Expression of Human α_2-Macroglobulin cDNA in Baby Hamster Kidney Fibroblasts: Secretion of High Levels of Active α_2-Macroglobulin†

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ABSTRACT: Human α_2-macroglobulin (α_2M) is a unique 720-kDa proteinase inhibitor with a broad specificity. Unlike most other proteinase inhibitors, it does not inhibit proteolytic activity by blocking the active site of the proteinase. During complex formation with a proteinase, α_2M entraps the proteinase molecule in a reaction that involves large conformational changes in α_2M. We describe the molecular cloning of α_2M cDNA from the human hepatoblastoma cell line HepG2. The cDNA was subcloned under control of the adenovirus major late promoter in a mammalian expression vector and introduced into the baby hamster kidney (BHK) cell line. Transformed clones were isolated and tested for production of human α_2M with a specific enzyme-linked immunosorbent assay. Human recombinant α_2M (rα_2M), secreted and purified from isolated transfected BHK cell lines, was structurally and functionally compared to α_2M purified from human serum. The results show that rα_2M was secreted from the BHK cells as an active proteinase-binding tetramer with functional thiol esters. Cleavage reactions of rα_2M with methylamine and trypsin showed that the recombinant product, which was correctly processed at the N-terminus, exhibited molecular characteristics similar to those of the human serum derived reference. Moreover, rα_2M–trypsin complex bound to purified human placental α_2M receptor with an affinity indistinguishable from that of a complex formed from serum-derived α_2M and trypsin.

The proteinase-binding plasma glycoprotein α_2-macroglobulin (α_2M)[1] inhibits proteinases from the four major classes EC 3.4.21–24 (Barrett & Starkey, 1973; Harpel, 1973). For a recent review of the biochemistry and biology of α_2M and related macroglobulins, see Sottrup-Jensen (1987).

Human α_2M is a 720-kDa homotetramer, whose 180-kDa subunits contain 1451 residues. The primary structure has been determined (Sottrup-Jensen et al., 1984b; Jensen & Sottrup-Jensen, 1986), and a human cDNA encoding pre-α_2M has been cloned from a liver library and its sequence determined (Kan et al., 1985).

Both 1:1 and 2:1 proteinase–α_2M complexes can be formed, but contrary to "classical" proteinase inhibitors, the α_2M-bound proteinase is still active, especially toward small synthetic substrates (Mehl et al., 1964; Ganrot, 1966). Proteinase binding by α_2M has been described by the "trap" hypothesis (Barrett & Starkey, 1973) in which proteolytic cleavage of a particular exposed peptide stretch near the middle of the 180-kDa subunit (the "bait" region) results in a conformational change of the α_2M tetramer (Barrett et al., 1979), thereby entrapping the proteinase.

However, a major fraction, typically >80% of the trapped proteinase, is covalently bound (Salvesen et al., 1981; Sottrup-Jensen et al., 1981; Pizzo et al., 1986). This results from reaction between ϵ-amino groups on the proteinase and an internal β-cysteinyl-γ-glutamyl thiol ester present in each α_2M subunit (Sottrup-Jensen et al., 1980; Salvesen et al., 1981; Howard, 1981). The ϵ-lysyl(proteinase)–γ-glutamyl(α_2M) bonds formed are stable. The thiol ester structure, formed from the side chains of Cys-949 and Glx-952 (Sottrup-Jensen et al., 1984b), can slowly be cleaved by small nitrogen nucleophiles such as methylamine, and it is a unique structure found only in the α-macroglobulins and in the complement proteins C3 and C4 (Tack et al., 1980; Harrison et al., 1981). The presence of active thiol esters in these proteins is revealed by a characteristic pattern of heat fragmentation (Harpel et al., 1979; Howard et al., 1980; Tack et al., 1980; Harrison et al., 1981).

While plasma α_2M is synthesized in the liver (Schreiber, 1987) by hepatocytes (Petersen et al., 1988b), other sites of synthesis exist including fibroblasts (Mosher et al., 1977) and monocytes/macrophages (Hovi et al., 1977).

α_2M–proteinase complexes are rapidly cleared from the circulation (Ohlsson, 1971; Imber & Pizzo, 1981) by high-affinity receptors on hepatocytes (Gliemann et al., 1983; Davidsen et al., 1985; Petersen et al., 1988a), macrophages (Debanne et al., 1975), fibroblasts (Van Leuven et al., 1979), and syncytiotrophoblasts (Jensen et al., 1988). A major part of the receptor recognition determinants is located in the C-terminal 138-residue domain, that can be specifically removed (Sottrup-Jensen et al., 1986; Van Leuven et al., 1986).

