thus prepared were designated as pEα_1PI (for normal α_1-PI), pEα_1PIM/R (mutant α_1-PI/Met^{352}→Arg), pERCM (C3), and pERSA (albumin).

Transfection of COS-1 cells and analysis of expressed proteins. Each plasmid (20 μg) was transfected into 5 x 10^6 COS-1 cells using an electroporation apparatus (Gene Pulser, BioRad) as described previously (14, 18). The transfected cells were cultured in Dulbecco's modified Earle's medium containing 10% fetal calf serum in 10-cm dishes for 48 h. The cells were labeled for 4 h at 37°C with [^{35}S]methionine (100 μCi/dish) in 5 ml of methionine-free Eagle's minimum essential medium. Cell lysates and media were prepared and subjected to immunoprecipitation with IgG monospecific for each antigen. The immunoprecipitates were analyzed by SDS-PAGE (10% gels for α_1-PI and 7.5% gels for C3) or by electrofocusing (pH 5-8), followed by fluorography (9, 13, 14). Rat hepatocytes in a primary culture were also labeled with [^{35}S]methionine (19, 20). ^{35}S-Labeled proteins secreted into medium were isolated by immunoprecipitation and used as molecular mass markers or authentic mature forms in PAGE as indicated.

Antibodies. Monospecific antibodies against rat α_1-PI (21) and albumin (22) were raised in rabbits as described previously. Anti-rat C3 was obtained from Cappel Laboratories (West Chester, PA).

RESULTS

The rat α_1-PI cDNA was modified by site-directed mutagenesis to encode its variant (Met^{352}→Arg) which corresponds to the human α_1-PI Pittsburgh (Met^{358}→Arg) (2, 3). An expression plasmid containing the mutant cDNA (pEα_1-PIM/R) was constructed and transfected into COS-1 cells. Labeleing experiments of the transfected cells demonstrated that the variant α_1-PI (Met^{352}→Arg) was effectively expressed and secreted as a form with 55 kDa (Fig. 1, lane 3), slightly smaller than the inhibitor (56 kDa) secreted from rat hepatocytes (lane 5). Since the normal counterpart (lane 2) expressed in the COS-1 cells had the same molecular mass as that of the variant, the small difference in molecular mass may be due to a possible difference in glycosylation of the inhibitor between the COS-1 cells and rat hepatocytes.

When the rat C3 cDNA was transfected into COS-1 cells, C3 was synthesized as a single polypeptide with about 180 kDa, pro-C3 (Fig. 2, lane 3), which was processed into the α and β subunits and secreted into the medium (lane 4). Although in cultured rat hepatocytes pro-C3 was completely processed to the subunits prior to secretion (lane 11), a significant amount of pro-C3 was

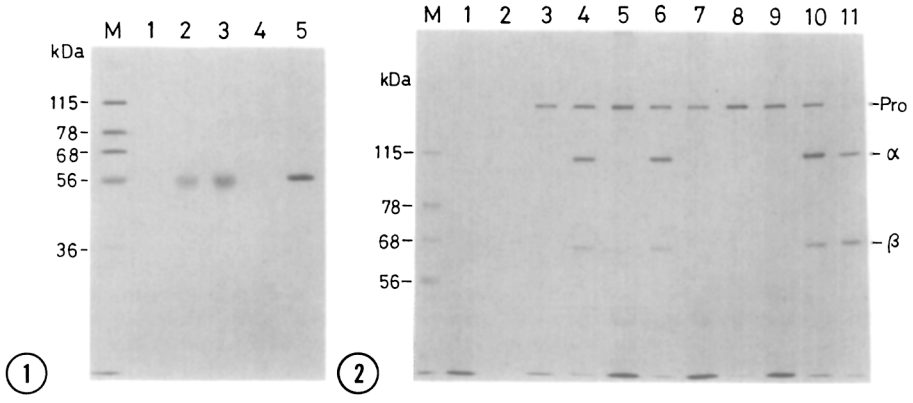

Fig. 1. SDS-PAGE of normal and mutant α_1-PIs expressed in the transfected cells. pSV-cDNA constructs encoding the indicated products were transfected into COS-1 cells. The transfected cells were incubated with [^{35}S]methionine at 37°C for 4 h. α_1-PI secreted into medium was immunoprecipitated with anti-(α_1-PI) IgG and analyzed by SDS-PAGE (10% gel)/fluorography. Lane 1, mock; lane 2, pEα_1PI (for normal α_1-PI); lane 3, pEα_1PIM/R (α_1-PI/Met^{352} → Arg); lane 4, pEα_1PIM/R(r) containing the α_1-PIM/R cDNA in reverse orientation; lane 5, α_1-PI secreted by rat hepatocytes. Lane M, ^{35}S-labeled marker proteins.

