In vitro biosynthesis of the human cell surface receptor for transferrin

C. Schneider, U. Asser, D.R. Sutherland and M.F. Greaves

Membrane Immunology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

Received 6 May 1983

The human cell surface receptor for transferrin is a transmembrane phosphoglycoprotein composed of two disulphide linked and apparently identical subunits of M_r 90 000. Using an affinity purified, polyclonal rabbit antibody, we have studied the in vitro biosynthesis of this receptor. The primary translation product, synthesised in a rabbit reticulocyte lysate programmed with human placental RNA, appears to have the same M_r (78 000) as the unglycosylated molecule immunoprecipitated from tunicamycin-treated cells. In the presence of a dog pancreatic microsomal system the cell free system accurately reproduces the glycosylation and the asymmetric transmembrane integration

Human transferrin receptor *In vitro translation* *In vitro processing* *Asymmetric transmembrane integration* *Human placenta RNA*

1. INTRODUCTION

The in vitro synthesis of many secretory proteins and of some membrane glycoproteins has been studied in detail [1,2]: a cell-free system reproduced cell features required for the synthesis of protein and its insertion into the endoplasmic reticulum.

We have used a monoclonal antibody (OKT9) to study the human, cell-surface receptor for transferrin. This receptor is a transmembrane phospho–glycoprotein composed of two disulphide linked subunits of 90 kDa. It has 3 asparagine-linked glycan chains [3] and covalently-bound fatty acid [4]. Many of these features are common to other membrane glycoproteins (e.g., H-2, HLA, viral glycoproteins) for which details of in vivo and in vitro biosynthesis are known [5,6].

Abbreviations: SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DTT, dithiothreitol; NEN,*N*-ethyl maleimide; M_r *N* relative molecular mass

Here, we examine the in vitro biosynthetic product from rabbit reticulocyte lysate programmed with human placental RNA. Using an affinity purified polyclonal antibody raised in a rabbit against the SDS–PAGE purified, reduced and alkylated transferrin receptor, we characterise the in vitro synthesised molecule and follow its processing and membrane insertion.

2. MATERIALS AND METHODS

Human placentas were obtained from University College Hospital, London (courtesy of Professor Fairweather).

2.1. *Preparation of anti-transferrin receptor antibody*

Transferrin receptor was purified from foetal liver or placenta by cross-linked immunoaffinity chromatography [7]. The eluate from the affinity column was dialysed against PBS containing 1% SDS, concentrated by pressure dialysis, reduced in the presence of 20 mM DTT at 100°C for 4 min, cooled at 4°C and alkylated by adding 70 mM

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00145793/83/$3.00

NEM for 2 h at 4°C [8]. The sample was then separated after boiling in SDS sample buffer containing 1% β-mercaptoethanol, by preparative SDS–PAGE on 7% polyacrylamide, 2 mm thick slab gels [9]. At the end of the run the gels were stained in Coomassie brilliant blue 0.2% (w/v) – water for 30 min and destained in water. The band containing the transferrin receptor was cut out, homogenised in 1 ml PBS and emulsified in 1.5 ml complete Freund's adjuvant. Equivalents 100 μg of transferrin receptor were injected subcutaneously into 15 sites on the back of a female New Zealand rabbit. Booster injections in incomplete Freund's adjuvant were given at 1 week intervals for 3 weeks. The rabbits were boosted after a further month and bled 2 weeks later.

2.2. *Affinity purification of rabbit antibodies to denatured transferrin receptor*

Transferrin receptor was purified by gel electrophoresis as above. The gel slices were homogenised and extracted overnight in 1% Li-dodecylsulphate (LDS), 1 M LiCl, 50 mM triethanolamine (TEA) (pH 7.5). After overnight dialysis the material containing about 500 μg pure transferrin receptor was added to 500 mg Eupergit C according to the manufacturer's protocol (Rohm Pharma Weiterstadt). Aliquots (1 ml) of the rabbit serum were passed through the column and after washing the column with 10 column vol. (30 ml) of PBS containing 0.05% NP40, the antibody was eluted by 0.1 M HCl and immediately neutralised with Tris to pH 7.0. It was dialysed overnight against PBS and stored in 0.2% gelatin at −20°C.