In order to investigate heterologous expression of biologically active α_2M, we have isolated an α_2M cDNA from the human HepG2 cell line and expressed it in a hamster kidney cell line. We report here the characterization of purified recombinant α_2M, produced at high levels, and demonstrate that it is a proteinase-binding tetramer containing functional thiol esters

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[1] Abbreviations: α_2M, α_2-macroglobulin; hα_2M, human α_2-macroglobulin; rα_2M, recombinant α_2M; FCS, fetal calf serum; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase; ELISA, enzyme-linked immunosorbent assay; STI, soybean trypsin inhibitor.

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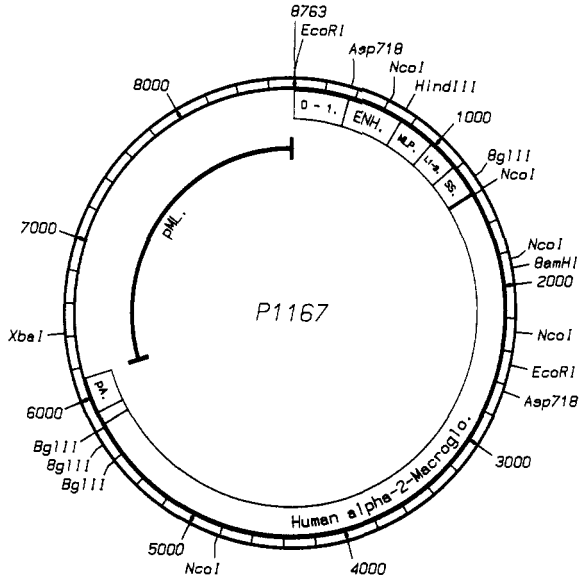

FIGURE 1: Structure of the mammalian expression vector pl167 used to obtain expression of human α_2M in BHK cells. The human α_2M cDNA is cloned under control of the adenovirus major late promoter and the SV40 polyadenylation signal. See Materials and Methods for a detailed description of, and reference to, the structural and functional elements of the vector.

and receptor-binding domains. The availability of recombinant α_2M will eventually allow a deeper characterization of structural and functional aspects of α_2M through protein engineering.

Materials and Methods

Molecular Cloning and Sequencing of Human α_2M cDNA. The human hepatoblastoma cell line HepG2 (American Type Culture Collection HB 8065) was used as a source for mRNA preparation.

Total RNA was isolated by the guanidinium thiocyanate method (Chirgwin et al., 1979) and purified by CsCl gradient centrifugation. mRNA was isolated by use of an oligo(dT)–cellulose column (Aviv & Leder, 1972). A cDNA library was constructed in the pCDVI-PL/pSP62-K2 vectors (Noma et al., 1986) by use of described methods (Okayama & Berg, 1982, 1983). The library was screened by standard colony hybridization technique (Maniatis et al., 1982).

A 20-mer oligonucleotide mixture, 5'(CCT/CTTCATG/ATCT/CTCT/CTGT/CTT)3', complementary to the human α_2M mRNA in the region encoding amino acid residues Lys_{493}-Gln-Glu-Asp-Met-Lys-Gly (Sottrup-Jensen et al., 1984b) was used as hybridization probe. A colony that showed hybridization was isolated, and the cDNA insert of the corresponding plasmid (designated pα_2M) from this isolate was sequenced by dideoxy sequencing according to the procedures of Tabor and Richardson (1987).

Construction of a Mammalian Expression Vector for α_2M. α_2M cDNA was subcloned in a mammalian expression vector, pD5, under control of the adenovirus 2 major late promoter (Ad 2 MLP). The adenovirus–promoter-based vector was constructed by K. L. Berkner (ZymoGenetics Inc., Seattle, WA); a detailed description of the functional elements in this expression vector has been published (Powell et al., 1986; Boel et al., 1987). The α_2M mRNA transcribed from the resulting 8.76-kb plasmid (designated pl167, Figure 1) has the adenovirus 2 late tripartite leader (L1-3) at its 5' end together with an mRNA splice signal (SS). At the 3' end of the construct, transcription is terminated with the SV40 late termination and polyadenylylation signals. 5' to the Ad 2 MLP, the construct includes the SV40 enhancer (ENH) and the 0 to 1 (0 – 1) map units of DNA from adenovirus 5.

Expression of Human α_2M in BHK Cells. For expression of human α_2M in cultured BHK cells (Syrian hamster kidney; Waechter & Baserga, 1982), the expression vector pl167 was cotransfected with pDHFR-I (Berkner & Sharp, 1984) into subconfluent cells by the calcium phosphate mediated transfection procedure (Graham & van der Eb, 1973). In the transfection experiment, the molar ratio of pl167 to pDHFR-I was 10:1. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

Forty-eight hours after transfection, cells were trypsinized and diluted into medium containing 400 nM methotrexate (MTX). After 10–12 days, individual colonies were cloned and expanded separately. The expanded cultures were propagated for 24 h as described above, and producer clones were identified with an ELISA (Petersen et al., 1985) directed against human α_2M secreted into the growth medium. The catching antibody used in the assay was anti-α_2M A033, and the peroxidase-conjugated antibody was anti-α_2M PE326, both from DAKOPATTS A/S, Copenhagen, Denmark.

