RESEARCH COMMUNICATION

A conserved RGD (Arg-Gly-Asp) motif in the transferrin receptor is required for binding to transferrin

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The transferrin receptor contains a highly conserved Arg-Gly-Asp (RGD) sequence in the C-terminal region where transferrin is thought to bind. RGD sequences are commonly involved in cell adhesion. This sequence is crucial for transferrin binding, suggesting possible evolutionary links between molecules mediating iron uptake and cell adhesion.

Key words: affinity binding, conformational epitopes, iron uptake, secreted alkaline phosphatase fusion protein.

INTRODUCTION

The major mechanism for the uptake of iron by vertebrate cells involves the binding of diferric transferrin to a high-affinity cell-surface receptor. This is followed by endocytosis into an acidic intracellular compartment where iron is released and transported into the cytoplasm. Apotransferrin bound to the receptor is then transported back to the cell surface, where the pH returns to neutrality. Apotransferrin has decreased affinity for the receptor at pH 7.4 and is therefore released into the circulation, allowing a new cycle to begin [1–5].

The human transferrin receptor is a type II membrane protein, with an N-terminal cytoplasmic domain of 61 amino acids, a 28-amino-acid transmembrane region (amino acids 62–89) and a 671-amino-acid extracellular C-terminal region (amino acids 90–760) [6,7]. Whereas the intracellular N-terminal sequences that are involved in receptor endocytosis have been intensively studied [8], much less is known about the extracellular domain and the requirements for binding of transferrin. Glycosylation at Asn^{727} is required for folding, processing and cell-surface expression [9]. Chimaeric receptors between human and chicken receptors have implicated the C-terminal 192 amino acids (amino acids 569–760, encoded by exons 17–19) in the binding of transferrin [10,11].

We have observed the presence of an Arg-Gly-Asp (RGD) sequence at amino acids 646–648 (human numbering system) in all transferrin receptors so far sequenced, including human, mouse, rat, Chinese hamster and chicken. RGD sequences are functionally important in the binding of many proteins to cells, such as fibrinogen to the platelet integrin gpIIb/IIIa and fibronectin to its cognate receptor [12]. The RGD recognition motif has also been exploited by a number of pathogenic organisms. Attachment of foot-and-mouth-disease virus to cells involves an RGD motif on the virus envelope protein [13]. The disintegrin family of anticoagulant proteins from snake venom bind to gpIIb/IIIa antagonists via RGD motifs [14], and the related anticoagulant leech proteins decorsin and ornatin act in a similar manner [15].

We now present evidence that the RGD sequence of the transferrin receptor plays an important role in the binding of transferrin.

EXPERIMENTAL

The vector pSEAP2 Control (Clontech Inc., Palo Alto, CA, U.S.A.), which contains a secretory form of human placental alkaline phosphatase driven by the simian-virus-40 (SV40) promoter, was extensively modified to create a generally useful vector for making secreted alkaline phosphatase fusion proteins. A unique endonuclease-*Nhe*I site at position 25 (just 5′ of the SV40 promoter) was eliminated by cutting, filling in and blunt ligation. A unique *Eco*RI site at position 260, just 5′ of the initiation codon of alkaline phosphatase, was also eliminated. A *Bam*HI site at the 3′ end of the SV40 enhancer (position 2302) was eliminated by partial digestion, filling in and blunt-ended religation. A second *Bam*HI site in the coding region of human placental alkaline phosphatase was eliminated by changing a single base without changing the resulting amino acid sequence. The stop codon of alkaline phosphatase was replaced by a short polylinker (*Bam*HI, *Eco*RI, *Xba*I) to allow insertion of fusion proteins. The new vector was named pSEAP2M (M = modified).

A similar construct was made in which the non-cleavable signal/transmembrane sequence of the transferrin receptor was replaced with the cleavable signal sequence of influenza haemagglutinin [16] in the vector pCI-neo (Promega) in which the *Bam*HI site at the the end of the neomycin-resistance gene of the vector had been eliminated by cutting, filling in and religation. This construct was also efficiently secreted and bound transferrin, but lacked the alkaline phosphatase tag.

The cDNA clone pcDTR1 encoding the human transferrin receptor [17] was generously provided by Dr. Lukas Kühn (Harvard University, Cambridge, MA, U.S.A.). The sequence Glu^{93}–Pro^{94}, which lies just outside the cell membrane, was changed to Asp-Pro to allow for the creation of a *Bam*HI site. The resulting clone was cut with *Bam*HI near the 5′ end, and *Xba*I, which cuts just 3′ of the stop codon of the transferrin receptor.

Abbreviations used: RGD (one-letter amino acid code), Arg-Gly-Asp; SV40, simian virus 40.

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Ligation into pSEAP2M generated an in-frame fusion protein with secretory alkaline phosphatase at its 5′ end, followed by the extracellular domain of the human transferrin receptor (lacking only the three juxtamembranous extracellular amino acids 90–92) at its 3′ end. This construct was named pSEAP2M-HTFR. The fusion protein was efficiently secreted.

