PROTEOLYSIS AT A LYSINE RESIDUE ABOLISHES THE RECEPTOR-RECOGNITION SITE OF α_2-MACROGLOBULIN COMPLEXES

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1. Introduction

α_2-Macroglobulin is a unique protease inhibitor in the circulation of vertebrates by its steric mode of complexation of virtually all endoproteases [1]. The α_2M–protease complexes are rapidly cleared from blood and body fluids by receptor-mediated endocytosis by fibroblasts [2,3], macrophages [4,5] and Küpffer cells [6]. The receptor is highly discriminative and recognizes specifically α_2M–protease complexes [3,5].

Reaction with methylamine [7] transforms α_2M to a conformation indistinguishable from that of α_2M–protease complexes, by criteria of rate electrophoresis and isoelectric focusing [8]. Furthermore, the methylamine-derivative (α_2M · MA) is recognized and internalized by fibroblasts [8,9] and macrophages [10,11]. The receptor-recognition site is thus part of the α_2M polypeptide chains [8], hence complexes of any protease bound to α_2M will be cleared by receptor-mediated endocytosis [2].

To examine the structure recognized by the cellular receptor on complexed α_2M, we have prepared monoclonal antibodies towards methylamine-inactivated α_2M. A hybridoma (F2B2) produced an antibody specifically reacting with complexed α_2M (either protease or methylamine generated) thereby preventing receptor-mediated endocytosis of the complex [12]. Fab portions derived from the antibody (F2B2) were equally potent in inhibiting receptor-mediated endocytosis. This was taken as evidence that the antigenic site recognized by the antibody was part of the receptor-recognition site on the α_2M complexes. Furthermore proteolysis of α_2M complexes by trypsin abolished both receptor-mediated endocytosis of, and antibody binding to, the complexes [12]. Since this proteolysis seemed very limited, further characterization of the receptor-recognition site on α_2M complexes by this approach was indicated [12].

Here, we have examined the proteolysis of α_2M complexes, probed by receptor-mediated endocytosis and F2B2 antibody binding. The results indicate a lysine residue to be involved in both phenomena.

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2. Materials and methods

Endoproteinase Lys-C was from Boehringer. Trypsin (TPCK-treated) was from Worthington. 2,4,6-Trinitrobenzenesulfonate (TNBS) was from Sigma. Carrier-free $Na^{125}I$ was from the Radiochemical Centre, Amersham. Cyclohexane-1,2-dione was from Aldrich.

2.1. *Receptor-mediated endocytosis*

Normal human skin fibroblasts (NHF) were cultured as in [13]. The purification of α_2M from human plasma, the iodination and assay for receptor-mediated endocytosis are published procedures [3,8,14].

2.2. *Derivatization of α_2M*

Plasma α_2M was treated with 0.2 M methylamine in Tris–HCl (50 mM, pH 8.0) for 3 h at 37°C. The protein was isolated by gel filtration (ACA22, Ultrogel, LKB).

Trinitrobenzoylation of α_2M · MA was done essentially as in [15], except that $NaHCO_3$ buffer (0.1 M) was used and 1 mM TNBS. The reaction was followed

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spectrophotometrically at 367 nM. Reductive methylation of α_2M · MA was done as in [16,17]. Arginine residues were blocked with cyclohexane-1,2-dione, as in [18], in 0.2 M borate buffer (pH 8.5) overnight at room temperature.

2.3. *Rate electrophoresis*

Native proteins were examined by rate electrophoresis on 5% polyacrylamide gels [8,17].

2.4. *Trypsin activity*

Enzymatic activity of trypsin was measured spectrophotometrically as in [8]. Benzoyl-arginine–*p*-nitroanilide (BAPNA) was the substrate and activity is expressed as increase in absorbance per minute at 410 nm, pH 8.2 and 37°C.