The ELISA was specific for human α_2M [detection limit 1–2 μg/L (Petersen et al., 1985)] and did not detect α_2M in medium supplemented with 10% FCS, or in BHK cell conditioned medium. Of 24 isolated MTX-resistant clones, 16 produced detectable amounts of recombinant α_2M. Two cell lines that secreted 12.3 mg/L (K16-6) and 19.1 mg/L (K17-6) in the supernatant (grown in a six-well NUNC-plate) over a 48-h period were expanded for large-scale production of recombinant human α_2M.

Purification of Recombinant Human α_2M (rα_2M). The cell lines K16-6 and K17-6 were each expanded in a 10-double tray (NUNC, Denmark) with a growth surface of 6000 cm^2. At 80% confluency, the medium on the cells was changed from 10% FCS to 2% FCS. After 48 h of growth in medium with 2% FCS, the medium was removed, and the cells were washed twice with serum-free medium supplemented with 5 μg/mL human insulin and 5 μg/mL transferrin. Cells were then grown under serum-free conditions for 4–5 days with a change of medium every 2 days. Conditioned medium was pooled and analyzed for rα_2M by ELISA. The pooled conditioned medium from cell lines K16-6 and K17-6 contained 7.15 and 21.5 mg/L rα_2M, respectively.

rα_2M was purified by Zn^{2+} chelate chromatography (Porath et al., 1975; Sottrup-Jensen et al., 1983). Briefly, the conditioned medium (820 mL) was loaded onto a 10-mL column packed with Zn^{2+}–iminodiacetic acid–Sepharose 4B equilibrated with 25 mM Tris-HCl, pH 8.0, and washed with 100 mL of phosphate-buffered saline (PBS), pH 7.2 until $A_{280} <$ 0.036. A second wash with 20 mM sodium phosphate/500 mM NaCl, pH 6.2, was performed until $A_{280} < 0.033$. The flow rate was 100 mL/h, and 3-mL fractions were collected. rα_2M was eluted with 100 mM Na_2-EDTA, pH 7.0, at a flow rate of 40 mL/h. During elution, 1-mL fractions were collected. The rα_2M-containing fractions were concentrated to approximately 1 mL on an Amicon device equipped with a PM 10 membrane and then loaded onto a Superose 12 gel filtration column equilibrated with 25 mM Tris-HCl/150 mM NaCl, pH 8.0. In several experiments, the yield of pure rα_2M was 60–80%. The rα_2M-containing fractions from this column were pooled and stored at −20 °C until analysis.

Trypsin Protection Assay and N-Terminal Sequence Analysis of rα_2M. Assay of trypsin bound to α_2M and protected from inhibition by STI was done essentially as described (Ganrot, 1966; Sottrup-Jensen et al., 1981). Briefly, 100 μL

Table I: Silent Codon Changes in Human α_2M cDNA Isolated from Liver and HepG2 Libraries

codon	liver[a]	HepG2[b]
413 (Asn)	AAC	AAT
495 (Phe)	TTT	TTC
750 (Gly)	GGG	GGT
796 (Leu)	CTT	CTC
835 (Leu)	CTT	CTA
1266 (Ala)	GCC	GCA
1296 (Asn)	AAT	AAC
1326 (Thr)	ACC	ACA
1442 (Leu)	CTC	CTG
1460 (Ile)	ATC	ATT

[a] Kan et al. (1985). [b] Present work.

of α_2M (in 25 mM Tris-HCl/150 mM NaCl, pH 8.0) was mixed with 30 μL of trypsin (0.5 mg/mL in 20 mM sodium acetate, pH 5.0). After incubation for 2 min, 30 μL of 1 mg/mL STI (in PBS) was added. Ten-microliter aliquots were removed after 2 and 4 min and mixed with 750 μL of 0.12 mM S-2222 (*N*-benzoyl-L-Ile-L-Glu-Gly-L-Arg-*p*-nitroanilide, dissolved 0.1 M sodium phosphate pH 8.0, and 5% dimethyl sulfoxide). The initial rate of substrate hydrolysis was determined (A_{405}/min).

Amino-terminal sequence analysis was performed as described (Sottrup-Jensen et al., 1984a) using a Beckman 890C instrument.

Binding of rα_2M–Trypsin and rα_2M–Methylamine to the α_2M Receptor. The approximately 440-kDa purified α_2M receptor (Moestrup & Gliemann, 1989) was prepared by affinity chromatography from detergent-solubilized human placental membranes as described previously (Jensen et al., 1989). The receptor protein (100–150 fmol) was immobilized on nitrocellulose disks (diameter 6 mm) and incubated for 16 h at 4 °C in 200 μL of buffer with 4 fmol of ^{125}I-labeled serum-derived human α_2M–trypsin complex and varying concentrations of unlabeled hα_2M–trypsin (Jensen et al., 1989), rα_2M–trypsin, or rα_2M–methylamine. Competition assays using rat liver (Gliemann et al., 1989) or human placental (Jensen et al., 1989) membranes were performed as described previously.