2.3 *Preparation of RNA*

Placental tissue was washed in cold PBS several times to remove blood, and then homogenised in 7 M urea–3 M LiCl in a Sorvall-omnimixer for 3 min at 1 g of tissue/10 ml LiCl–urea solution. The homogenate was left overnight at 4°C and then centrifuged at 10000 × g in a SS-34 Sorvall for 20 min. The pellet was extracted twice with phenol–chloroform (1:1, v/v) and the final aqueous layer was made 0.2 M in ammonium acetate (pH 5) and 70% in ethanol, and left at −20°C.

2.4 *Cell-free translation*

Rabbit reticulocyte was used as recommended by the supplier [New England Nuclear (NEN) Boston MA] and supplemented with 6 mM 2-aminopurine. Total RNA (15 μg) was added for each 25 μl translation mixture. After translation for 90 min at 31°C, the reaction was stopped by adding 1 vol. 2% SDS in 10 mM EDTA (pH 7.4) and immediately boiled at 100°C for 1 min. When dog pancreatic microsomal membranes were used (also obtained from NEN), the reaction mixture was instead layered on 5% sucrose in 0.1 N NaOH and centrifuged at 100000 × g in a Beckman airfuge for 30 min. The pellet (containing the membranes) was resuspended in 1% SDS–10 mM EDTA and immediately boiled at 100°C for 3 min.

2.5. *Trypsin digestion*

For the post-translational trypsin digestion of the products synthesised in the presence of dog pancreatic microsomes, the reaction was stopped by adding $CaCl_2$ to a final concentration of 5 mM. The trypsin was added at 500 μg/ml to 5 × 25 μl reaction mixture and left at 4°C for 1 h. Digestion was terminated by addition of soybean trypsin inhibitor (5 mg/ml) and EDTA 10 mM followed by SDS solubilisation.

2.6. *Immunoprecipitation*

Lysate (150 μl; 3 × 25 μl lysate equivalents; see section 2.4) was diluted 6-fold in 2.5% NP40 200 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.02% NaN_3. Normal rabbit serum (10 μl) was added and after 1 h at 4°C, 100 μl of heat-killed, formalin-fixed *Staphylococcus aureus* Cowan I strain (SaCl) was added and left for a further 30 min. The bacteria were pelleted in an Eppendorf microfuge and the preclearance was repeated twice. Finally, 20 μl of affinity-purified antibodies (about 5 μg of pure antibody) were added together with 5 mg cytochrome c 0.5 M NaCl. The antibodies were left overnight at 4°C and then precipitated by adding 50 μl of a 50% protein A Sepharose-4BCl slurry for 30 min. The beads were washed twice in 1 ml of 0.5 M NaCl, 10 mM Tris, 0.5% NP40 and once in 0.15 M NaCl, 10 mM Tris (pH 8.1), 0.5% NP40, 0.1% SDS boiled for 2 min in gel sample buffer [9] and supernatants analysed by SDS–PAGE [9] and fluorography [11].

2.7. *Endoglycosidase (endo-H) digestion*

HSB-2 cells (a T-lymphoblastoid cell-line) were pulse-labelled with [^{35}S]methionine for 10 min. Cell lysis and immunoprecipitation with OKT9 was performed as in [3]. When rabbit antibody was used, the cell lysate was adjusted to 1% (w/v) SDS and boiled at 100°C for 1 min as above. Processing of immune complexes and endo-H digestion were performed as in [12, 13].

2.8 *Tunicamycin treatment*

Washed HSB-2 cells were suspended in methionine-free Eagle's E4 medium (5×10^6/ml) supplemented with tunicamycin (a gift from Dr Hamill, Ely Lilly Corp.) at 10 μg/ml. The cells were incubated at 37°C for 2 h before addition of [^{35}S]methionine (4 mCi/10^8 cells) and then further incubated for 10 min or 2 h before extraction and immune complex formation with specific antibodies as above.