3. Results

3.1. *Proteolysis of the receptor-recognition site*

As described, α_2M complexes stored with excess free trypsin showed a selective loss of the F2B2 determinant [12]. A major drawback for further analysis of the proteolytic process was its sluggishness primarily because extensive autodigestion of trypsin occurred [12]. Therefore, we examined conditions under which the proteolysis of the receptor-recognition site would become amenable to analysis. We preferred to use α_2M–methylamine (α_2M · MA) complexes, to circumvent primary proteolysis of the bait region in α_2M–protease complexes [19].

Advantage was taken of the well-known effect of Ca^{2+} on the autodigestion of trypsin. α_2M · MA was incubated at room temperature with 20% (w/w) trypsin with and without 20 mM Ca^{2+}. Samples, taken after different time intervals (up to 20 h), were treated with STI and examined by rate electrophoresis (5% polyacrylamide gels), with and without preincubation with the monoclonal antibody F2B2.

The α_2M · MA complexes migrated normally in rate electrophoresis, and showed only a minor increase in mobility, even after 20 h incubation with trypsin (fig.1A). Nevertheless, a progressive loss of F2B2 determinant had occurred and a complete loss of binding was observed in the 20 h sample containing Ca^{2+} (fig.1B). As judged from these gels no extensive loss of α_2M was apparent, which was confirmed by rocket immunoelectrophoresis: 90% or more of the original amount of α_2M was recovered after 20 h incubation in all samples (not shown).

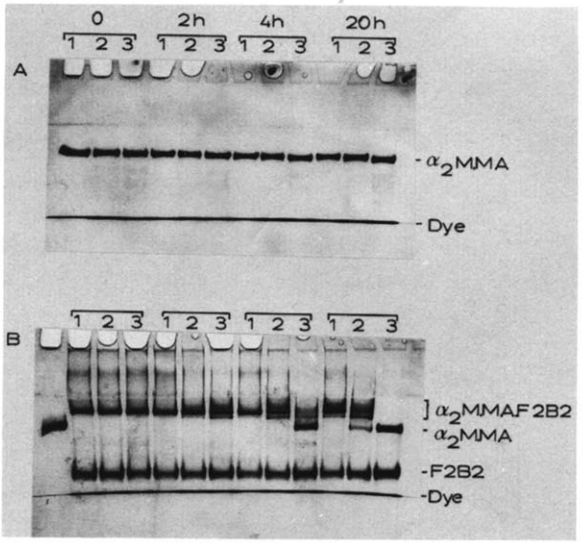

Fig.1. Time course of tryptic loss of F2B2-antigenic site on α_2M · MA. Effect of calcium. α_2M · MA (1 mg/ml) was incubated with trypsin: (1) at 50 μg/ml; (2) at 200 μg/ml; (3) at 200 μg/ml plus 20 mM $CaCl_2$. The mixtures were buffered at pH 8.0 (50 mM Tris–HCl) and kept at 37°C. At the time periods indicated, portions (20 μg α_2M) were taken and either examined (A) directly by rate electrophoresis (5% polyacrylamide gels) or (B) incubated with the monoclonal antibody F2B2 (30 μg IgG) for 10 min at room temperature before rate electrophoresis. The first lane in panel B contained the α_2M · MA, prepared as described in the text, not treated with trypsin or F2B2. Note the difference in pattern of the 20 h samples in panel B: sample (3) (trypsin + Ca^{2+}) no longer bound the F2B2-antibody. The mobility of the proteins is indicated at the right and is similar in the following figures.

3.2. *Identification of the amino acid residue involved*

The narrow specificity of trypsin for arginine and lysine residues allowed us to obtain some information on the amino acid residue(s) actually involved in the proteolysis of the F2B2 determinant.

In preliminary experiments, the arginine side chains on α_2M · MA complexes were derivatized with cyclohexane-1,2-dione [18]. No effect was observed on binding of the monoclonal antibody to the derivatives, while the loss of the determinant by trypsin proceeded, if anything, somewhat faster than on the untreated α_2M · MA complexes (not shown). Receptor-mediated endocytosis of these complexes was also unaffected (table 1).