Results and Discussion

Characterization of an α_2M cDNA from HepG2 Cells. The isolated plasmid pα_2M had a cDNA insert of approximately 4.6 kb including the entire coding region. In addition, the insert contained sequences derived from the 5′ and 3′ untranslated regions of the α_2M mRNA molecule. The amino acid sequence of the human α_2M, as deduced from the cDNA in pα_2M, was in agreement with the published sequence (Sottrup-Jensen et al., 1984b; Jensen & Sottrup-Jensen, 1986). Codon 1000 (numbered from the initiating methionine codon in the signal peptide) was found to be ATC encoding isoleucine and not GTC (encoding valine) as found in an α_2M cDNA synthesized from human liver mRNA (Kan et al., 1985). In the α_2M cDNA sequence from the HepG2 library, 10 silent changes were present compared with the cDNA sequence obtained from the liver library (Kan et al., 1985) (Table I).

Level of α_2M Expression in Transformed BHK Cells. Levels of α_2M present (after 48-h growth) in fully supplemented medium ranged from 0 to 21 mg/L in the 24 cell lines investigated. Several cell lines, including K16-6 and K17-6, continued to produce α_2M for several days under serum-free conditions. As judged from SDS–PAGE analysis of conditioned medium from the high producer cell line K17-6 (data not shown), strong bands attributable to α_2M were present. From a separate Mono Q ion-exchange run (data not shown), approximately 25% of the protein present was α_2M. Recombinant α_2M (rα_2M) isolated from cell lines K16-6 and K17-6 was compared with a reference preparation of human α_2M (hα_2M) prepared from serum (Sand et al., 1985).

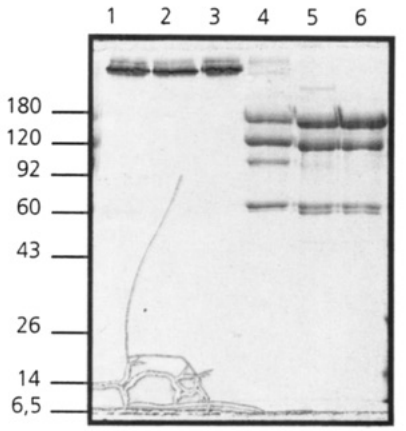

FIGURE 2: SDS–PAGE of purified hα_2M and rα_2M: lanes 1 and 4, hα_2M; lanes 2 and 5, rα_2M K16-6; lanes 3 and 6, rα_2M K17-6. Lanes 1–3 were unreduced; lanes 4–6 were reduced. The samples were analyzed on a 10–20% gel after thermal fragmentation (see text). The size and position of markers are shown (reduced human α_2M subunit, 180 kDa; large heat fragment from hα_2M, 120 kDa; human plasminogen, 92 kDa; small heat fragment from α_2M, 60 kDa; ovalbumin, 43 kDa; bovine chymotrypsinogen, 26.5 kDa; lysozyme, 14 kDa; bovine pancreatic trypsin inhibitor, 6.5 kDa). hα_2M contained a small amount of bait region cleavage fragments (approximately 85 kDa).

Thermal Fragmentation and Methylamine-Induced Thiol Ester Cleavage. When heated to 95 °C for 15 min, the thiol ester isomerizes to a lactam structure containing an internal pyroglutamic acid residue and a CysH residue (Howard et al., 1980; Khan & Erickson, 1982). Subsequent peptide cleavage at the N-terminal side of the internal pyroglutamic acid residue results in fragmentation of the 180-kDa α_2M monomer into two fragments of 120 kDa (N-terminal) and 60 kDa (C-terminal) seen in reducing SDS–PAGE (Harpel et al., 1979; Howard et al., 1980). Figure 2 shows an analysis of rα_2M from two cell lines and of hα_2M after heat treatment. All preparations showed a high degree of heat fragmentation. In the nonreduced samples, the molecules migrated as the 360-kDa disulfide-bridged α_2M dimer. In hα_2M (lane 4), a minor fragment migrating slightly faster than the 60-kDa fragment was seen. A similar component was present at higher levels in rα_2M (lanes 5 and 6). As discussed previously (Van Leuven et al., 1986; Sottrup-Jensen et al., 1986), the fast migrating component contains a variant lacking most of the complex carbohydrate group attached to Asn-1401 (Sottrup-Jensen et al., 1984b). The finding here that the intensity of this band is increased in the rα_2M preparation relative to hα_2M, and the fact that the apparent sizes of the rα_2M subunit and its large and small heat fragments are slightly lower than those of the reference preparation, indicates that rα_2M is underglycosylated, presumably affecting most if not all of the eight carbohydrate groups in the α_2M subunit (Sottrup-Jensen et al., 1984b). Minor differences in glycosylation between human plasma coagulation factor VIIa and recombinant factor VIIa expressed in BHK cells have been reported (Thim et al., 1988). After methylamine-induced cleavage of the thiol ester, thermal fragmentation of α_2M can no longer be observed (Harpel et al., 1979; Howard et al., 1980). Figure 3 shows an SDS–PAGE run in which hα_2M and rα_2M had been pretreated with 0.2 M methylamine for 2 h. Upon reduction, methylamine-treated α_2M and rα_2M migrated as a single 180-kDa monomer species, containing negligible amounts of the heat cleavage fragments. From these results, we conclude

