Receptor recognition sites reside in both lobes of human serum transferrin

Anne B. MASON^{*1}, Beatrice M. TAM[†], Robert C. WOODWORTH^{*}, Ronald W. A. OLIVER[‡], Brian N. GREEN^{‡§}, Lung-Nan LIN^{||}, John F. BRANDTS[¶], Kerry J. SAVAGE[†], Janet A. LINEBACK^{**} and Ross T. A. MACGILLIVRAY[†]

^{*}Department of Biochemistry, University of Vermont, College of Medicine, Burlington, VT 05405, U.S.A., [†]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3, [‡]Biological Materials Analysis Research Unit, Department of Biological Sciences, University of Salford, Salford M5 4WT, U.K., [§]VG Organic Ltd., Altrincham, Cheshire WA14 5RZ, U.K., ^{||}Department of Chemistry, University of Massachusetts at Amherst, Lederle Graduate Research Center, Amherst, MA 01003, U.S.A., [¶]Microcal, 22 Industrial Drive East, Northampton, MA 01060, U.S.A., and

^{**}Department of Medical Laboratory Sciences, Florida International University, Miami, FL 33199, U.S.A.

The binding of iron by transferrin leads to a significant conformational change in each lobe of the protein. Numerous studies have shown that the transferrin receptor discriminates between iron-saturated and iron-free transferrin and that it modulates the release of iron. Given these observations, it seems likely that there is contact between each lobe of transferrin and the receptor. This is the case with chicken transferrin, in which it has been demonstrated unambiguously that both lobes are required for binding and iron donation to occur [Brown-Mason and Woodworth (1984) *J. Biol. Chem.* **259**, 1866–1873]. Further support to this contention is added by the ability of both N- and C-domain-specific monoclonal antibodies to block the binding of a solution containing both lobes [Mason, Brown and Church (1987) *J. Biol. Chem.* **262**, 9011–9015]. In the present study a similar conclusion is reached for the binding of human serum

transferrin to the transferrin receptor. With the use of recombinant N- and C-lobes of human transferrin produced in a mammalian expression system, we show that both lobes are required to achieve full binding. (Production of recombinant C-lobe in the baby hamster kidney cell system is reported here for the first time.) Each lobe is able to donate iron to transferrin receptors on HeLa S₃ cells in the presence of the contralateral lobe. The results are not identical with the chicken system, because the C-lobe alone shows a limited ability to bind to receptors and to donate iron. Further complications arise from the relatively weak re-association between the two lobes of human transferrin compared with the re-association of the ovotransferrin lobes. However, domain-specific monoclonal antibodies to either lobe block the binding of N- and C-lobe mixtures in the human system, thus substantiating the need for both.

INTRODUCTION

The transferrins are glycosylated metal-binding proteins that function in the transport of iron to cells and as bacteriostatic agents in a variety of biological fluids [1–4]. The present-day 80 kDa proteins seem to have evolved by gene duplication, giving rise to two globular lobes, each containing a deep cleft capable of binding a metal ion. The binding clefts are each defined by two domains joined by a hinge region. In all transferrins for which crystallographic data are available, each ferric ion is directly co-ordinated to the side chains of two tyrosine residues, one histidine residue, one aspartic residue and two oxygen atoms from the synergistic carbonate anion [5–8].

To deliver iron to cells, diferric serum transferrin binds with high affinity to receptors that reside on the plasma membrane of actively dividing cells. The number of receptors is regulated by iron through an iron-responsive element in the 3′-non-coding region of the transferrin receptor mRNA [9–12]. In the transferrin cell cycle, the transferrin receptor complex is taken into the cell by receptor-mediated endocytosis. Once inside the cell, and apparently with the participation of the receptor [13–17] and a change in pH [18–20], the iron is removed from the transferrin, which then returns to the cell surface still bound to receptor. At the neutral extracellular pH, the receptor releases the apo-transferrin, allowing the cycle to continue. Although little is

known at the molecular level about which regions of transferrin and receptor interact, the receptor does discriminate between iron-bound and iron-free transferrin [18]. The structural studies document the large conformational changes in each lobe that accompany iron binding [5–8]. To explain the discrimination by the receptor, and as pointed out by Baker et al. [5], receptor contact seems likely to involve both domains in a lobe and might involve both lobes. Support for this latter view derives from studies from our laboratory that suggest that both the N- and C-lobes of ovotransferrin contain recognition sites that are required to attain physiological levels of binding; there is little or no binding or iron donation when a single lobe is present [21,22]. This idea has been reinforced by studies that showed that domain-specific monoclonal antibodies to either lobe block binding of an equimolar mixture of isolated N- and C-lobes [23]. These studies involved proteolytically prepared N- and C-lobes from chicken ovotransferrin (oTF) binding to receptors on chick embryo reticulocytes. More recently, the same results have been obtained with recombinant ovotransferrins [24]. In contrast with the ovotransferrin work, one paper claims that, for human transferrin interacting with human-derived cells, the ‘primary receptor recognition site’ resides in the C-lobe [25]. A second study indicates that the N-terminal domain alone might contact the transferrin receptor [26]. To clarify these conflicting results, we have used recombinant N- and C-lobes of human transferrin

Abbreviations used: BHK cells, baby hamster kidney cells; DMEM-F12, Dulbecco's modified Eagle's medium–Ham F-12; hTF, human serum transferrin; hTF/2N, recombinant N-lobe of human serum transferrin; hTF/2C, recombinant C-lobe of human serum transferrin; oTF, chicken ovotransferrin.

¹ To whom correspondence should be addressed.

to study the interaction with the transferrin receptor on HeLa S₃ cells. Successful production of the C-lobe is reported here for the first time. We conclude that the human transferrin receptor recognizes regions in both lobes of transferrin.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium–Ham F-12 (DMEM-F12) nutrient mixture was obtained with and without Phenol Red from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The same medium with Phenol Red as well as the serum replacement Ultrosor G and antibiotic–antimycotic solution (100×) were from Gibco-BRL–Life Technologies (Gaithersburg, MD, U.S.A.). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA, U.S.A.) and was tested before use to ensure the adequate growth of baby hamster kidney (BHK) cells. Corning expanded surface roller bottles, Wheaton Omnivials and Dynatech Removawells were obtained from a local distributor. Na¹²⁵I and ⁵⁹FeCl₃ were from DuPont–NEN®. Human serum transferrin (hTF) was purchased from Boehringer Mannheim. The chromatographic resin, Poros 50 HQ and the QE column were from PerSeptive Biosystems. Sephacryl S-100 HR and Sephadex G-75 were from Pharmacia. Methotrexate from Cetus was purchased at a local hospital pharmacy. Centricon 10 and 30 microconcentrators, YM-10 and YM-30 ultrafiltration membranes and a spiral cartridge concentrator (CH2PRS) fitted with an S1Y10 cartridge were from Amicon. Rabbit anti-(mouse IgG) was purchased from Southern Biological Associates. All chemicals and reagents were of A.R. grade.