3. RESULTS

3.1. *Identification of the transferrin receptor as synthesised in vitro*

A rabbit antiserum to the purified, denatured transferrin receptor subunit was produced as in section 2. This antiserum was shown to immunoprecipitate the transferrin receptor from SDS-denatured lysates labelled with [^{35}S]methionine (fig. 1A, track a); the characteristics are the same as that immunoprecipitated by the monoclonal antibody OKT9 [3]. The rabbit antibody was in some respects a superior reagent for analysing the tunicamycin-treated form of the receptor as the 'denaturing' treatment of the cell lysate seemed to clear the background of non-specifically immunoprecipitated (presumably aggregated) labelled proteins.

The in vitro synthesis of the transferrin receptor subunit could be demonstrated by immunoprecipitation of total translation products from a rabbit reticulocyte lysate programmed with human placental RNA. This primary translation product has the same app. M_r (~78 000; fig. 1B, track c) as the polypeptide immunoprecipitated from cells pulse-labelled for 10 min in the presence of tunicamycin (fig. 1B, track b). When cells are labelled for 2 h in the presence of tunicamycin,

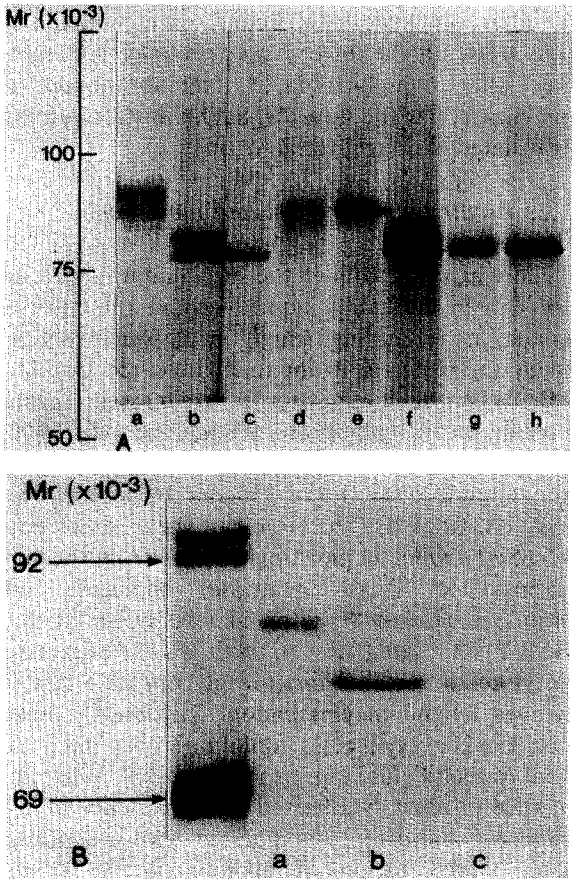

Fig. 1. Autoradiograph of [^{35}S]methionine-labelled transferrin receptor immunoprecipitated with affinity-purified rabbit antibody. (A) Immunoprecipitates from HSB-2 cells [^{35}S]methionine labelled for 2 h in the absence (track a) or in the presence (track b) of 10 μg tunicamycin/ml. Immunoprecipitate of transferrin receptor synthesised in vitro in the absence (track c) or in the presence (track d) of dog pancreas microsomal membranes (membranes were pelleted through a 5% sucrose layer/0.1 N NaOH and solubilised in 1% SDS). HSB-2 cells were pulse-labelled for 10 min with [^{35}S]methionine: the immunoprecipitated transferrin receptor (track e) was digested with 0.1 mV (track f) or 1 mV (track h) of endoglycosidase-H. Transferrin receptor synthesised in vitro in the presence of dog pancreas microsomal membranes was digested with 1 mV of endoglycosidase-H (track g). All samples were run on a gradient gel of 7–12% acrylamide. (B) Transferrin receptor immunoprecipitated from HSB-2 cells labelled for 10 min in the absence (track a) or in the presence (track b) of 10 μg tunicamycin/ml. Track c, transferrin receptor immunoprecipitated from in vitro-translated placental RNA. All samples were run on 8% SDS–polyacrylamide gel, 30 cm long.

another polypeptide is immunoprecipitated (fig. 1A, track b); the lower band, probably representing the non-glycosylated, membrane-inserted precursor, has the same M_r as the in vitro primary translated product (fig. 1A, track c).

