Phenylalanine Hydroxylase Deficiency Caused by a Single Base Substitution in an Exon of the Human Phenylalanine Hydroxylase Gene†

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ABSTRACT: A novel restriction fragment length polymorphism in the phenylalanine hydroxylase (PAH) locus generated by the restriction endonuclease *Msp*I was observed in a German phenylketonuria (PKU) patient. Molecular cloning and DNA sequence analyses revealed that the *Msp*I polymorphism was created by a T to C transition in exon 9 of the human PAH gene, which also resulted in the conversion of a leucine codon to a proline codon. The effect of the amino acid substitution was investigated by creating a corresponding mutation in a full-length human PAH cDNA by site-directed mutagenesis followed by expression analysis in cultured mammalian cells. Results demonstrate that the mutation in the gene causes the synthesis of an unstable protein in the cell corresponding to a CRM^- phenotype. Together with the other mutations recently reported in the PAH gene, the data support previous biochemical and clinical observations that PKU is a heterogeneous disorder at the gene level.

Phenylketonuria (PKU) is a human genetic disorder characterized by a deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1). This disorder is transmitted in an autosomal recessive number and is a common inborn error of metabolism with a frequency of about 1 in 10000 births among Caucasians. The disease state is characterized by an accumulation of phenylalanine in the serum, and patients develop severe mental retardation (Folling, 1934). A phenylalanine-restricted diet (Bickel et al., 1957) administered to PKU patients during the first decade of life has been shown to be effective in correcting the biochemical and clinical phenotypes. Previous biochemical and clinical observations have suggested that PAH deficiency is a very heterogeneous disease (O'Flynn et al., 1980). The wide distribution of plasma phenylalanine levels measured during the screening of newborns and upon the administration of a defined protein load is an expression of this heterogeneity (Trefz et al., 1985). These data collectively suggest the existence of multiple mutations in the PAH gene and the possibility of compound heterozygosity in the patients. The isolation of a full-length human PAH cDNA (Kwok et al., 1985) has provided a critical reagent to begin the characterization of PKU at the molecular level.

Hybridization analysis using the PAH cDNA clone as the probe has revealed extensive restriction fragment length polymorphism (RFLP) in the human PAH locus (Woo et al., 1983; Lidsky et al., 1985). RFLP haplotype analysis of the PAH locus in a Northern European population has revealed the presence of several prevalent haplotypes comprising 90% of all mutant alleles (Chakraborty et al., 1987). An association between certain prevalent mutant haplotypes and various hyperphenylalaninemic phenotypes has also been observed (Güttler et al., 1987). These data demonstrate that DNA haplotypes may be associated with specific molecular defects as had been demonstrated in the Thalassemias (Orkin et al., 1982). More recently, two specific mutations in the PAH gene have been reported (DiLella et al., 1986b, 1987) which are tightly linked to the respective RFLP haplotypes due to linkage disequilibrium. In this paper, we report a third PKU mutation in the PAH gene that is associated with a novel RFLP.

Experimental Procedures

Materials

Restriction enzymes were purchased from New England Biolabs and used according to manufacturers' specifications. *Escherichia coli* DNA polymerase I (Klenow fragment) was obtained from Boehringer Mannheim, while ^{32}P- and ^{35}S-labeled nucleotides were a product of Amersham. Nitrocellulose membranes employed for library screening and Southern Western blot analysis were supplied by Schleicher & Schuell. The M13 replicative form (RF) DNAs and M13 sequencing primers were purchased from P-L Biochemicals. AuroProbe BLplus-IntenSG II was purchased from Janssen Life Sciences Products.

Clinical Data. The patient (HS3) was found to have PKU through diagnosis in the newborn screening program and was treated early by a phenylalanine-restricted diet. Diagnosis of "classical PKU" was made at the age of 6 months by employing the following criteria: (1) plasma phenylalanine levels over 20 mg % after an oral protein load (180 mg/kg body weight of phenylalanine over 3 days; Blaskovics et al., 1974); (2) no detectable in vitro enzyme activity of phenylalanine hydroxylase (liver needle biopsy material; Bartholome et al., 1975); and (3) no measurable in vivo hydroxylation (intravenous deuteriated phenylalanine load; Trefz et al., 1979). Psychomotoric development of the patient was normal (Hannover-Wechsler-Intelligenztest fuer das Vorschulalter; Schuck & Eggert, 1975) and revealed above-average results at the age of 6 years, although plasma phenylalanine levels

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have frequently reached levels above 10 mg during infancy. This was the result of a very low phenylalanine tolerance (10–13 mg/kg body weight of phenylalanine since the age of 4 years), where even very rigid dietary treatment did not result in plasma phenylalanine levels below 8 mg %.

