Isolation and renaturation of α_2 -macroglobulin receptor from diploid human fibroblasts

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α_2 -Macroglobulin receptor was extracted from human diploid fibroblasts and purified by affinity chromatography in a single step. The receptor had mol.wt. 125 000 after sodium dodecyl sulphate(SDS)/polyacrylamide-gel electrophoresis. The isolated receptor was separated by SDS/polyacrylamide-gel electrophoresis, transferred on to nitrocellulose sheets and subsequently renatured, as shown by a specific binding test, by incubation with Nonidet P40.

α_2 -Macroglobulin (α_2 M) is one of the major human serum proteinase inhibitors. It is a high-molecular-weight (725 000) glycoprotein consisting of four identical subunits (James, 1980). α_2 M is a potent inhibitor of nearly all endopeptidases examined. The formation of the proteinase– α_2 M complex requires the splitting of a sensitive peptide bond within the α_2 M. This leads to a drastic conformational change and results in a physical trapping of the proteinase (Starkey & Barrett, 1977).

Human fibroblasts express specific binding sites for α_2 M on their plasma membranes. Binding studies with cultured human fibroblasts have shown that the receptor preferentially binds the α_2 M–proteinase complex, whereas native α_2 M has a much lower affinity (Van Leuven *et al.*, 1979). The receptor has recently been solubilized from mouse tumour fibroblasts (Hanover *et al.*, 1983).

In the present paper we describe the isolation of the receptor from human diploid fibroblasts. After transfer on to nitrocellulose sheets and subsequent renaturation, the isolated receptor bound the α_2 M–proteinase complex.

Experimental

Cell culture

Human diploid fibroblasts were obtained from young healthy volunteers by skin biopsy and grown in DMEM medium (Gibco, Wiesbaden, Germany)

Abbreviations used: SDS, sodium dodecyl sulphate; DMEM, Dulbecco's minimal essential medium; α_2 M, α_2 -macroglobulin; α_2 M–T, α_2 M–trypsin complex; IgG, immunoglobulin G.

supplemented with 10% (v/v) foetal-calf serum (Gibco), 2% pyruvate, 2 mM-glutamine and non-essential amino acids (Sando & Neufeld, 1977). The cells were maintained at 37°C under CO₂/air (1:9).

Labelling of cells

After preincubation of fibroblasts for 1 h in leucine-free DMEM medium, the cellular proteins were labelled with [³H]leucine by adding, per 680 cm² roller bottle, 750 μ Ci of L-[4, 5-³H]leucine (130 Ci/mmol) (Amersham Buchler, Braunschweig, Germany). Cells were labelled for 16–24 h. α_2 M–T was prepared as described by Van Leuven *et al.* (1979). The biologically active human α_2 M used in all experiments was kindly provided by Dr. N. Heimbürger and Dr. U. Vogel (Behringwerke, Marburg, Germany). Protein A (Pharmacia, Frieburg, Germany) was labelled by reductive methylation as described by Jantoft & Dearborn (1979).

Isolation of the receptor

The α_2 M–T receptor was isolated from the subconfluent [³H]leucine-labelled human fibroblasts grown in roller bottles. Cells were scraped from the bottles with a rubber policeman and washed three times with serum-free DMEM medium. After centrifugation (250 g for 10 min at 4°C) the cell pellet was resuspended in 30 ml of the same medium without serum and supplemented with 250 μ g of freshly prepared α_2 M–T complex. After incubation for 4 h at 4°C with shaking, the cells were washed four times with ice-cold phosphate-buffered saline (1.5 mM-KH₂PO₄/8.4 mM-Na₂HPO₄/2.7 mM-KCl 0.14 M-NaCl/1 mM-CaCl₂, pH 7.4) to remove any