3.2. *In vitro glycosylation and membrane insertion*

Core glycosylation to the high mannose precursor can be faithfully reproduced in the cell-free system by including rough microsomal vesicles from dog pancreas in the translation reaction [14, 15]. When such microsomes are present during translation of placental RNA, the M_r 78 000 primary translation product (fig. 1A, track c) is converted into a series of 3 higher M_r polypeptides (fig. 1A, track d). The largest of these has an app. M_r 85 000 which is indistinguishable from the core glycosylated (high mannose) molecule immunoprecipitated from an in vivo pulse labelled HSB-2 cell lysate (fig. 1A, track e). The oligomannosyl cores of the immature glycoprotein can be cleaved by the enzyme endoglycosidase-H; digestion yields polypeptides containing only the proximal *N*-acetyl glucosamine residues [12]. When the in vitro product synthesised in the presence of dog pancreas microsomes is digested with a supraoptimal concentration of endoglycosidase-H, the polypeptide which is produced (fig. 1A, track g) has the same M_r as the 10 min in vivo pulse-labelled molecule treated in parallel with endo-H (fig. 1A, track h). In addition, the two digested products comigrate at the same position in a two-dimensional isoelectric focusing/SDS–PAGE gel system (not shown).

The other polypeptides present in the in vitro product synthesised in the presence of dog pancreas microsomes (fig. 1A, track d) correspond to the stepwise addition of oligomannosyl core units. Treating the in vivo synthesised molecule immunoprecipitated after a 10 min pulse with suboptimal concentration of endoglycosidase-H results in a similar pattern of intermediate bands (fig. 1A, track f).

Insertion of the translated product into the rough endoplasmic reticulum could be reproduced in the cell free system by including dog pancreas microsomes in the translation reaction. The translation mixture containing the dog pancreas microsomes was pelleted through a 5% sucrose layer containing 0.1 N NaOH. This treatment strips off all the extrinsic non-integrated synthesised proteins but the transferrin receptor subunit can still be immunoprecipitated from the solubilised pellet. Proof that the products were co-translationally integrated into the dog pancreas microsomal membranes in a transmembrane configuration comes from an experiment in which the post-translational products were incubated with trypsin. Each of the intact, membrane integrated polypeptides (fig. 2, track b) appeared to be digested to a corresponding membrane-protected

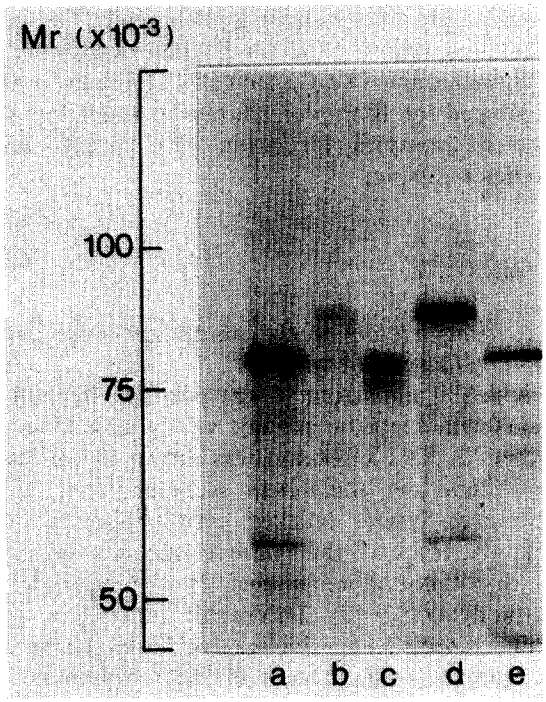

Fig. 2. Transferrin receptor immunoprecipitated with the rabbit antibody from a reticulocyte lysate translation system in the absence (track e) or in the presence (track b) of dog pancreas microsomal membranes during translation of human placental RNA. Track (c), in vitro translation of placental RNA in the presence of dog pancreas microsomes followed by trypsin digestion (section 2) and immunoprecipitation of the transferrin receptor. Track d, in vivo synthesised transferrin receptor after immunoprecipitation with monoclonal antibody OKT9 from a 10 min labelled [^{35}S]methionine HSB-2 cell lysate and (track a) digestion of the same with 1 mV of endoglycosidase-H. All samples were run on a gradient gel of 7–12% acrylamide.