When the lysine residues on α_2M · MA were derivatized (either by trinitrobenzoylation or by reductive methylation), binding of the monoclonal antibody to these complexes was abolished. The same treatment

Table 1
Effect of derivatization of α_2M on binding of monoclonal antibody F2B2 and on receptor-mediated endocytosis

Additions	F2B2 binding	Receptor-mediated endocytosis (% inhibition)
α_2M	–	4
α_2M · MA	+	59
α_2M · MA–trypsin	–	6
α_2M · MA–TNB	–	9
α_2M · MA–(*N,N'*)dimethyl	–	14
α_2M · MA–CHD	+	61

Normal human fibroblast monolayers were incubated at 37°C for 30 min with 50 μg/ml ^{125}I-labeled α_2M · trypsin complexes in serum-free medium without or with addition of the derivatives listed, at 200 μg/ml. Results are expressed relative to control (no addition) and are the mean of duplicate determinations on separate cell layers. Binding of the monoclonal antibody F2B2 was examined by rate electrophoresis on 5% polyacrylamide gels [12]. Derivatives of α_2M were prepared as described in the text: α_2M · MA was either treated with 20% (w/w) trypsin or derivatized with 2,4,6-trinitrobenzenesulfonate (α_2M · MA–TNB), $NaBH_4$ and formaldehyde (α_2M · MA–(*N,N'*)dimethyl) or cyclohexane-1,2-dione (α_2M · MA–CHD). Protein was freed from excess reactant by gel filtration

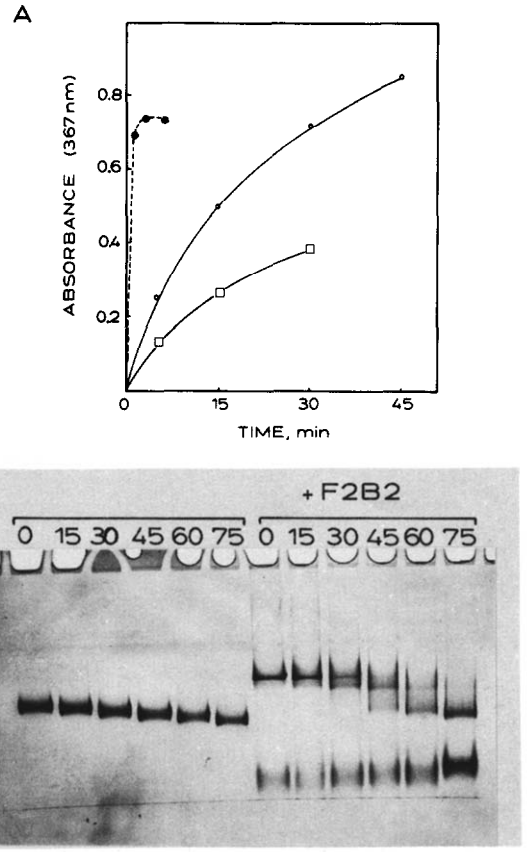

Fig.2. Trinitrobenzoylation of α_2M · MA. (A) α_2M · MA was derivatized with 2,4,6-trinitrobenzenesulfonate (1 mM final conc.) in 0.1 M $NaHCO_3$, at room temperature. The extent of reaction was measured spectrophotometrically at 367 nm. Two concentrations of α_2M · MA were examined: 0.22 mg/ml (3×10^{-7} M (□)) and 0.44 mg/ml (6×10^{-7} M (○)). Lysine (0.03 mM (●)) was treated with TNBS under similar conditions. (B) α_2M · MA (0.5 mg/ml) in 0.1 M $NaHCO_3$ was treated with TNBS (1 mM). At the time points indicated, lysine (100 mM, pH 8.0) was added (5 mM final conc.) to stop the reaction. For the zero time point lysine was added prior to TNBS. Rate electrophoresis (5% polyacrylamide gels) of the derivatives without (left) and after addition of the monoclonal antibody F2B2 (right) to the same samples, is shown.

also affected receptor-mediated endocytosis (table 1). In fact, the very close correlation between normal binding of the monoclonal antibody on the one hand and inhibition of receptor-mediated endocytosis on the other [12] was considerably extended by these findings.