Methods

Southern Blotting. Genomic DNA was isolated from white blood cells, digested with restriction enzymes, electrophoresed on 0.8% agarose gels, transferred to nitrocellulose membranes, and hybridized to a ^{32}P nick-translated PAH cDNA probe (2×10^8 cpm/μg) as described previously (Woo et al., 1983).

Construction and Screening of a Genomic DNA Library from the PKU Patient. High molecular weight genomic DNA (50 μg) isolated from lymphocytes of the PKU patient (HS3) was digested to completion with the restriction endonuclease *Eco*RI. DNA fragments were dephosphorylated with calf intestine alkaline phosphatase (Frischauf et al., 1983) and ligated to the phage vector λ2001 digested with *Eco*RI (Karn et al., 1984): λ2001 is a vector that confers the Spi selection, such that only recombinant phages can grow in P2 lysogen-containing host cells. The ligated DNA was packaged in vitro by using extracts prepared according to the procedure of Hohn (1979), yielding a total of 1.2×10^6 recombinant phages. The library was screened by using the in situ amplification method of Woo (1979). The probes utilized in screening the library were derived from a full-length PAH recombinant plasmid designated phPAH247 (Kwok et al., 1985).

Clone Characterization and DNA Sequence Analysis. DNA from the plaque-purified recombinants was prepared according to the liquid lysate procedure of Blattner et al. (1977). Cloned inserts were electrophoretically analyzed with respect to both size and the presence of the new *Msp*I restriction site following digestion with *Eco*RI or *Eco*RI/*Msp*I, respectively. The identity of the inserted DNA was verified by Southern blotting. An internal 550 base pair (bp) *Bam*HI fragment was shown to contain the new *Msp*I restriction site. This 550 bp *Bam*HI fragment was inserted into the *Bam*HI site of the M13 vector mp18 and subjected to DNA sequence analysis by the dideoxy chain termination method of Sanger et al. (1977), utilizing the modifications described by Biggin et al. (1983).

Site-Directed Mutagenesis of PAH cDNA. The base substitution was generated in an M13mp18 recombinant containing the full-length PAH cDNA insert by site-specific mutagenesis. The double-priming approach was employed, in which one of the two priming oligonucleotides contains the single base change reflecting the mutation. The mutagenic primer was 5′-phosphorylated, and the two primers were annealed to the single-stranded M13mp18 PAH DNA, followed by repair synthesis using *E. coli* DNA polymerase I (Klenow fragment) in the presence of T4 DNA ligase was described by Zoller and Smith (1984). The DNA was used to transform competent *E. coli* TG-1 host cells. Seventy M13mp18 transformants were obtained and screened by using the mutant oligonucleotide, resulting in five positive clones. The authenticity of the mutant clone was established by DNA sequence analysis of the mutagenized site.

Expression Analysis of the Mutant Recombinant. The mutant PAH recombinants were amplified in TG-1 cells following infection. Double-stranded RF DNA was prepared according to Messing (1983) followed by *Eco*RI excision of the 2.3-kilobase (kb) inserts from both the mutant and normal PAH cDNA recombinants. The eukaryotic expression recombinants were constructed by ligation of the gel-purified cDNA fragments into the *Eco*RI site of the expression vector

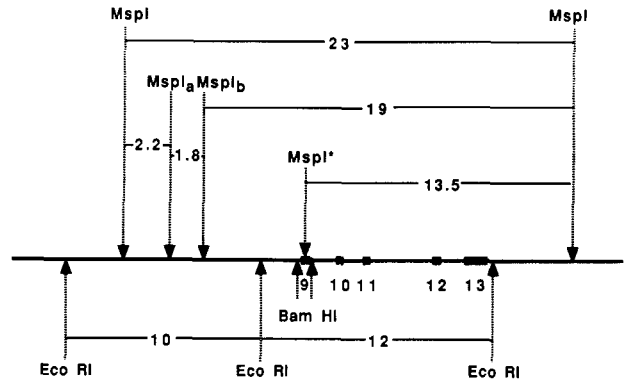

FIGURE 1: Schematic representation of the 3′ end of the human PAH gene, showing the 10- and 12-kb fragments generated by the restriction endonuclease *Eco*RI. *Msp*I$_a$ and *Msp*I$_b$ represent the known polymorphic *Msp*I sites in the gene, and the novel polymorphic *Msp*I site is indicated by the asterisk. The fragment sizes are shown in kilobases, and exons 9–13 of the gene in the 12-kb *Eco*RI fragment are shown as boxes.