A monoclonal antibody, designated E-8, was prepared in our laboratory from murine ascites fluid generously provided by Dr. James D. Cook and co-workers (University of Kansas Medical Center, Kansas City, KS, U.S.A.). The antibody is specific to the C-terminal lobe of human transferrin; a complete description of this antibody and other monoclonal antibodies specific to the N-lobe of hTF has been given [27].

All oligonucleotides were synthesized on an Applied Biosystems Model 391 DNA Synthesizer. After deprotection, short oligonucleotides (less than 25 nt in length) were concentrated in a Speed Vac Vacuum Centrifugation System (Savant) and used without further purification. Longer oligonucleotides were purified by reverse-phase chromatography on C₁₈ columns (SepPak) as described previously [28].

Molecular biology

Several different constructs were made in our attempts to express the C-lobe of hTF (hTF/2C) in BHK cells. C-lobe 1 was designed to place the transferrin signal peptide adjacent to the codon for Ala-334 in the bridging peptide (residues 331–339) of transferrin (Figure 1). Oligo 1 (Table 1) contained an *Sma*I site, DNA coding for the complete signal peptide of transferrin, and six amino acids of the C-lobe starting with the codon for Ala-334. Oligo 2 was designed to anneal to the 3' region of the transferrin and also contained an *Sma*I site. Oligos 1 and 2 were used to amplify the corresponding DNA fragment by using PCR. The DNA fragment was digested with *Sma*I, purified by gel electrophoresis and ligated into the *Sma*I site of Bluescript. This plasmid was called BS hTF/2C, and was used for further manipulations (see the next section). The *Sma*I fragment was isolated from BS hTF/2C and ligated into the *Sma*I site of the expression vector pNUT; the correct orientation of the fragment

in pNUT was confirmed by DNA sequence analysis before transfection of the BHK cells.

C-lobe 2 (Figure 1) was subsequently designed with four amino acid residues of hTF (Val-Pro-Asp-Lys) placed between the signal peptide and the codon for Thr-336. Oligo 3 (Table 1) was used as a mutagenic primer in the *dut*[−]*ung*[−] method [28] with BS hTF/2C as the template; oligo 3 contained DNA coding for residues −13 to −19 of the signal peptide and residues 1–4 of transferrin followed by residues 336–341 of the C-lobe. The resulting DNA fragment in Bluescript was designated BS hTF/2C VPDK. A similar strategy with oligo 4 (Table 1) was used to produce C-lobe 3 (Figure 1), which contained the N-terminal six residues of hTF.

At this point, the complete nucleotide sequence of BS hTF/2C VPDK was determined: a point mutation was discovered at the codon for residue 351 that changed the codon from a glutamic residue to a glycine. Because of the lack of suitable restriction sites for removing the mutation by replacing the mutated region with the corresponding region from the transferrin cDNA sequence, the glycine mutation was back-mutated by using a PCR-based method [29] with oligo 5 and an *Sma*I–*Hpa*I fragment from BS hTF/2C VPDK. The resulting *Sma*I–*Hpa*I fragment containing the correct codon for Glu-351 was subcloned back into BS hTF/2C VPDK, and the complete nucleotide sequence of the insert was determined to confirm the presence of the back-mutation and the absence of other mutations. The *Sma*I fragment was then excised from BS hTF/2C VPDK and ligated into the *Sma*I site of pNUT.

To assess the effect(s) of the mutation at residue 351 and the inclusion of the cryptic signal peptidase cleavage site, C-lobe 5 was constructed in which the four residues from the N-lobe were deleted from C-lobe 1 (Figure 1). Again, a PCR-based mutagenesis procedure [29] was used to delete the DNA coding region for the four amino acids: oligo 5 (Table 1) and the plasmid BS hTF/2C were used for the mutagenesis. After the success of the deletion and the absence of other mutations had been confirmed, the fragment was ligated into pNUT.

Expression vector and cell culture

BHK cells were grown in DMEM-F12 medium with 5% (v/v) fetal bovine serum and transfected as previously described [30]. Selection of transfected cells with methotrexate, and expansion to roller bottles, have also been described in detail [28,31]. Better adhesion of the cells to the roller bottles was achieved by using DMEM-F12/5% fetal bovine serum through two to three medium changes before switching to DMEM-F12/1% Ultrosor-G. Antibiotic–antimycotic solution (1×) was present in all media.

Isolation and characterization of the recombinant N- and C-lobes of transferrin

Isolation and purification of the N-lobe of human transferrin has been described [30,31]. The same general strategy, i.e. both anion exchange and gel filtration, was used to purify the C-lobe. Briefly, after addition of PMSF and sodium azide and a saturating amount of Fe(NTA)₂ (where NTA is nitrilotriacetate), the harvested medium was reduced in volume and exchanged into 5 mM Tris/HCl buffer, pH 8.0, by using a spiral cartridge. At this stage the samples were kept frozen until a total of four or five batches had accumulated. These were thawed, pooled and subjected to chromatography on a Poros 50 HQ anion-exchange column (2.5 cm × 40 cm). After centrifugation at 5900 g at 4 °C

Figure 1 Schematic representation of different fragments of recombinant hTF expressed in BHK cells

The regions of transferrin cDNA included in the expression vectors are represented by the horizontal bars. The signal peptide is shown by a solid black bar, the N-lobe by a bar with horizontal hatching, the bridging region by a bar with diagonal hatching, and the C-lobe by an open bar. Amino acid residue numbering is from the cDNA sequence [35]. Sites of glycosylation are indicated by CHO. The presence of the glycine mutation at residue 351 is indicated by G351; the attachments of the signal peptide coding region to the codon for Ala-334 and Thr-336 are indicated by A334 and T336 respectively. The numbers of residues separating the signal peptide and the bridging peptide in C-lobes 1–4 are given under the horizontally hatched bar. See the text for further details.