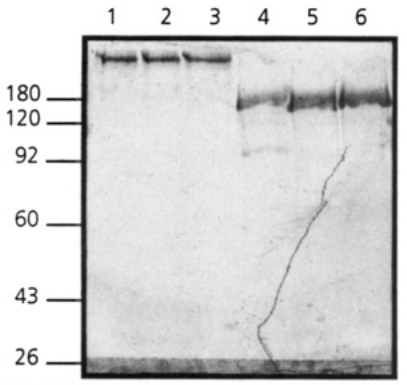

FIGURE 3: SDS–PAGE analysis of hα_2M and rα_2M after treatment with methylamine. Before electrophoresis, the α_2M preparations were heated to 95 °C for 15 min (thermal fragmentation, see text). The position and size (kilodaltons) of electrophoretic markers are shown. Lanes 1 and 4, hα_2M; lanes 2 and 5, rα_2M K16-6; lanes 3 and 6, rα_2M K17-6. Lanes 1–3 were not reduced; lanes 4–6 were reduced.

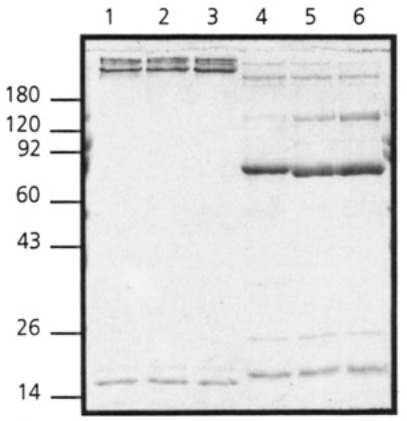

FIGURE 4: SDS–PAGE analysis of trypsin-treated hα_2M and rα_2M. The position and size (kilodaltons) of electrophoretic markers are shown. Lanes 1 and 4, hα_2M; lanes 2 and 5, rα_2M K16-6; lanes 3 and 6, rα_2M K17-6. Lanes 1–3 were not reduced; lanes 4–6 were reduced. The α_2M preparations were treated with bovine trypsin (65% active) for 2 min at a trypsin:α_2M molar ratio of approximately 2:1. Fragments from inactive trypsin are seen near the dye front, and trypsin dissociated from the complexes by reducing SDS is seen as a faint band (reducing lanes).

that rα_2M contains functionally active thiol esters and that their reactivity toward methylamine is similar to those of authentic α_2M.

Cleavage Pattern upon Reaction with Trypsin. In this reaction, trypsin will cleave at its target site(s) in the bait region of α_2M, and the resulting cleavage products will migrate as an approximately 85-kDa band under reducing conditions. Figure 4 shows the result of such an analysis of hα_2M and of the two rα_2M preparations. For each preparation, bands corresponding to reaction products larger than the 360-kDa α_2M dimer were seen under nonreducing conditions (Wang et al., 1983; Sottrup-Jensen et al., 1981). As revealed after reduction, bait region cleavage (Harpel, 1973; Barrett & Starkey, 1973; Swenson & Howard, 1979) was nearly complete in hα_2M, but in the rα_2M preparations, approximately 5–10% intact 180-kDa subunit was present. Since hα_2M was partially cleaved in its bait region, the rα_2M preparations are essentially as sensitive to trypsin cleavage as authentic α_2M.

The 85-kDa fragments derived from the recombinant material migrated slightly faster than the human standard, again suggesting that the recombinant material is underglycosylated.

When α_2M is treated with methylamine, α_2M changes conformation from a "slow" form to a "fast" form (Barrett et al., 1979; Van Leuven et al., 1981). In this conformation, it can no longer rapidly form complexes with proteinases such as trypsin (Barrett et al., 1979; Wang et al., 1981).