p91023(B) (Wong et al., 1985), and transformed HB101 cells were selected on tetracycline (7.5 μg/mL) LB plates. The orientation of the inserted cDNA was determined by digestion with the restriction endonuclease *Xho*I. DNA from recombinant plasmids containing the normal and mutant cDNA inserts in the sense orientation with respect to the adenovirus major late promoter in the vector was used for transfection into COSM6 cells according to Wong et al. (1985). RNA was isolated from the transfected cells and analyzed by dot-blot and Northern blot hybridization as described previously (Ledley et al., 1985). Cellular protein extracts were analyzed immunologically by Western blot using an affinity-purified goat antibody to rat PAH (Robson et al., 1982) and Immunogold silver-staining rabbit anti-goat IgG. The protein extracts from the transfected cells were assayed for PAH activity as previously described (Ledley et al., 1985).

RESULTS

A Novel MspI Polymorphism in the PAH Gene. During RFLP haplotyping analysis of a German PKU family (HS), a novel RFLP generating by *Msp*I was observed. There are two known polymorphic *Msp*I sites in the human PAH gene (Figure 1): *Msp*I$_a$ results in the 23-kb versus 19-kb polymorphism, and *Msp*I$_b$ causes a 4.0-kb versus 2.2-kb + 1.8-kb polymorphism (Lidsky et al., 1985). The positions of these two polymorphic sites in the PAH gene have subsequently been defined (DiLella et al., 1986a). In this family, both the father and the affected child showed a hybridizing band of 13.5 kb in length (Figure 2). Since the patterns of other restriction endonuclease digests remain unchaged (data not shown), the new *Msp*I band does not appear to be the result of gross deletion or insertion in the gene. Instead, it was likely that one of the paternal PAH genes contained a point mutation within the 23-kb *Msp*I fragment, generating an additional *Msp*I site. Because this was the allele that had been transmitted to the PKU child, it was necessary to determine if this was a structural mutation and whether this mutation caused PAH deficiency.

Molecular Cloning and Sequence Characterization of the Mutation. The map positions of the established polymorphic *Msp*I sites as well as their flanking *Msp*I sites in the PAH gene are known. Since one of the large polymorphic *Msp*I fragments (either 23 or 19 kb) had decreased in length, it is likely that the new *Msp*I site is located within the 12-kb *Eco*RI fragment (Figure 1). A genomic DNA library was thus constructed from *Eco*RI-digested DNA of the PKU patient,

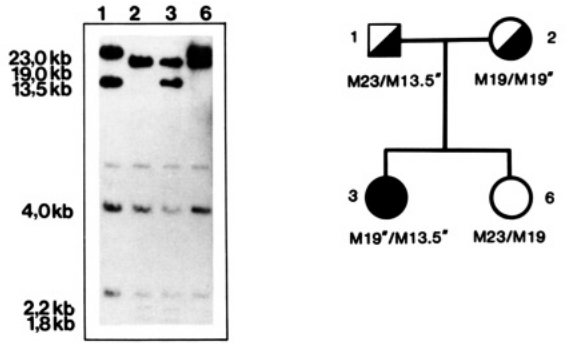

FIGURE 2: Southern blot analysis of a German PKU family (HS), showing the new *Msp*I RFLP in the PAH locus. DNA was isolated from lymphocytes of the family members. Ten micrograms of the genomic DNAs was digested with the restriction endonuclease *Msp*I and subjected to Southern blot analysis using phPAH 247 as the hybridization probe. Lane 1, father; lane 2, mother; lane 3, affected child; lane 4, unaffected sibling. The segregation of the mutant PAH alleles (quotation marks) with the appropriate polymorphic bands is shown schematically on the righ-hand panel.

resulting in 1.2×10^6 recombinants. Screening of the library using a nick-translated cDNA fragment corresponding to exons 4–13 of the gene yielded 152 signals. Fifty-six primary signals were rescreened by using a cDNA probe corresponding to exons 6–13 of the gene, and 42 recombinants were detected. Of these, 35 were isolated and analyzed by *Eco*RI digestion: 33 contained the 12-kb and 2 the 10-kb *Eco*RI fragments.