unbound $\alpha_2\text{M-T}$. The cell pellet was gently lysed in an equal volume of 10 mM-potassium phosphate buffer (pH 7.4)/1% Nonidet P40/1% albumin/2 mM- CaCl_2 , for 30 min at 0°C with shaking. Intact cells and cellular debris were removed by centrifugation at $20\,000g$ for 30 min. Portions of the resultant supernatant were subsequently incubated either with 20 mg of protein A-Sepharose (Pharmacia), preloaded with a specific rabbit antiserum against human $\alpha_2\text{M}$ (Behringwerke), or preloaded with rabbit preimmune serum. The binding of the solubilized receptor- $\alpha_2\text{M-T}$ complex to protein A-Sepharose was performed in 10 mM-potassium phosphate buffer (pH 7.4)/1% albumin/0.25% Nonidet P40/1 mM- CaCl_2 . After a 3 h incubation at 4°C the protein A-Sepharose was washed with the binding buffer until no more radioactivity could be detected in the supernatant. After three subsequent washing steps with 0.125 M-Tris/HCl buffer, pH 6.8, the bound receptor was eluted by boiling the protein A-Sepharose in $10\,\mu\text{l}$ of SDS solubilizer (Laemmli, 1970).

SDS/polyacrylamide-gel electrophoresis was performed, under reducing conditions, as described by Laemmli (1970) with ^{14}C -labelled molecular-weight-standard proteins: phosphorylase *b* (mol.wt. 92 000), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000), and lysozyme (14 000) (Amersham Buchler). Fluorography of the SDS/polyacrylamide gels was performed as described by Laskey & Mills (1975). In order to renature the $\alpha_2\text{M}$ receptor the protein was isolated from unlabelled cells as described above. After SDS/polyacrylamide-slab-gel electrophoresis the gel was incubated for 2 h in 4 M-urea. The proteins were subsequently electrophoretically transferred on to nitrocellulose sheets in 4 M-urea at 6 V/cm for 16 h (Burnette, 1981). After transfer the nitrocellulose sheet was briefly washed with 50 mM-potassium phosphate buffer, pH 7.4. This step and all other incubations were done at 4°C . The receptor was renatured by incubating the nitrocellulose sheet in 50 mM-potassium phosphate buffer (pH 7.4)/3% (w/v) bovine serum albumin (to block unspecific binding sites on nitrocellulose)/0.25% Nonidet P40/1 mM- CaCl_2 for 24 h with several changes of the buffer. After incubation of the nitrocellulose sheet the renatured receptor was detected with $100\,\mu\text{g}$ of $\alpha_2\text{M-T}$ complex in 12 ml of 50 mM-potassium phosphate buffer (pH 7.4)/0.25% Nonidet P40/1% bovine serum albumin/1 mM- CaCl_2 , followed by further incubation with either specific rabbit anti-(human $\alpha_2\text{M}$) antiserum or rabbit preimmune serum for another 4 h in the same buffer. Finally the nitrocellulose sheet was incubated with 3×10^5 c.p.m. of ^{14}C -labelled protein A (sp. radioactivity 60 000 c.p.m./ μg) in 12 ml of 50 mM-potassium phosphate buffer (pH 7.4)/1% bovine serum albumin/0.25% Nonidet P40/1 mM- CaCl_2 for 30 min, rinsed

in 50 mM-potassium phosphate buffer/1 mM- CaCl_2 , pH 7.4, dried and exposed to a Kodak XR film.

Results and discussion

Human fibroblasts were exposed to $\alpha_2\text{M-T}$ complex for 4 h at 4°C to achieve complete saturation of the $\alpha_2\text{M}$ receptors before subsequent lysis in a Nonidet P40-containing buffer (see the Experimental section). The conditions for solubilization used were so gentle that the $\alpha_2\text{M-T}$ -receptor complex was not destroyed but rather was enriched in the supernatant and could be separated from the bulk of the insoluble proteins. Nonidet P40 proved to be the most suitable detergent for the isolation of the receptor under the conditions tested.