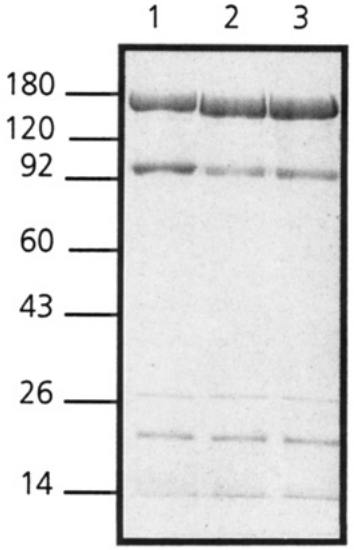

FIGURE 5: SDS–PAGE analysis of methylamine-treated hα_2M and rα_2M further reacted with bovine trypsin. The position and size (kilodaltons) of electrophoretic markers are shown. Lane 1, hα_2M; lane 2, rα_2M K16-6; lane 3, rα_2M K17-6. All samples were reduced. The α_2M preparations were first treated with 0.2 M methylamine for 2 h and then incubated with bovine trypsin (65% active) for 20 min at a trypsin:α_2M molar ratio of approximately 2:1. Nonbound trypsin and fragments from inactive trypsin are seen near the dye front.

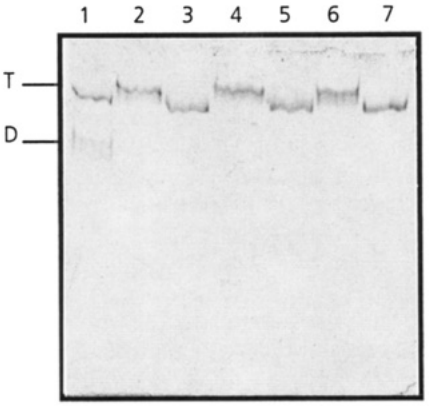

FIGURE 6: Nondenaturing PAGE of hα_2M and rα_2M before and after treatment with methylamine. Lanes 2 and 3, hα_2M; lanes 4 and 5, rα_2M K16-6; lanes 6 and 7, rα_2M K17-6. Native preparations were loaded in lanes 2, 4, and 6, and methylamine-treated preparations (0.2 M, 2 h) were loaded in lanes 3, 5, and 7. For comparison, purified human pregnancy zone protein (Sand et al., 1985) was loaded in lane 1. This macroglobulin has both a tetrameric (T) and a dimeric (D) conformation. The samples were analyzed on a 5–10% discontinuous gel according to the procedure of Van Leuven et al. (1981).

Figure 5 shows the results of a set of experiments that were run in parallel to the experiments shown in Figure 4. However, before reaction with trypsin, the native human α_2M and the rα_2M used in this experiment had been treated with methylamine. Under these conditions, both the hα_2M and the rα_2M showed a marked decrease in reactivity toward trypsin (more than 80% of the hα_2M and rα_2M monomers migrated as a 180-kDa subunit), and no high molecular weight reaction products were seen.

Mobility Shifts. The results shown in Figures 2–5 indicated that there was no significant difference between rα_2M and hα_2M, and their ability to undergo the characteristic "slow" to "fast" form conformational change (Barrett et al., 1979; Van Leuven et al., 1981) was therefore compared. Figure 6 shows the conformational change, as it appears after reaction of hα_2M and rα_2M with methylamine. For each preparation,

Table II: Comparison of the Trypsin Protection Capacity of rα_2M and hα_2M[a]

prepn of α_2M	A_{405}/min[b]	α_2M in cuvette (μg)	act. (A_{405}/min^{-1} μg^{-1})
hα_2M	0.140	5.00	0.028
rα_2M K16-6	0.111	4.62	0.024
tα_2M K17-6	0.119	4.87	0.024

[a] Adapted from Ganrot (1966). See Materials and Methods for details. [b] Average of values determined after 2- and 4-min reaction with STI.

methylamine induced the same conformational change. Separate experiments with trypsin (not shown) resulted in a similar conformational change. By comparison with hα_2M, the rα_2M was judged to be a 720-kDa tetramer (see also below).

Gel Chromatography of α_2M. The human reference α_2M preparation and the rα_2M preparation were analyzed on a 24-mL Superose 6 column equilibrated and eluted with 25 mM Tris-HCl/125 mM NaCl, pH 8.0, at a flow rate of 1 mL/min. On this column, native tetrameric α_2M eluted at 11.5 mL, while native dimeric PZP eluted at 14.2 mL (unpublished). The elution profiles were similar to those seen earlier on TSK-G-3000 SW columns (Sand et al., 1985). The elution positions of the rα_2M preparations were found to be identical with that of α_2M (not shown) in accordance with the results of PAGE (Figure 6), and showed that rα_2M was secreted from BHK cells in a tetrameric configuration.

Trypsin Protection Analysis. Trypsin bound to α_2M retains its catalytic capacity toward low molecular weight substrates such as S-2222. If trypsin is efficiently complexed with α_2M, it will be protected against a high molecular weight inhibitor like STI. The properties of rα_2M were compared with those of the hα_2M preparation. The results of a protection assay are given in Table II. From this, we conclude that rα_2M has essentially the same protection capacity for trypsin against STI as hα_2M.