To distinguish the two alleles with and without the new *Msp*I site, eight phage clones containing the 12-kb insert were double digested with *Eco*RI and *Msp*II. Three clones were resistant to *Msp*I digestion, while five clones yielded fragments of 11.5 and 0.5 kb. The result also indicated that the new *MspI* restriction site must be located at either end of the fragment and is most likely to be near the 5′ end of the 12-kb *Eco*RI fragment in order to generate the 13.5-kb *Msp*I fragment (Figure 1). The clone was digested with *Bam*HI, which cuts the vector at the cloning site and the insert twice at its 5′ end, generating three fragments of 300 bp, 550 bp, and 11 kb in length. The 550 bp fragment was known to contain exon 9 of the gene (DiLella et al., 1986a) and shown to contain the new *Msp*I site by restriction digestion of gel-purified DNA (data not shown). Thus, the 550 bp *Bam*HI fragments from both alleles were subcloned into the vector M13mp18 for sequence analysis. The results revealed that the new *Msp*I site was generated by a T to C transition (Figure 3), corresponding to nucleotide 1154 in the cDNA (Kwok et al., 1985) which is within exon 9 of the human PAH gene. The point mutation caused the conversion of a leucine codon to a proline codon at residue 311 of the enzyme.

Nucleotide Substitution in the PAH Gene Is a PKU Mutation. To investigate whether the amino acid substitution is responsible for PKU, expression studies in cultured mammalian cells by gene transfer were carried out after creating the corresponding mutation in the full-length PAH cDNA by site-directed mutagenesis. Both the mutant and normal PAH cDNA inserts were excised from the respective recombinants and subcloned into the eukaryotic expression vector p91023(B). The constructs were then transfected into the Simian kidney cell line COSM6 in a transient expression assay. After 72 h, the cells were harvested, and crude protein extracts of the cells were assayed for PAH enzymatic activity. The results showed that the extract from cells transfected with the normal cDNA insert was capable of converting [^{14}C]phenylalanine into [^{14}C]tyrosine, while that from cells transfected with the mutated cDNA insert did not (Figure 6A).

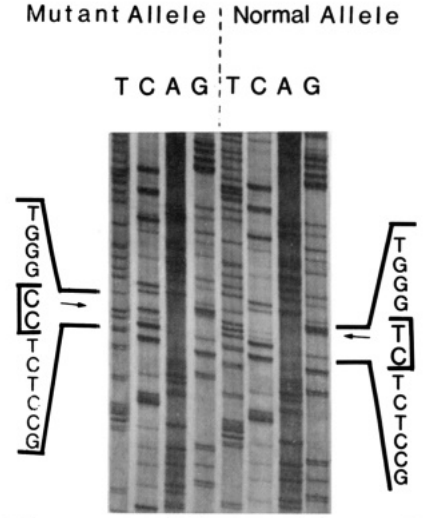

FIGURE 3: DNA sequence of a fragment of the normal and mutant human PAH genes, showing a T to C transition within exon 9 of the gene. The mutant allele is shown on the left and the normal allele on the right.

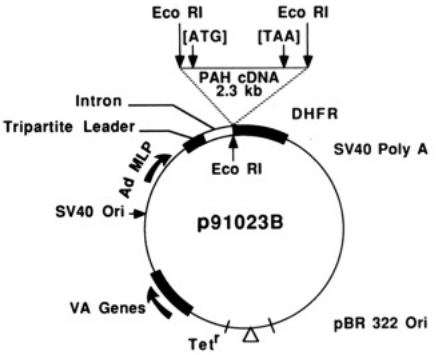

FIGURE 4: Schematic representation of the normal and mutant PAH cDNA fragments inserted into the *Eco*RI site of the eukaryotic expression vector p91023(B).