Table 1 Synthetic oligonucleotides used in cloning hTF/2C

Oligo 1	5'-ACACCCGGGAAGATGAGGCTCGCCGTGGGAGCCCTGCTGGTCTGCGCCGCTCTGGGGCTGTGTCTGGCTGCCCAACAGATGAATGCAA-3'
Oligo 2	5'-ACACCCGGGGCCCTACCTCTGAGATTTA-3'
Oligo 3	5'-GGCTTGCATTTCATCTGTTTATCAGGGACAGCCAGACAGCCCCA-3'
Oligo 4	5'-GGCTTGCATTTCATCTGCACAGTTTATCAGGGACAGCCAGACAGCCCCA-3'
Oligo 5	5'-AGCCACACGAGAGGCTCAA-3'

for 15 min, the clarified sample was applied to the Poros column with a Pharmacia P-1 pump at a rate of approx. 10 ml/min; 8 ml fractions were collected. Elution from the column involved a single step of 180 mM Tris/HCl buffer, pH 8.0. All fractions containing pink colour were pooled, concentrated to less than 10 ml and loaded on a Sephacryl S-100 HR column (5 cm × 80 cm) equilibrated and run in 0.1 M NH₄HCO₃ as described [31]. Two passages over the Sephacryl column and over another smaller gel-filtration column (Sephadex G-75 Superfine, 2.5 cm × 90 cm) were needed before the final anion-exchange column. The final purification step involved use of a Poros QE/M (10/100) column run on a PerSeptive Biosystems Sprint chromatography system. The column was equilibrated and run in 50 mM Tris/Bis-tris-propane buffer, pH 8.0, at a rate of 7 ml/min. A linear gradient of 0–400 mM NaCl in the same buffer over 5 column volumes was used to develop the column. Fractions of 2 ml were collected. The Sprint system allows simultaneous monitoring of pH, conductivity and absorbance at 280 nm. The Poros resins accelerate the anion-exchange steps, owing to the faster flow rates at which the columns can be run. The homogeneity of the samples was assessed by gel electrophoresis as described [28].

N-terminal sequence

The N-terminal sequence of hTF/2C was determined by Dr. Steve Smith (Protein Chemistry Laboratory, UTMB Cancer Center, Galveston, TX, U.S.A.).

Electrospray MS analysis

Samples were analysed on a Micromass Quattro II mass spectrometer (Micromass, Altrincham, Greater Manchester, U.K.). The details of the analysis procedure have been described [28].

Radioimmunoassay of hTF/2C

The competitive solid-phase immunoassay used to determine the concentration of hTF/2C in the culture medium and at various stages of the purification has been discussed previously [31]. Initially holo-hTF was used as the standard. The use of antibodies specific to the N-lobe allowed discrimination between the secreted hTF/2C and the hTF that is present in the serum replacement.

Titration calorimetry studies

The reassociation experiments between the two lobes of hTF were performed on a Microcal MCS ultrasensitive isothermal calorimeter (ITC) with Observer software for instrument control and data acquisition (MicroCal, Northampton, MA, U.S.A.). Measurements were made in 0.1 M Hepes, pH 7.5, containing 25 mM NaHCO₃. Details of the reassociation experiments between the two lobes of ovotransferrin have been described [32].

Cell-binding experiments

HeLa S₃ cells were a gift from Dr. Joan Moehring (Department of Microbiology, University of Vermont, College of Medicine, Burlington, VT, U.S.A.). The protocols for preparing the cells, conducting the binding experiments and analysing the data have been described [28,33]. Briefly, iron-saturated hTF and the N- and C-lobes of hTF were iodinated by the McFarlane procedure as previously described [31]. Specific radioactivities were typically between 25 000 and 40 000 c.p.m./pmol. To label the samples with ⁵⁹Fe, iron was removed from 200 µg of hTF or of N- and C-lobes, followed by exchange of the apo-proteins into 20 mM Hepes, pH 7.4, containing 20 mM NaHCO₃ and 150 mM NaCl. After the addition of 5 molar equivalents of nitrilotriacetate, sufficient ⁵⁹FeCl₃ (25.95 mCi/mg) was added to saturate each protein. After incubation at room temperature for 1 h and at 4 °C overnight, the samples were washed and exchanged into the same buffer in Centricon microconcentrators. The specific radioactivity was approx. 600 c.p.m./pmol.

For the experiments in which the ability of hTF and the N- and C-lobes to bind to and/or donate iron to HeLa S₃ cells was tested, the cells were prepared as described [28]. Amounts of protein as indicated in the Results section were added to cell suspensions (approx. 2 × 10⁷ cells/ml) in Joklik's minimum essential medium containing 20 mM Hepes and 2 % (w/v) BSA, pH 7.4. Triplicate aliquots of 50 µl were removed at each time point and processed [28]. The results were fitted to the first-order rate equation with the AXUM program (version 3.0). This gave estimates of the on rate and the number of transferrin molecules per cell at the plateau.

For the experiments to test the ability of domain-specific monoclonal antibodies to block binding, the cells were incubated for 20 min with 10 mM NH₄Cl to inhibit iron removal from the transferrin in subsequent incubations. A suspension of cells (300 µl containing 1.4 × 10⁶ cells) was added to Omnivials (Wheaton) containing 40 pmol of radioiodinated N- or C-lobe, alone or in the presence of equimolar unlabelled contralateral lobe with or without 20 or 40 pmol of antibody in a total volume of 100 µl. After incubation at 37 °C for 30 min with gentle shaking, portions of the cell suspension (three portions, 100 µl in each) were washed and assayed as described above.

RESULTS

Expression, isolation and characterization of hTF/2C

As shown in Figure 1, we have achieved the efficient expression of several forms of human transferrin with the pNUT-BHK cell expression system. Efficient expression of human transferrin and of the N-terminal lobe of transferrin by BHK cells with the pNUT vector has been demonstrated in previous studies [28,30]. Important features of the pNUT vector include a mouse metallothionein promoter to induce cDNA transcription in the presence of heavy metals, pUC18 sequences to allow replication and selection in *Escherichia coli* and mutated dihydrofolate reductase cDNA driven by an SV40 early promoter to allow a one-step selection of cells with large numbers of copies of pNUT with

methotrexate [30]. Initial experiments involved the mutation of transferrin cDNA by introducing two stop codons after the codon for Asp-337; this residue is in the middle of the peptide that links the N- and C-lobes of transferrin [30]. This construct, containing the transferrin signal peptide, directed the secretion of the transferrin N-lobe into the tissue culture medium at levels up to 20 mg/l [30]. As with the native N-lobe, the recombinant N-lobe (hTF/2N) is not glycosylated. Subsequent optimization has increased the expression levels of the human N-lobe to 55–120 mg/l [31].