Fig. 2. Processing of pro-C3 expressed in the transfected cells with or without co-transfection with α_1-PI plasmids. COS-1 cells transfected with the indicated plasmid(s) were incubated with [^{35}S]methionine at 37°C for 4 h. Cell lysates (lanes 1, 3, 5, 7 and 9) and media (lanes 2, 4, 6, 8 and 10) were prepared and subjected to immunoprecipitation with anti-C3 IgG. The immunoprecipitates were analyzed by SDS-PAGE (7.5% gel)/fluorography. Lane M, marker proteins; lane 1 and 2, mock; lanes 3 and 4, pERCM (for C3); lanes 5 and 6, pERCM and pEα_1PI (normal α_1-PI); lanes 7 and 8, pERCM and pEα_1PIM/R (α_1-PI/Met^{352} → Arg); lanes 9 and 10, pERCM and pEα_1PIM/R(r); and lane 11, mature C3 secreted by rat hepatocytes. Pro, α and β indicate the precursor and subunits of C3, respectively.

secreted without being processed in the transfected cells (lane 4). Such an incompleteness in proteolytic processing was also observed for other products over-expressed in transfected COS-1 cells (18, 23, 24). We then examined the effect of protease inhibitors on the processing of pro-C3 by co-transfection with each cDNA. The expression of the normal α_1-PI caused no significant effect on the processing of pro-C3 (Fig. 2, lanes 5 and 6). However, in the cells co-transfected with pEα_1PIM/R, only the pro-C3 form was detected in both the cells and medium (lanes 7 and 8), indicating that the mutant α_1-PI (Met^{352} →Arg) expressed inhibited the proteolytic processing of pro-C3.

Essentially the same results were obtained for the proteolytic processing of proalbumin with or without co-expression of the protease inhibitor (Fig. 3). In cells transfected with pERSA alone, proalbumin expressed was processed to serum albumin (lanes 3 and 4), although approximately a half amount of the

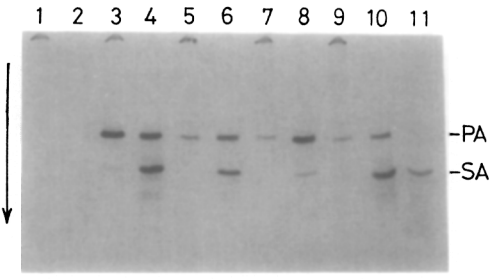

Fig. 3. Processing of proalbumin expressed in the transfected cells with or without co-transfection with α_1-PI plasmids. COS-1 cells transfected with the indicated plasmids were incubated with [^{35}S]methionine at 37°C for 4 h. Cell lysates (lanes 1, 3, 5, 7, and 9) and media (lanes 2, 4, 6, 8, and 10) were prepared and subjected to immunoprecipitation with anti-RSA IgG. The immunoprecipitates were analyzed by polyacrylamide gel electrofocusing (pH 5-8)/fluorography. Lanes 1 and 2, mock; lanes 3 and 4, pERSA (for albumin); lanes 5 and 6, pERSA and pEα_1PI (normal α_1-PI); lanes 7 and 8, pERSA and pEα_1PIM/R (α_1-PI/Met^{352} → Arg); lanes 9 and 10, pERSA and pEα_1PIM/R(r); and lane 11, mature albumin secreted by rat hepatocytes. PA and SA represent proalbumin and serum albumin, respectively. An arrow indicates a pH gradient from 8 to 5.

proform was secreted without being processed (lane 4), as observed above in the processing of pro-C3 (Fig. 2). This processing was strongly inhibited by co-expression of the mutant α_1-PI (Met^{352}→Arg) (Fig. 3, lanes 7 and 8), in contrast to no significant effect by the normal α_1-PI (lanes 5 and 6). However, the inhibition by the mutant inhibitor was not so complete as that observed for pro-C3 (Fig. 2, lane 8). The reason for such a difference is not clear at present.

DISCUSSION

A variety of secretory proteins are synthesized as proprotein precursors which are subsequently processed intracellularly. The most frequent sites of cleavage in all precursor proteins is at pairs of basic amino acids such as Arg-Arg or Lys-Arg (25). In fact, both pro-C3 and proalbumin have the sequence Arg-Arg at their cleavage site. A proalbumin processing endopeptidase has been partially characterized as a Ca^{++}-dependent serine protease which is localized in the Golgi and secretory vesicle membranes (6, 26). This endopeptidase seems to be different from another Ca^{++}-dependent endopeptidase cleaving preferentially on the C-terminal side of Lys-Arg (27, 28).

The present study demonstrated that two independent cDNAs were effectively co-transfected into the same cells. This allowed us to detect two intracellular

reactions occurring between a putative convertase and the exogenously introduced substrates (pro-C3 and proalbumin) or inhibitors (normal and variant α_1-PI). COS-1 cells used here were found to possess at least the Arg-Arg-specific processing endopeptidase, since both pro-C3 and proalbumin expressed in the transfected cells were processed to their mature forms. This activity was not inhibited by the normal α_1-PI, but strongly inhibited by the α_1-PI variant (Met^{352}→Arg), indicating that the variant inhibitor obtained by site-directed mutagenesis can be used for characterization of the convertase which has not yet been purified from any tissues.

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