In order to determine whether the absence of detectable enzymatic activity is secondary to the lack of PAH mRNA or the protein, steady-state levels of the mRNA and PAH in the transfected cells were determined. Northern as well as dot-blot analyses of serially diluted RNA samples from cells transfected with both the normal and mutant cDNAs showed that their PAH mRNA contents were both qualitatively and quantitatively comparable (Figure 5). Cellular extracts were also assayed for the presence of immunoreactive PAH by Western analysis. The extract of cells transfected with the normal cDNA clone displayed a band at about 50 kilodaltons (kDa) (Figure 6B, lanes 2–6) which is identical with the native protein synthesized in a human hepatoma cell line (Figure 6B, lane 1). An equal amount of protein extract from cells transfected with the mutant PAH cDNA clone did not result in any immunoreactive PAH (Figure 6B, lanes 7 and 8). Since the detection limit of the analysis was a hundredfold below the amount of normal protein used in this analysis (Figure 6B, lanes 3–6), it can be estimated that cells transfected with the mutant cDNA contained less than 1% of cytoplasmic PAH relative to that of the normal cDNA.

Discussion

While performing RFLP haplotype analysis of the PAH locus in a German PKU family, we observed a novel *Msp*I site in one of the mutant alleles. This site was created by a T to C transition in exon 9 of the gene and resulted in the substitution of leucine by proline at residue 311 of the protein.

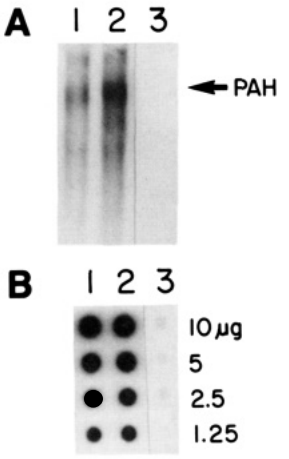

FIGURE 5: Analysis of PAH RNA in COSM6 cells transfected with the normal and mutant PAH cDNAs. (Panel A) Northern blot hybridization using PAH cDNA as the probe. Ten micrograms of total cellular RNA was applied in each lane. Lane 1, normal PAH cDNA; lane 2, mutant PAH cDNA; lane 3, mock-transfected cells. (Panel B) Quantitative analysis by dot-blot hybridization. Serially diluted RNA samples containing 10, 5, 2.5, and 1.25 μg of total RNA were applied from normal PAH cDNA (column 1), mutant PAH cDNA (column 2), and mock-transfected cells (column 3).

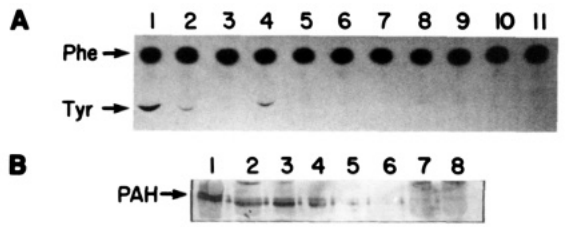

FIGURE 6: (Panel A) PAH activity assay using crude cellular extracts of transfected cells in the presence and absence of the synthetic cofactor 6-methyl-5,6,7,8-tetrahydropterin as noted under Experimental Procedures. The assay measures the conversion of [^{14}C]phenylalanine to [^{14}C]tyrosine. Crude human liver extract (150 μg) served as a positive control (lane 1). 500 μg of protein from cells transfected with the normal PAH cDNA + cofactor (lanes 2 and 4) and without cofactor (lanes 3 and 5); 500 μg of protein from cells transfected with the mutant PAH cDNA + cofactor (lanes 6 and 8) and without cofactor (lanes 7 and 9); mock-transfected cellular extract + cofactor (lane 10) and without cofactor (lane 11). (Panel B) Western blot analysis by Immunogold silver staining of PAH protein expressed by the transfected cells. Lane 1, crude human liver extract (15 μg). Lanes 2–6 contained 400, 80, 16, 3.2, and 0.64 μg of protein extract from COSM6 cells transfected with normal PAH cDNA, respectively. Lanes 7 and 8 contained 400 μg each of protein from cells transfected with mutant PAH cDNA.

Mutagenesis and expression analyses have verified that the amino acid substitution caused the lack of accumulation of the corresponding protein and the nucleotide substitution must represent a novel mutation in the PAH gene that causes PKU. Since L-prolyl residues cannot occur within a right-handed α-helix (Schimmel & Flory, 1968), its presence can cause sharp turns in an α-helix and dramatically alter the secondary structure of a protein, which can affect the active sites of enzymes and/or their intracellular stabilities.