A similar strategy was used to express the full-length human transferrin in the same expression system (Figure 1). Again, the transferrin signal peptide was used to direct the secretion of the protein into the tissue culture medium at levels up to 125 mg/l. Like native hTF, the recombinant transferrin was glycosylated. However, as revealed by electrospray MS, the glycosylation pattern was far more complex and heterogeneous than that found in the native protein [28]. To circumvent this heterogeneity, the two N-linked asparagine residues (Asn-413 and Asn-611) were mutated to aspartic residues. Although initially levels of 25 mg/l were obtained and reported [28], subsequent runs have yielded levels up to 95 mg/l (A. B. Mason, unpublished work).

To obtain expression of hTF/2C, the initial strategy was to place the transferrin signal peptide adjacent to the codon for Ala-334 (Figure 1). After ligation into pNUT and transfection into BHK cells, no expression of hTF/2C could be measured. The lack of protein expression might have been due to either the lack of cleavage of the signal peptide in the chimaeric junction in C-lobe 1 or a problem with the glycosylation and/or secretion. Because secretion of glycosylated full-length transferrin was successful, the first possibility seemed more likely. The C-lobe 2 construct therefore contained the first four residues from the N-lobe placed upstream of Thr-336 in the bridging peptide; the rationale was that the placement of this sequence would mimic the signal peptide cleavage site found in native human transferrin. However, no C-lobe was detected in the tissue culture medium of BHK cells transfected with this construct. C-lobe 3, containing the first six residues from the transferrin N-lobe, was then made; again there was no detectable protein secretion. At this point the complete sequence of the hTF/2C cDNA was determined and a point mutation of Glu-351 to glycine was found. Presumably this mutation had occurred during one of the DNA manipulation steps. After back-mutation of this codon to that for a glutamic residue, the construct C-lobe 4 (Figure 1) was ligated into pNUT and transfected into BHK cells. This construct led to the successful expression of the C-lobe described in this paper. One further construct (C-lobe 5) was made to query the role of the Gly-351 mutation compared with the presence of the four residues from the N-lobe. In C-lobe 5, the signal peptide sequence was placed adjacent to Ala-334. After transfection into BHK cells, the C-lobe 5 construct led to the secretion of detectable levels of hTF/2C that were slightly lower than those observed for C-lobe 4. From these results we conclude that the presence of the Gly-351 mutation was sufficient to block the expression and/or secretion of the C-lobe from BHK cells. The inclusion of four residues from the transferrin N-lobe led to the expression of the human transferrin C-lobe. The construct containing the back-mutation, but lacking the four residues, also led to the expression of C-lobe at a level of production that was slightly less. The limited number of production runs with each construct preclude definitive statements as to the importance of these extra residues at the N-terminus.

With the C-lobe 4 construct, a maximum of approx. 12 µg/ml hTF/2C was secreted into the medium by the BHK cells.

Figure 2 SDS/PAGE of hTF/2N and hTF/2C under reducing conditions

Lane 1, Bio-Rad low-molecular-mass standards with masses (from top to bottom) of 97.4 (faint band), 66.2, 45, 31, 21.5 and 14.4 kDa; lane 2, holo-hTF; lane 3, hTF/2N; lane 4, hTF/2C. Approx. 3 μ g of each protein was loaded on the gel. The calculated molecular masses are 37151 Da for hTF/2N [40] and 43078 Da for hTF/2C.

Purification of the C-lobe was difficult. According to the assay, hTF/2C accounted for approx. 12% of the starting A_{280} units. Unlike the situation with the smaller hTF/2N, where baseline resolution can be achieved, the gel-filtration columns only partly resolved the C-lobe from the hTF in the serum replacement. The sample was chromatographed on both the gel-filtration and the QE/M column multiple times until purity, as ascertained by SDS/PAGE, was attained. The yield of pure C-lobe was very low, 3 mg total or 16% of the C-lobe at the beginning of the purification for one preparation and 10 mg or 20% for a second preparation.

An analysis of the purified hTF C-lobe by SDS-PAGE and comparison with full-length hTf and with hTF/2N is presented in Figure 2. The somewhat indistinct appearance of the C-lobe band results from the extensive and heterogeneous glycosylation of the recombinant sample (see below). The concentrations of N- and C-lobe were determined from the millimolar absorption coefficient at 280 nm. For the recombinant hTF N-lobe the experimentally derived value of $38.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [30] was used. This is in excellent agreement with the calculated value of $38.36 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [34]. The calculated value of $46.76 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was used for the recombinant C-lobe [34].

Analysis of the N-terminal sequence revealed the following amino acid sequence for the first seven residues: Val-Pro-Asp-Lys-Thr-Asp-Glu. These results confirm both that the signal peptide in the original construct was correctly cleaved and that the recombinant C-lobe contains the first four residues from the transferrin N-lobe attached to Thr-336 [35]. These results are consistent with the DNA manipulations described earlier.

The molecular mass calculated for the recombinant polypeptide containing four residues from the N-lobe and residues 336–679 is 38665.89 [35]. In most serum transferrins two biantennary glycans are attached to Asn residues at positions 413 and 611 [36], resulting in an additional mass of 4412.02 to give a total of 43077.91. Eight experimental masses from electrospray MS analysis were found; they ranged from 42043.6 to 43957.6. Assuming a single polypeptide of molecular mass 38665.89, the recombinant C-lobe preparation comprised eight different glycan species ranging in mass from 3377.7 to 5291.7. The results are consistent with our previous findings for recombinant hTF expressed in BHK cells, in which it was noted that the glycosylation pattern is very complex and does not resemble the pattern found in hTF isolated from human serum [28]. For recombinant

Figure 3 Progress curves for the binding of hTF, and hTF/2N and hTF/2C, to HeLa S_3 cells at 37 °C

The samples are as follows: Fe^{125}I -hTF (100 nM) (●); Fe^{125}I -hTF/2N (200 nM) + Fe-hTF/2C (200 nM) in the absence (■) or the presence (□) of a 50-fold molar excess of Fe_2 -hTF; Fe^{125}I -hTF/2C (200 nM) alone (▼), in the presence of equimolar Fe-hTF/2N (▲) or in the presence of equimolar Fe-hTF/2N and excess Fe_2 -hTF (△). Experimental points are means $\pm$ S.E.M. for measurements made in triplicate. Abbreviation: Tf, transferrin.

Table 2 Kinetic parameters for binding of hTF and N- and C-lobes of hTF to receptors on HeLa S_3 cells

The progress curves are shown in Figure 3. See the legend for Figure 3 for concentrations of sample and details of the assay for binding sites.