If α_2M is treated with methylamine before incubation with trypsin, its protection capacity is decreased dramatically (Barrett et al., 1979; Sottrup-Jensen et al., 1980; Howard et al., 1980). In a similar assay to that described above (data not shown), methylamine-treated hα_2M only retained 17% of its protection capacity, while K16-6 and K17-6 rα_2M retained 16% and 14%, respectively. Thus, the protection capacity of rα_2M was similarly dependent on the presence of active thiol esters as that of hα_2M.

Receptor-Binding Affinity. The "fast" form of hα_2M, e.g., hα_2M–trypsin and hα_2M–methylamine, binds to the purified human placental α_2M receptor with high affinity (Jensen et al., 1989). Figure 7 shows the ability of rα_2M–trypsin and rα_2M–methylamine, as compared with hα_2M–trypsin, to compete with a trace amount of ^{125}I-labeled hα_2M–trypsin for binding to nitrocellulose-immobilized human placental α_2M receptor. The rα_2M derivatives are fully receptor-active since they can displace the bound tracer completely at high concentrations, and the affinities are not different from that of hα_2M–trypsin. The concentrations causing half-maximal displacement of ^{125}I-hα_2M–trypsin were calculated from five experiments: hα_2M–trypsin, 354 ± 25 pM; rα_2M–trypsin, 391 ± 31 pM; rα_2M–methylamine, 435 ± 34 pM (mean values ± 1 SD). Analogous experiments were carried out using receptor preparations from human placental and rat liver membranes, and similar results were obtained (not shown).

Amino-Terminal Sequence Analysis of rα_2M. α_2M characterized in the present investigation could only be bovine

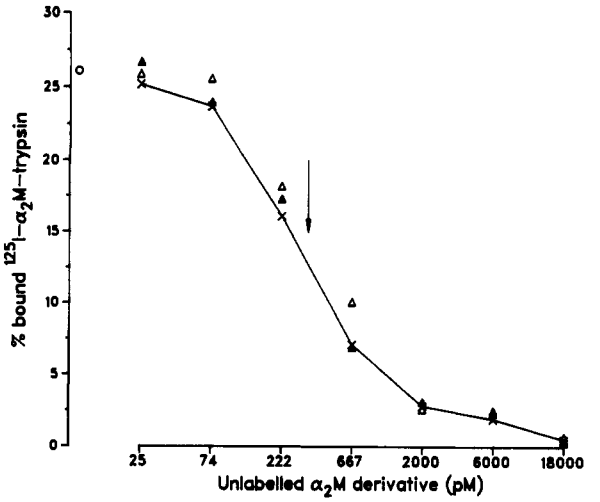

FIGURE 7: Competition of hα_2M–trypsin, rα_2M–trypsin, and rα_2M–methylamine with ^{125}I-labeled hα_2M–trypsin for binding to purified α_2M receptor. ^{125}I-Labeled hα_2M–trypsin (20 pM) was incubated with nitrocellulose-immobilized human placental α_2M receptor protein in the absence (○) or presence (×, ▲, △) of unlabeled ligand. The amount of radioactivity (1.4% of the added tracer) associated with the nitrocellulose in the presence of a saturating concentration (150 nM) of hα_2M–trypsin was regarded as nonspecific binding and was subtracted from all measurements. Unlabeled ligands are hα_2M–trypsin (×), rα_2M–trypsin (▲), and rα_2M–methylamine (△). The arrow shows the hα_2M–trypsin concentration (335 pM) causing half-maximal displacement of bound tracer. The results are the mean value of four replicate incubations from one representative experiment.

(contaminant from FCS), hamster (endogenous product from the BHK cell line), or human (derived from expression of the transfected plasmid p1167). The ELISA assay never detected any α_2M in nontransfected BHK cell conditioned medium, with or without added fetal calf serum. To characterize the amino-terminal processing of the recombinant product, sequence analysis was carried out on K16-6 and K17-6 rα_2M as described (Sottrup-Jensen et al., 1984a) for 12 cycles. The identity of the detected amino acid derivative in each cycle (data not shown) was in agreement with the amino-terminal sequence of human α_2M: Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val-. No sequence corresponding to bovine α_2M (Ala-Val-Asp-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val-) (unpublished results) was detected.

Conclusions

The present study demonstrates that baby hamster kidney fibroblasts transfected with a human α_2M cDNA cloned under control of the adenovirus 2 major late promoter can synthesize and secrete human recombinant α_2M. Clones capable of producing more than 20 mg of rα_2M per liter of growth medium were identified, and rα_2M production was high even when minimal medium devoid of fetal calf serum was used. Recombinant α_2M was purified by Zn^{2+} chelate affinity chromatography followed by a single gel chromatography step in a yield of 60–80%.