It has recently been reported that proteolytic cleavage of a 5-kDa fragment from the carboxyl terminus and a 11-kDa fragment from the amino terminus of native PAH (52 000 daltons per subunit) did not abolish the enzymatic activity of the PAH molecule (Iwaki et al., 1986). Thus, the catalytic determinants of the enzyme must reside within an internal 36-kDa fragment. The amino acid substitution observed in exon 9 of the mutant gene occurred about 14 kDa from the carboxyl terminus and lies within the enzymatic core of the protein.

The absence of any detectable steady-state PAH in the cells transfected with the mutant cDNA can be explained by the production of either an unstable PAH molecule or a structurally altered protein such that it is nonimmunoreactive with the antiserum. The former possibility is more likely for two reasons: (i) known leucine by proline substitutions in the α- and β-globin chains have been shown to result in unstable protein (Goosens et al., 1982; Bunn et al., 1977); and (ii) the antibody against PAH is a polyclonal antiserum preparation that is likely to recognize multiple epitopes and is indeed capable of detecting subfragments of the enzyme. Thus, the new mutation in the PAH gene reported in this paper corresponds to a CRM$^-$ phenotype.

Recently, a splicing mutation involving a GT to AT transition at the donor splice site of intron 12 in the PAH gene has been identified to be a PKU mutation, resulting in the skipping of the preceding exon during pre-mRNA splicing and the synthesis of an unstable protein (DiLella et al., 1986; Marvit et al., 1987). A second PKU mutation involving a missense mutation of Arg^{408} to Trp^{408} as the result of a T to C transition in exon 12 of the PAH gene has also been characterized (DiLella et al., 1987). The current report establishes a third mutation in the PAH gene that causes PKU and provides additional support to the concept that PKU is a heterogeneous disorder at the gene level.

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Binding of α_2-Macroglobulin–Thrombin Complexes and Methylamine-Treated α_2-Macroglobulin to Human Blood Monocytes†

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ABSTRACT: The binding of α_2-macroglobulin (α_2M) to human peripheral blood monocytes was investigated. Monocytes, the precursors of tissue macrophages, were isolated from fresh blood by centrifugal elutriation or density gradient centrifugation. Binding studies were performed using ^{125}I-labeled α_2M. Cells and bound ligand were separated from free ligand by rapid vacuum filtration. Nonlinear least-squares analysis of data obtained in direct binding studies at 0 °C showed that monocytes bound the α_2M–thrombin complex with a K_d of 3.0 ± 0.9 nM and the monocyte had 1545 ± 153 sites/cell. Thrombin alone did not compete for the site. Binding was divalent cation dependent. Direct binding studies also demonstrated that monocytes bound methylamine-treated α_2M in a manner similar to α_2M–thrombin. Competitive binding studies showed that α_2M–thrombin and methylamine-treated α_2M bound to the same sites on the monocyte. In contrast, native α_2M did not compete with α_2M–thrombin for the site. Studies done at 37 °C suggested that after binding, the monocyte internalized and degraded α_2M–thrombin and excreted the degradation products. Receptor turnover and degradation of α_2M–thrombin complexes were blocked in monocytes treated with chloroquine, an inhibitor of lysosomal function. Our results indicate that human monocytes have a divalent cation dependent, high-affinity binding site for α_2M–thrombin and methylamine-treated α_2M which may function to clear α_2M–proteinase complexes from the circulation.

α_2-Macroglobulin (α_2M)[1] is a plasma proteinase inhibitor that binds proteolytic enzymes of all four classes (Barrett & Starkey, 1973). α_2M–proteinase complexes are bound, internalized, and degraded by tissue macrophages of the mouse and rabbit (Debanne et al., 1976; Kaplan & Nielsen, 1979a,b; Imer & Pizzo, 1981). The goal of this study was to determine whether the human peripheral blood monocyte, the precursor of the tissue macrophage (Ebert & Florey, 1939), has a

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M-Ma, methylamine-treated α_2M; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HBSS, Hank's balanced salt solution supplemented with $NaHCO_3$ (4 mM) and HEPES (10 mM), pH 7.1; HBSS–BSA, HBSS with 0.5% bovine serum albumin; EDTA, disodium ethylenediaminetetraacetate; TCA, trichloroacetic acid.

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