Sample	$10^{-5} \times$ Binding sites per cell	K_{on} (min^{-1})	R^2
Fe^{125}I -hTF	9.10 ± 0.40	0.14 ± 0.02	0.99
Fe^{125}I -hTF/2N + Fe-hTF/2C	3.71 ± 0.30	0.25 ± 0.09	0.95
Fe^{125}I -hTF/2N + Fe-hTF/2C + Fe_2 -hTF	0.07*		
Fe^{125}I -hTF/2C + Fe-hTF/2N	3.11 ± 0.17	0.35 ± 0.09	0.97
Fe^{125}I -hTF/2C + Fe-hTF/2N + Fe_2 -hTF	0.16*		
Fe^{125}I -hTF/2C	1.45 ± 0.16	0.22 ± 0.09	0.93

* Binding sites per cell found at the 20 min time point.

full-length hTF, 16 different glycans ranging in mass from 3689.9 to 5658.9 were found.

Cell-binding experiments

A study to test the ability of the N- and C-lobes to bind to transferrin receptors on HeLa S_3 cells was performed. The binding profiles are shown in Figure 3 and the kinetic parameters derived from this study are given in Table 2. Significant binding of radioiodinated N-lobe in the presence of equimolar unlabelled C-lobe was observed (each lobe at a concentration of 200 nM). This binding was decreased to 2% by excess holo-hTF. Likewise, significant binding was observed for radioiodinated C-lobe in the presence of unlabelled N-lobe. This binding decreased to 5% in the presence of excess hTF. Binding at this concentration of combined lobes was approximately one-third of that measured for holo-hTF. Under these same conditions there was significant binding of the C-lobe alone, although it was half of the amount seen for the C-lobe in the presence of the N-lobe.

Figure 4 Uptake of ^{59}Fe from hTF, and hTF/2N and hTF/2C, to HeLa S_3 cells at 37°C

The samples are as follows: $^{59}\text{Fe}_2\text{-hTF}$ (120 nM) (●); $^{59}\text{Fe-hTF/2N}$ (200 nM) + Fe-hTF/2C (200 nM) (■); $^{59}\text{Fe-hTF/2N}$ alone (□); $^{59}\text{Fe-hTF/2C}$ (200 nM) + Fe-hTF/2N (200 nM) (▲); $^{59}\text{Fe-hTF/2C}$ (200 nM) + Fe-hTF/2N (2000 nM) (▼); $^{59}\text{Fe-hTF/2C}$ (200 nM) alone (△). Experimental points are means $\pm$ S.E.M. for measurements made in triplicate.

An uptake study to test the ability of ^{59}Fe -labelled hTF, hTF/2N and hTF/2C to donate iron to HeLa S_3 cells was undertaken. The results are presented in Figure 4. Iron was donated in a linear fashion over the time course of the study. At a ratio of 1:1 of the N- and C-lobes the rate is similar regardless of which lobe has the radiolabelled iron. Iron donation from ^{59}Fe -labelled N-lobe (in the presence of C-lobe) was approx. 30% of the uptake from ^{59}Fe -labelled hTF. The theoretical maximum is 50%. No iron was donated by ^{59}Fe -hTF/2N alone. Iron donation from ^{59}Fe -labelled C-lobe (in the presence of

Figure 5 Progress curves for the binding of radioiodinated hTF, and hTF/2N and hTF/2C, to HeLa S_3 cells at 37°C

The samples are as follows: $\text{Fe}_2^{125}\text{-hTF}$ (●); $\text{Fe}_2^{125}\text{-hTF}$ in the presence of excess unlabelled $\text{Fe}_2\text{-hTF}$ (○); $\text{Fe}_2^{125}\text{-hTF/2N}$ alone (◇), in the presence of unlabelled Fe-hTF/2C (■) or in the presence of Fe-hTF/2C and excess unlabelled $\text{Fe}_2\text{-hTF}$ (□); $\text{Fe}_2^{125}\text{-hTF/2C}$ alone (▼) or in the presence of excess unlabelled $\text{Fe}_2\text{-hTF}$ (▽); $\text{Fe}_2^{125}\text{-hTF/2C}$ in the presence of unlabelled Fe-hTF/2N (▲) or in the presence of unlabelled Fe-hTF/2N and excess unlabelled $\text{Fe}_2\text{-hTF}$ (△). Holo-hTF was present at approx. 100 nM. Radioiodinated hTF/2N and hTF/2C were present at approx. 300 nM and the unlabelled contralateral lobes were present at approx. 3 μM . Experimental points are means $\pm$ S.E.M. for measurements made in triplicate. Abbreviation: Tf, transferrin.

Table 3 Kinetic parameters for binding of hTF and the N- and C-lobes of hTF to receptors on HeLa S_3 cells

The progress curves are shown in Figure 5. See the legend to Figure 5 for concentrations of sample and details of the assay.

Sample	$10^{-5} \times$ Binding sites per cell	K_{on} (min^{-1})	R^2
$\text{Fe}_2^{125}\text{-hTF}$	6.69 ± 0.19	0.32 ± 0.06	0.98
$\text{Fe}_2^{125}\text{-hTF} + \text{Fe}_2\text{-hTF}$	0.48*		
$\text{Fe}_2^{125}\text{-hTF/2N} + \text{Fe-hTF/2C}$ (1:10)	5.78 ± 0.17	0.43 ± 0.08	0.98
$\text{Fe}_2^{125}\text{-hTF/2N} + \text{Fe-hTF/2C}$ (1:10) + $\text{Fe}_2\text{-hTF}$	0.30		
$\text{Fe}_2^{125}\text{-hTF/2N}$	0.97		
$\text{Fe}_2^{125}\text{-hTF/2C} + \text{Fe-hTF/2N}$ (1:10)	6.59 ± 0.26	0.23 ± 0.05	0.96
$\text{Fe}_2^{125}\text{-hTF/2C} + \text{Fe-hTF/2N}$ (1:10) + $\text{Fe}_2\text{-hTF}$	0.61		
$\text{Fe}_2^{125}\text{-hTF/2C}$	1.89 ± 0.19	0.21 ± 0.17	0.64
$\text{Fe}_2^{125}\text{-hTF/2C} + \text{Fe}_2\text{-hTF}$	0.58–1.43†		

* Binding sites per cell at the 30 min time point.

† The range of binding sites/cell at the various time points.

unlabelled N-lobe) was approx. 40% of the uptake from holo-hTF. The amount of iron donated from the C-lobe alone was significant (about half of that found for the C-lobe in the presence of equimolar N-lobe). The amount of iron donated by the ^{59}Fe -hTF/2C could be increased substantially (to approx. 60% of holo-hTF uptake) by the presence of 10-fold molar excess of unlabelled N-lobe.