The extent of derivatization of α_2M · MA complexes with trinitrobenzenesulfonate was followed spectrophotometrically at 367 nm, while in parallel, the derivative was examined by rate electrophoresis on 5% polyacrylamide gels. Whereas TNBS reacted practically immediately with lysine, reaction with α_2M · MA continued for at least 45 min (fig.2A). This was accompanied with a progressive increase in electrophoretic mobility of α_2M · MA (fig.2B) and more importantly, with a progressive decrease in binding of the monoclonal antibody F2B2. After 75 min reaction with TNBS subsequent binding of the antibody was nearly completely abolished (fig.2B).

A further indication of the presence of a lysine-residue in the F2B2 determinant was obtained with endoproteinase Lys-C, a serine endoproteinase of bacterial origin specific for lysine residues. This protease was very active in provoking proteolytic loss of the F2B2 determinant on α_2M · MA complexes (fig.3). Incubation of α_2M · MA with endoproteinase Lys-C, inhibited with TLCK or aprotinin, did not abolish the subsequent formation of F2B2-α_2M · MA complexes.

4. Discussion

The proteolytic degradation of the receptor-recognition site on α_2M · methylamine complexes was

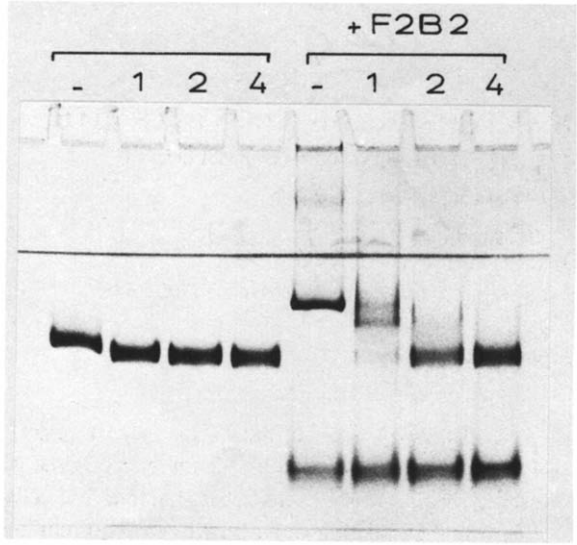

Fig.3. Proteolytic loss of F2B2-determinant by endoproteinase Lys-C. α_2M · MA (1 mg/ml) was incubated with endoproteinase Lys-C (2 mg lyophilisate/ml) in Tris · HCl (50 mM) pH 8.0 at 37°C. After 1, 2 and 4 h, the enzyme was inactivated by addition of TLCK (2 mM final conc.). Control (lane denoted by –) was incubated without the enzyme under otherwise identical conditions. Rate electrophoresis (5% polyacrylamide gel) is shown for these samples (left) and after treatment with the monoclonal antibody F2B2 (right). Note the minor increase in mobility of the protease treated α_2M · MA, and the practically complete loss of F2B2-determinant after 4 h incubation.

probed by a monoclonal antibody and by receptor-mediated endocytosis. Trypsin digestion of these complexes (in their native conformation without denaturation) was very limited, even after prolonged incubation. Incubation with Ca^{2+} accelerated the degradation of the receptor-recognition site, probably by protecting trypsin towards autodigestion.

Derivatization of the lysine residues, but not the arginine residues, on α_2M · methylamine blocked binding of the monoclonal antibody as well as receptor-mediated endocytosis. A lysine-specific endoprotease from bacterial origin proved as potent as trypsin in degrading the receptor-recognition site. These data indicated the presence of at least 1 lysine residue at or near the receptor-recognition site. This lysine residue seemed not abnormally reactive, as a close parallel was observed between overall trinitrobenzoylation and loss of F2B2 binding.

These data extend the analogy between the receptor-recognition site and the F2B2 antigenic determinant. Both were similarly affected by the different modifications, as listed in table 1. This extensive evidence that the F2B2-antigenic determinant is at least part of the receptor-recognition site on α_2M complexes will allow us to locate this site, along the α_2M polypeptide chain.

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