fragment M_r 78 000 reduced in size by about 6 kDa/fig. 2, track c). This trypsin cleavage product is easily distinguishable from the one obtained by treating detergent-lysed [^{35}S]methionine pulse-labelled cells with trypsin (M_r 67 000; not shown). In fact, there is a trypsin-sensitive cleavage site on the extracellular facing side of the mature molecule which originates a M_r 70 000 trypsin-resistant fragment [3].

4. DISCUSSION

Here, we have examined the cell free synthesis, glycosylation and membrane insertion of the human cell surface receptor for transferrin. This membrane protein exists as a disulphide-linked dimeric complex. It has been assumed, although no direct proof exists, that the two M_r 90 000 subunits are identical [3]. The data reported here are in accord with this interpretation since only one major primary translation product could be detected.

It is now well established that secretory proteins and some membrane proteins are synthesised on polyribosomes bound to the endoplasmic reticulum. The formulation and subsequent elaboration of the signal hypothesis [1] has led to the view that the specificity of this interaction is associated with the amino-terminal sequence of the nascent chain, usually associated with the presence of a detachable, hydrophobic 'signal' sequence. There appears to be no absolute requirement for a detachable signal sequence, as ovalbumin, and some membrane proteins such as rhodopsin [16] and *Escherichia coli* lactose permease [17] are synthesised and exported without removal of a peptide.

We were not able to detect a difference in M_r for the primary translation product of the transferrin receptor synthesised in a cell free system that might reflect the existence of a detachable signal peptide; i.e., the 'in vivo' unglycosylated, but membrane-inserted protein, (immunoprecipitated from pulse-labelled cells preincubated in tunicamycin), had the same relative mobility as the primary translation product. Furthermore, by adding dog pancreas microsomes, no apparent processing to a smaller M_r, representing the integrated, unglycosylated and signal sequence cleaved intermediate, could be detected.

Our failure to detect such a difference in relative mobility representing a cleavable signal peptide could be explained as:

(i) The possible increase of hydrophobicity contributed by the signal peptide resulting in increased SDS-binding and a higher mobility of the protein in SDS–PAGE; the resulting small difference in mobility may not be resolvable by this technique;
(ii) The possibility that the precursor peptide is retained in vivo in the presence of tunicamycin as suggested in the case of the IgM μ chain [18].

Explanation (ii) seems unlikely, since the same result is obtained in a 10 min pulse or a 2 h labelling in the presence of tunicamycin; furthermore the polypeptide so obtained has an $M_r \sim 500$ lower than the endo-H-treated high-mannose form of the molecule, accounting for 3 *N*-acetylglucosamine residues still remaining after digestion. Alternatively, the transferrin receptor may provide another example of a membrane protein with no cleavable signal sequence. At present we cannot distinguish between these interpretations. Sequencing of cloned cDNA corresponding to the transferrin receptor mRNA should provide a definitive answer.

Finally, we were able to demonstrate the membrane integration and glycosylation of the transferrin receptor in vitro by adding dog pancreas microsomes to the reticulocyte lysate. Both processes appeared to be identical to the 'in vivo' biosynthesis of the receptor. Due to the slower kinetics of the in vitro system it was possible to identify all the intermediates in the core glycosylation process, including a population of non-glycosylated molecules.

ACKNOWLEDGEMENTS

We are grateful to Miss U. Asser for helpful technical assistance, to Dr M.J. Owen for helpful suggestions and to the staff of University College Hospital, Obstetrics Department, for placentas. We wish to thank Mrs J. Needham for her patience in typing this manuscript.

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