A third, more extensive, study was performed to establish the specificity of the binding. Samples labelled with ^{125}I were made to a final concentration of 300 nM for the radiolabelled lobes. The amount of unlabelled lobe was increased 10-fold to try to promote association and binding. The results of the binding study are shown in Figure 5 and the binding data are given in Table 3. A similar amount of binding was observed for the combined lobes to that for holo-hTF, regardless of which lobe was labelled. Excess hTF decreased the binding of labelled protein to background levels (less than 10% of that seen in the absence of hTF). Although significant binding was observed for the C-lobe alone (25.5% of the combined sample), holo-hTF was not very effective at decreasing this binding, especially at the early time points.

To test further the requirement for both lobes in specific binding to the receptor, domain-specific monoclonal antibodies were used to determine whether binding could be blocked. In previous studies the monoclonal antibodies HTF.14 (specific for the N-lobe) and E-8 (specific for the C-lobe) were able to decrease the binding of radioiodinated $\text{Fe}_2\text{-hTF}$ to HeLa S_3 cells to 7.1% and 4.3% of control respectively, at an equimolar ratio of hTF to monoclonal antibody (A. B. Mason and R. C. Woodworth, unpublished work). By following an identical protocol it was found that when a subsaturating amount of radioiodinated N-lobe (40 pmol) was incubated with C-lobe (40 pmol) in the presence of equimolar HTF.14 or E-8, binding decreased to less than 10% of that measured in the absence of antibody (Table 4). Binding of the N-lobe alone was 8%, such that 100% inhibition was approached regardless of whether the antibody was bound to the N-lobe or to the C-lobe.

Similar results were obtained in the inverse experiment (Table 4). Either antibody decreased the binding of radiolabelled hTF/2C to the background level. The result of binding of 40 pmol of radioiodinated hTF/2C alone, in the presence of N-lobe and in the presence of 20 and 40 pmol of HTF.14, is shown. Also given is the result when 40 pmol of C-lobe was incubated

Table 4 Ability of domain-specific monoclonal antibodies to block binding of recombinant radioiodinated N- and C-lobes to transferrin receptors on HeLa S₃ cells

Fe¹²⁵I-hTF/2N (40 pmol) was incubated alone or in the presence of 40 pmol of Fe-hTF/2C with either 20 or 40 pmol of antibody HTF.14, which is specific for the N-lobe of hTF, or 20 or 40 pmol of the antibody E-8, which is specific for the C-lobe of hTF. Cells were added and incubated for 30 min at 37 °C. Aliquots of the cell suspension were removed and processed as described in the Materials and methods section. Experimental points are the means $\pm$ S.E.M. for measurements made in triplicate. The results of incubating 40 pmol of Fe¹²⁵I-hTF/2C in the absence or presence of 40 pmol of Fe-hTF/2N with the antibody against the N-lobe, HTF.14, present at two different concentrations are given. Also shown are the results of incubating 40 pmol of Fe¹²⁵I-hTF/2C alone, in the presence of 400 pmol of Fe-hTF/2N and with 20 or 40 pmol of antibody E-8, which is specific for the C-lobe. For equimolar Fe¹²⁵I-hTF/2C + Fe-hTF/2N the transferrin molecules per cell were 338385 ± 8145 . For equimolar Fe¹²⁵I-hTF/2C + Fe-hTF/2N the transferrin molecules per cell were 340921 ± 31799 , and for Fe¹²⁵I-hTF/2C + Fe-hTF/2N at a ratio of 1:10 the control value was 696831 ± 31474 transferrin molecules per cell.

Sample	Antibody	Molar ratio of antibody to antigen	Binding (% of control)
Fe ¹²⁵ I-hTF/2N + Fe-hTF/2C (1:1)	None	—	100.0 $\pm$ 2.3
	Anti-N-lobe	1:2	32.9 $\pm$ 1.9
	Anti-N-lobe	1:1	10.8 $\pm$ 7.2
	Anti-C-lobe	1:2	26.7 $\pm$ 0.9
	Anti-C-lobe	1:1	7.1 $\pm$ 1.1
Fe ¹²⁵ I-hTF/2N alone	None	—	7.6 $\pm$ 1.1
Fe ¹²⁵ I-hTF/2C + Fe-hTF/2N (1:1)	None	—	100.0 $\pm$ 9.3
	Anti-N-lobe	1:2	66.9 $\pm$ 1.9
	Anti-N-lobe	1:1	37.3 $\pm$ 1.1
Fe ¹²⁵ I-hTF/2C alone	None	—	43.0 $\pm$ 1.8
Fe ¹²⁵ I-hTF/2C + Fe-hTF/2N (1:10)	None	—	100.0 $\pm$ 4.5
	Anti-C-lobe	1:2	32.8 $\pm$ 6.9
	Anti-C-lobe	1:1	13.2 $\pm$ 1.2
Fe ¹²⁵ I-hTF/2C alone	None	—	21.0 $\pm$ 0.9

Figure 6 Titration of iron-containing hTF/2C with multiple injections of Fe-hTF/2N at 18.3 °C and pH 7.5

The raw data are shown in the upper frame and the integrated heats and best-fit line are shown in the lower frame. The binding parameters are given in Table 5.

with 400 pmol of N-lobe to promote binding. The E-8 antibody caused a decrease in binding to a level lower than that observed with the C-lobe alone.

Table 5 Best values for fitting parameters for binding of recombinant ferric N-lobe of human transferrin to recombinant C-lobe

All measurements were performed in 100 mM Hepes, pH 7.5, containing 25 mM NaHCO₃. The C-lobe in the mixing chamber was titrated with the N-lobe as shown in Figure 6. The concentration of hTF/2N in the syringe was 0.649 mM for all three experiments. The concentration of hTF/2C was 0.031 mM at the two lower temperatures and 0.015 mM at 38 °C. Owing to low *C* values (i.e. concentration in the cell multiplied by the binding constant), the fitting of the data, especially at 38 °C, was poor, and the estimated error for the three parameters is probably 10% or higher.

Temperature (°C)	<i>n</i>	ΔH (kJ/mol)	<i>K</i> (10 ⁴ M ⁻¹)
18.3	0.91	−44.18	8.34
28.0	0.91	−48.70	4.16
38.0	0.99	−50.62	2.04

Re-association of N- and C-lobes

The association of the recombinant N- and C-lobes was measured by titration calorimetry at three different temperatures. The integrated ΔH values were fitted to a model on the basis of the assumption of a single set of sites. The data for the experiment at 18.3 °C are shown in Figure 6 and the parameters obtained from the curve-fitting for experiments at three different temperatures are given in Table 5. The results show a relatively weak affinity of hTF/2N and hTF/2C when compared with that found for the N- and C-lobes of oTF [32]. The association constant for the human lobe interaction was approx. one-eighth of that of the oTF lobe interaction (8.3×10^4 M⁻¹ at 18.3 °C compared with 6.9×10^5 M⁻¹ at 17 °C). The enthalpy changes for the human N- and C-lobes are also considerably lower, −44.2 kJ/mol at 18.3 °C as opposed to −73.2 kJ/mol at 17 °C for oTF. The ΔC_p for hTF is less than −418 J/°C per mol, compared with −1463 J/°C per mol for oTF.