The receptor was isolated and purified to homogeneity from the [^3H]leucine-labelled lysate by single-step chromatography on a protein A-Sepharose column preloaded with specific anti-(human $\alpha_2\text{M}$) IgG. The isolated receptor was homogeneous as judged by the fact that a single radiolabelled protein band was obtained on SDS/polyacrylamide-gel electrophoresis (Fig. 1, lane 2); this band corresponded to apparent mol.wt. 125 000.

In order to verify our results and to demonstrate the receptor character of the isolated membrane protein, we bound the $\alpha_2\text{M-T}$ complex to the isolated receptor after renaturation on nitrocellulose sheets. An unlabelled Nonidet P40 cell lysate was prepared and the receptor isolated by single-step chromatography as described above. The eluted receptor was subjected to SDS/polyacrylamide-gel electrophoresis and subsequently transferred on to a nitrocellulose sheet. Binding experiments showed that the receptor was biologically inactive after SDS/polyacrylamide-gel electrophoresis and transfer, and could only bind the $\alpha_2\text{M-T}$ complex after renaturation. Fig. 1 (lane 4) shows that the transferred, renatured receptor protein binds the $\alpha_2\text{M-T}$ complex, which is detected after binding of rabbit anti-(human $\alpha_2\text{M}$) IgG to the receptor-ligand complex followed by incubation with ^{14}C -labelled protein A. The renatured receptor co-migrated with the protein isolated directly from [^3H]leucine-labelled cells (Fig. 1, lane 2). Some minor bands were seen with mol.wts. of $\sim 50\,000$ – $60\,000$. These bands are non-specific, as can be seen in the control experiment (Fig. 1, lane 3). In this control experiment the nitrocellulose sheet was exposed to a high concentration of rabbit preimmune serum after the same renaturation procedure followed by incubation with $\alpha_2\text{M-T}$. Despite the huge amount of non-specific IgG used, no band of mol.wt. 125 000 could be detected. Only the non-specific lower-molecular-weight bands were seen in addition to the heavy and light chains of IgG, which bind labelled protein A.

Fig. 1. Electrophoretic analysis of purified and renatured α_2 M receptor

14 C-labelled marker proteins (lane 1), [3 H]leucine-labelled purified α_2 M receptor (lane 2), and unlabelled purified α_2 M receptor (lanes 3 and 4) were separated on an SDS/10% (w/v)-polyacrylamide slab gel. Lanes 1 and 2 were further processed for fluorography. Proteins separated on lanes 3 and 4 were transferred on to a nitrocellulose sheet and renatured. After incubation with α_2 M-T the nitrocellulose strips were either incubated with pre-immune serum (lane 3) or with specific anti-(human α_2 M) antibodies (lane 4) and further processed for autoradiography (see the Experimental section). The α_2 M receptor is indicated by down-pointing arrows.

It should be mentioned that renaturation could only be achieved under the experimental conditions described if the transfer was performed in 4 M-urea instead of the buffer system originally used for 'Western' blotting (Burnette, 1981).

The isolation procedure used for the α_2 M receptor resulted in the isolation of a single protein band. This

suggests that the receptor is either a monomer or a polymer of this polypeptide. On the other hand, we cannot completely rule out the possibility that the receptor consists of different subunits that could have been lost during the isolation procedure (e.g. solubilization and dissociation by Nonidet P40). Whatever the subunit composition is, our results demonstrate that only one subunit is necessary for the binding of the α_2 M-proteinase complex.

Similar results have been described for the acetylcholine receptor (Saitoh *et al.*, 1980). This receptor consists of three different subunits and only one of them can bind acetylcholine (or the acetylcholine analogue bungarotoxin).

Unambiguous evidence that the isolated protein is either the α_2 M-proteinase receptor or one of its subunits is that we were able to renature the protein (after SDS/polyacrylamide-gel electrophoresis and blotting) and bind the α_2 M-proteinase complex to the renatured receptor.

The simple isolation procedure from solubilized cells allows the purification of the α_2 M receptor in quantities sufficient for the production of monoclonal antibodies.

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