Recombinant α_2M, having the same N-terminal sequence as hα_2M, was very similar to hα_2M with regard to the following properties: (1) subunit size (approximately 180 kDa) and structure (dimer of disulfide-bridged dimers, approximately 720 kDa); (2) presence of active β-cysteinyl-γ-glutamyl thiol esters; (3) cleavage pattern upon interaction with trypsin; (4) methylamine- and trypsin-induced conformational change; (5) capacity for protecting bound trypsin from inhibition by STI; (6) inactivation of trypsin binding by methylamine; (7) receptor-binding activity of the trypsin-complexed and methylamine-treated forms.

Recombinant α_2M appeared to be underglycosylated, a property seen with other artificially expressed glycoproteins. The binding data with "fast" form rα_2M suggest that correct glycosylation is not essential for the conformation of the receptor binding domain(s). There appeared to be no difference in the content of internal thiol esters among rα_2M and hα_2M, i.e., one per 180-kDa subunit. The biosynthesis of this bond has not been clarified. The thiol-esterified Glx residue is encoded as a Gln residue (Kan et al., 1985), and, as discussed by Sottrup-Jensen (1987), the internal thiol ester may be formed in reactions reminiscent of transglutaminases, either in enzyme-catalyzed reactions or "spontaneously" during the folding of the α_2M subunit. If enzyme-catalyzed reactions are involved, the BHK cell apparently expresses the necessary enzyme(s) at adequate levels.

The availability of an expression system for human α_2M will be important for an in-depth characterization of the biosynthesis and the role of the internal thiol esters in the proteinase-binding mechanism. The function of the bait region in proteinase recognition and characterization of the determinants that specify receptor recognition can now be investigated by protein engineering.

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Ligand-Induced Myosin Subfragment 1 Global Conformational Change†

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ABSTRACT: The effects of selected ligands on the structure of myosin subfragment 1 (S1) were compared by using transient electrical birefringence techniques. With pairs of dilute solutions of S1 at 3.5 °C in low ionic strength (μ = 0.020 M) buffers that had matched electrical impedances, S1 with Mg^{2+}, MgADP, or MgADP·V_i bound was subjected to 6–7-μs external electrical fields in the Kerr law range. Specific Kerr constants and the rates of rotational Brownian motion after the electric field was removed were measured. Neither Mg^{2+} nor MgADP had a measurable effect on either observable, but when orthovanadate (V_i) bound S1·MgADP it decreased the rotational correlation coefficient from 267 ± 6 to 244 ± 10 ns. Parallel measurements of MgATPase activity indicated that S1·MgADP·V_i was greater than 95% inhibited. These results confirm the conclusion of Aguirre et al. [(1989) *Biochemistry 28*, 799] that V_i binding to S1·MgADP increases its rate of rotational Brownian motion and provide data that are more quantitatively correlated with S1 structure. The V_i-induced change in the rotational correlation coefficient is consistent with S1 becoming more flexible or more compact when V_i binds. Assuming that S1·MgADP·V_i is an analogue for S1·MgADP·P_i, the structural changes observed for S1–ligand complexes in solution are discussed in relation to possible structural changes of intermediates on the kinetic pathway of ATPase hydrolysis. A new model of force generation by S1 in muscle is hypothesized.

The nature of the adenosine 5′-triphosphate (ATP)[1]-driven structural change of the actomyosin complex, which generates force in muscle, is unknown. The binding to and hydrolysis of MgATP by actomyosin comprise the chemical event hypothesized to explain the increase in muscle tension that occurs with increasing overlap of the thick and thin filaments (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954). In solutions that approximate intracellular conditions, the kinetic pathway involves a cycle of myosin binding to and dissociating from actin for each MgATP hydrolyzed (Lymn & Taylor, 1971; Eisenberg & Kielley, 1973; Inoue & Tonomura, 1974). Force to slide filaments by one another in muscle is generated, presumably in an analogous cycle, by a myosin structural change between two bound states (Huxley, 1969; Huxley & Simmons, 1971; Nehei et al., 1974). This structural change is thought to be accompanied by an increase in the stability of the actomyosin complex and is usually characterized as some combination of the subfragment 1 (S1) portion of myosin rolling on actin, and/or S1 bending while bound, and/or actin rolling to rotate bound S1. Spectroscopically detected changes have been reported in the orientation of some but not all of the orientation-sensitive probes that have been attached to the S1 portion of myosin in fibers (Borejdo et al., 1979, 1982; Thomas & Cooke, 1980; Yanagida, 1981; Ajtai & Burghardt,

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[1] Abbreviations: M, myosin; S1, myosin subfragment 1; TEB, transient electric birefringence; Δn, birefringence; τ_L, rotational correlation coefficient (=$1/6\theta$, where θ is the rotational diffusion coefficient) obtained from the decay of the birefringence from a steady-state signal; K_{sp}, specific Kerr constant; K_a, association constant; χ_B, angular displacement of one segment of a rigid cylinder bent at the center; Δd, linear displacement of the tip of one segment of a rigid bent cylinder away from the axis of the other segment; V_i, orthovanadate; P_i, orthophosphate; N, nucleotide; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate.

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