DISCUSSION

Production and secretion of the C-lobe of hTF by transfected BHK cells is lower than previously reported for the N-lobe and for holo-hTF (Figure 1). It is not clear why the production of the C-lobe is so poor; speculative explanations include inefficient signal peptide processing, incorrect folding, or abnormal secretion related to the abnormal glycosylation in the heterologous expression system. The presence of four residues from the N-lobe might aid in the secretion of the C-lobe. A construct lacking the four residues was expressed at levels that were somewhat lower than those found for the construct containing them. However, owing to the limited number of production runs and the large number of variables involved in the expression system it is impossible to say anything definitive about whether the amino acids from the N-lobe are really necessary. As described in the Materials and methods section, our original constructs (one with no residues from the N-lobe and one with four residues from the N-lobe) contained a PCR-induced mutation in which the wild-type Glu-351 was changed to Gly. Transfections with plasmids containing these constructs were successful, i.e. cells survived selection with 500 μ M methotrexate, but no protein was present in the tissue culture medium. Only after correction of this mutation were we able to obtain measurable amounts of secreted protein.

The purification of hTF/2C is complicated by the complex glycosylation pattern, such that the resolution on all columns is affected. It is possible that the final purified sample is only a subset of the C-lobe, which does not co-elute with the holo-hTF. This would explain why there are half the number of glycosylation forms found for recombinant full-length hTF expressed in the BHK system [28]. The overlap with hTF (derived from the serum replacement) also partly accounts for the poor recovery of pure C-lobe. Previous studies in which the binding of commercially available hTF to HeLa S_3 cells was compared with recombinant 'hyper'-glycosylated and completely non-glycosylated hTF produced in the BHK cell system showed that the levels of binding were the same whether the sample was extensively glycosylated or completely without glycosylation [28]. This led to the conclusion that the glycosylation level has no effect on the interaction of hTF with the receptor.

The binding studies show clearly that the N-lobe will not bind to the hTF receptor in the absence of C-lobe, as has been reported previously [25,37]. A crucial difference from the previous work is the observation that the N-lobe will bind to HeLa-cell receptors in the presence of equimolar C-lobe (Figure 3). In addition, the amount of binding increases when a 10-fold excess of C-lobe is present (Figure 5). The binding is abolished by the presence of excess hTF and is inhibited by monoclonal antibodies that are specific to either the N-lobe or the C-lobe (Table 4). Iron donation from ^{59}Fe -labelled N-lobe (in the presence of equimolar C-lobe) is approx. 30 % out of a maximum of 50 % (Figure 4). This is similar to the amount of iron uptake found for the N-lobe of oTF (in the presence of C-lobe) [21].

The situation with the C-lobe is more complex. The C-lobe alone definitely shows binding, although the amount is considerably less than that seen when equimolar N-lobe is also present. Of considerable importance is the fact that the binding of the combination of N- and C-lobe is effectively inhibited by excess holo-hTF, whereas the binding of the C-lobe alone is only partly inhibited under the same conditions. This finding implies that some portion of the observed binding of the C-lobe alone is specific and some is non-specific. It is difficult to dissect the exact amount of each. Equally noteworthy is the fact that a monoclonal antibody (HTF.14) against the N-lobe decreases the binding of

radioiodinated C-lobe (in the presence of unlabelled N-lobe) to receptor, at an equimolar ratio of antigen to antibody, to the level found for the radioiodinated C-lobe alone.

The results from the titration calorimetry help to explain the binding results further. At the low sample concentrations used in the cell studies very little association of the lobes would be expected. By increasing the amount of unlabelled contralateral lobe 10-fold, binding was increased considerably. Equilibrium binding experiments with the two lobes would require high concentrations to promote association in order to saturate the receptor sites. The situation is complex because the K_d for the binding of transferrin to the receptor is high in the human system (approx. 20 nM), whereas the binding constant for the lobe re-association is low. The opposite situation exists for the chicken system, where the receptor binding is one-tenth as great (K_d = 200 nM) but the association constant for the lobe interaction is 8-fold higher. In earlier studies with oTF and the isolated lobes of oTF, and with hTF, it was concluded that the lobe interactions seemed to be marginally stronger for oTF than for hTF [38]. The results of the present work indicate that there is in fact quite a large difference in the interaction.

These conclusions are somewhat in contrast with those reported by others [25,26]. In the studies by Zak et al. [25], the results are complicated by the fact that two different cell systems were used. Iron uptake was examined only in HuH-7 cells (a cell line derived from human liver). The binding studies that yielded comparative data were done in K562 cells. Two major differences exist. In the present study the recombinant N- and C-lobes re-associated in solution, whereas the proteolytically derived N- and C-lobes did not (see below). In addition, the recombinant N-lobe bound to the transferrin receptor in the presence of the recombinant C-lobe but not in its absence. In contrast with these results, binding of the proteolytically derived N-lobe to the receptor could not be demonstrated even in the presence of excess proteolytic C-lobe [25]. Nevertheless, the presence of N-lobe led to a 10-fold increase in the binding constant obtained for the binding of the proteolytic C-lobe to K562 cells when compared with C-lobe alone, signifying that some interaction must be taking place. Of possible relevance to these findings is the work of Evans et al. [39], comparing the binding of the only known natural variant hTF with normal hTF to K562 cells. This variant has the conformation of monoferric hTF. Iron binds tightly to the N-lobe, with the accompanying closure of the cleft. Although iron can bind to the C-lobe it remains in the open conformation. The binding affinity of this variant hTF is tighter than that found for apo-hTF but less avid than that found for diferric hTF. As pointed out by Evans et al., if the receptor binding resides solely in the C-lobe, one would predict that the binding affinity should be that of apo-hTF.

The largest difference between the two studies is in the use of proteolytically derived C-lobe in the work of Zak et al. [25]. The proteolytic preparation contains, in addition to the C-lobe, the bridge (residues 331–339) and five residues from the N-lobe (residues 326–330) and an unknown amount of a peptide from the N-lobe disulphide-linked to Cys-331. The proteolytic N-lobe is also not a monodisperse preparation [30] and would overlap considerably (approx. 12 amino acids) with the C-lobe, perhaps precluding any association between the two as reported [25]. The recent X-ray crystal structure of oTF shows that the lobe-lobe interaction resides at the C-terminal portion of each [8]. A higher-resolution structure of human hTF [7] will be necessary to see whether the same is true in its case. Recent work from our laboratory with well characterized recombinant oTF fragments has shown that the two lobes of oTF must be able to associate in solution to bind to receptor [24].

In conclusion, the results in the present study support the hypothesis that both lobes of hTF contact the receptor. The binding studies with well-characterized recombinant N- and C-lobes of transferrin in conjunction with the antibody-blocking studies offer strong evidence for this contention. Binding to the specific transferrin receptor is the first step in the transport of iron into the cell. It is therefore critical to define the essential features of the recognition. We believe that the work in the present study establishes that contact areas in each lobe must be elucidated. This is a major goal of our laboratory.

This work was supported by USPHS grant R01 DK 21739 from the National Institute of Diabetes, and Digestive and Kidney Diseases.

REFERENCES

- Harris, D. C. and Aisen, P. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., ed.), pp. 239–351, VCH, New York
- Aisen, P. (1989) in *Iron Carriers and Iron Proteins*, (Loehr, T. M., ed.), pp. 353–371, VCH, New York
- Thorstensen, K. and Romslo, I. (1990) *Biochem. J.* **271**, 1–10
- Baker, E. N. (1994) *Adv. Inorg. Chem.* **41**, 389–463
- Baker, E. N., Anderson, B. F., Baker, H. M., Haridas, M., Jameson, G. B., Norris, G. E., Rumball, S. V. and Smith, C. A. (1991) *Int. J. Biol. Macromol.* **13**, 123–129
- Sarra, R., Garratt, R., Gorinsky, B., Jhoti, H. and Lindley, P. (1990) *Acta Cryst.* **B46**, 763–771
- Zuccola, H. J. (1993) Ph.D. thesis, Georgia Institute of Technology, Atlanta, GA, U.S.A.
- Kurokawa, H., Mikami, B. and Hirose, M. (1995) *J. Mol. Biol.* **254**, 196–207
- Owen, D. and Kuhn, L. C. (1987) *EMBO J.* **6**, 1287–1293
- Mullner, E. W. and Kuhn, L. C. (1988) *Cell* **53**, 815–825
- Casey, J. L., Hentze, M. W., Koeller, D. M., Caughman, S. W., Rouault, T. A., Klausner, R. D. and Harford, J. B. (1988) *Science* **240**, 924–928
- Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L.-N. L., Klausner, R. D. and Harford, J. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3574–3578
- Bali, P. K., Zak, O. and Aisen, P. (1991) *Biochemistry* **30**, 324–328
- Bali, P. K. and Aisen, P. (1991) *Biochemistry* **30**, 9947–9952
- Bali, P. K. and Aisen, P. (1992) *Biochemistry* **31**, 3963–3967
- Aisen, P. (1992) *Ann. Neurol.* **32** (Suppl.), S62–S68
- Egan, T. J., Zak, O. and Aisen, P. (1993) *Biochemistry* **32**, 8162–8167
- Klausner, R. D., Ashwell, G., van Renswoude, J., Harford, J. B. and Bridges, K. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2263–2266
- Sipe, D. M. and Murphy, R. F. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7119–7123
- Sipe, D. M. and Murphy, R. F. (1991) *J. Biol. Chem.* **266**, 8002–8007
- Brown-Mason, A. and Woodworth, R. C. (1984) *J. Biol. Chem.* **259**, 1866–1873
- Mason, A. B., Brown, S. A., Butcher, N. D. and Woodworth, R. C. (1987) *Biochem. J.* **245**, 103–109
- Mason, A. B., Brown, S. A. and Church, W. R. (1987) *J. Biol. Chem.* **262**, 9011–9015
- Mason, A. B., Woodworth, R. C., Oliver, R. W. A., Green, B. N., Lin, L.-N., Brandts, J. F., Savage, K. J., Tam, B. M. and MacGillivray, R. T. A. (1996) *Biochem. J.* **319**, 361–368
- Zak, O., Trinder, D. and Aisen, P. (1994) *J. Biol. Chem.* **269**, 7110–7114
- Orlandini, M., Santucci, A., Tramontano, A., Neri, P. and Oliviero, S. (1994) *Protein Sci.* **3**, 1476–1484
- Mason, A. B. and Woodworth, R. C. (1991) *Hybridoma* **10**, 611–623
- Mason, A. B., Miller, M. K., Funk, W. D., Banfield, D. K., Savage, K. J., Oliver, R. W. A., Green, B. N., MacGillivray, R. T. A. and Woodworth, R. C. (1993) *Biochemistry* **32**, 5472–5479
- Nelson, R. M. and Long, G. L. (1989) *Anal. Biochem.* **180**, 147–151
- Funk, W. D., MacGillivray, R. T. A., Mason, A. B., Brown, S. A. and Woodworth, R. C. (1990) *Biochemistry* **29**, 1654–1660
- Mason, A. B., Funk, W. D., MacGillivray, R. T. A. and Woodworth, R. C. (1991) *Protein Exp. Purif.* **2**, 214–220
- Lin, L., Mason, A. B., Woodworth, R. C. and Brandts, J. F. (1991) *Biochemistry* **30**, 11660–11669
- Penhallow, R. C., Brown-Mason, A. and Woodworth, R. C. (1986) *J. Cell. Physiol.* **128**, 251–260
- Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) *Protein Sci.* **4**, 2411–2423
- Yang, F., Lum, J. B., McGill, J. R., Moore, C. M., Naylor, S. L., van Bragt, P. H., Baldwin, W. D. and Bowman, B. H. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2752–2756
- Spik, G., Coddeville, B. and Montreuil, J. (1988) *Biochimie* **70**, 1459–1469
- Lineback-Zins, J. and Brew, K. (1980) *J. Biol. Chem.* **255**, 708–713
- Lin, L.-N., Mason, A. B., Woodworth, R. C. and Brandts, J. F. (1994) *Biochemistry* **33**, 1881–1888
- Evans, R. W., Crawley, J. B., Garratt, R. C., Grossmann, J. G., Neu, M., Aitken, A., Patel, K. J., Meilak, A., Wong, C., Singh, J. et al. (1994) *Biochemistry* **33**, 12512–12520
- Woodworth, R. C., Mason, A. B., Funk, W. D. and MacGillivray, R. T. A. (1991) *Biochemistry* **30**, 10824–10829