Clone pSEAP2M-HTFR was transfected into COS-7 cells by electroporation (300 V, 125 μF, time constant of 8–12 ms). Supernatants were harvested after 3 days. Supernatants were collected in Phenol Red-free medium containing 4 % (v/v) foetal-bovine serum {which contains bovine transferrin, but bovine transferrin has very low affinity for the human transferrin receptor (K_d = 590 nM as against 3.6 nM for human transferrin) [18] and did not interfere with this assay}. Expression of each construct was assessed by measuring total alkaline phosphatase activity in supernatants after destroying bovine alkaline phosphatase by heating supernatants to 65 °C for 30 min. Preliminary experiments (results not shown) showed that this treatment does not affect human placental alkaline phosphatase, nor does it denature the human transferrin receptor. As expected, placental alkaline phosphatase alone did not bind to transferrin-coated plates. Accordingly, the heat-treatment step was omitted in experiments testing specific binding to transferrin.

Secretion rates of wild-type receptor and all mutants were typically within 20 % of each other when assessed by measurement of alkaline phosphatase activity immunoprecipitable from non-heat-treated supernatants using the conformation-sensitive monoclonal anti-(transferrin receptor) antibody OKT9. Minor differences in enzyme activity between supernatants were equalized by appropriate dilution such that the final enzyme activity per unit volume was the same for all groups prior to all binding assays.

A simple enzyme-linked receptor-binding assay was set up by coating flexible polyvinyl plates with iron-loaded human transferrin (10 μg/ml in 50 μl of PBS overnight at room temperature) followed by washing in 1 % (w/v) non-fat powdered milk in PBS to saturate any remaining non-specific protein-binding sites. Plates were then incubated with 50 μl of appropriately diluted COS cell supernatant for 1 h at room temperature, followed by extensive washing in the same buffer. After a final wash in PBS lacking milk, alkaline phosphatase substrate (120 mM *p*-nitrophenyl phosphate in 50 μl of 2 M diethanolamine buffer, pH 9.5, containing 1 mM $MgCl_2$ and 20 mM L-homoarginine) was added and colour allowed to develop. Plates were read in a microplate reader (Bio-Rad) at 405 nm after 1 h, at which time all readings were within the linear region. The absorbance in the negative control group, with alkaline phosphatase vector with no insert, was identical with that of substrate alone, and was therefore defined as zero, and subtracted from all readings, and binding was normalized to 100 % of the wild-type receptor.

Site-directed mutagenesis was carried out with the Gene Editor system (Promega Inc., Madison, WI, U.S.A.) with double-stranded DNA as template. All constructs were verified by DNA sequencing, and two clones of each mutant were routinely tested with identical results.

In order to test the conformation of the mutant receptors, a panel of monoclonal antibodies to the human transferrin receptor was tested by immunoblotting on soluble wild-type human transferrin receptors that had been subjected to SDS/PAGE and transferred to nitrocellulose membranes. All monoclonal antibodies were available within the Authors' Laboratory. The antibodies were OKT9, N-L009, N-L025, N-L086, N-L115, N-L130, N-L134 and N-L135 [5]. All antibodies except N-L025 reacted with the non-reduced transferrin receptors, but only antibodies N-L130 and N-L134 reacted with the reduced receptors. It was concluded that all antibodies except these two recognized conformational epitopes.

Polyvinyl plates were then coated with 10 μg/ml Protein G (Pharmacia), washed, blocked with BSA, then incubated with 100 μl of diluted ascites containing at least 10 μg/ml of each antibody, washed again, incubated with 100 μl of the relevant COS cell supernatant, washed again, and substrate added. As for the transferrin-binding assay, minor adjustments were made by appropriate dilution of supernatants so that the enzyme concentration was the same in all groups.

RESULTS AND DISCUSSION

We have observed the presence of an RGD sequence at amino acids 646–648 (human numbering system) in all transferrin receptors so far sequenced, including those of human, mouse, rat, Chinese hamster and chicken (Figure 1). If we make the simplifying assumption that all amino acids are present at equal frequency, the odds of finding an RGD sequence by chance in any random tripeptide are about 1 in 20^3 (i.e. 1 in 8000). A protein of 760 amino acids has 758 tripeptides, as the amino acid sequence can be read three residues at a time in an overlapping fashion (i.e. residues 1/2/3, 2/3/4 … 758/759/760). The odds of finding the RGD sequence occurring would then be about 1:10 against. However, conservation of an RGD tripeptide does not, in itself, prove a role in ligand binding. We have therefore examined the functional significance of the RGD motif in the transferrin receptor using site-directed mutagenesis.

Novel approaches are required to allow functional assessment of transfected mutant transferrin receptors, because all dividing mammalian cells possess high levels of transferrin receptors on their surfaces [10]. Transfection of mammalian cells with mutant transferrin receptors would be complicated by binding of transferrin to endogenous receptors. We have therefore constructed a water-soluble secreted transferrin receptor in which the cytoplasmic tail and transmembrane region are replaced by secretory alkaline phosphatase (Figure 2). Constructs were transfected into COS-7 cells and the biological activity of the enzyme-labelled secreted receptors was evaluated.

Expression of secreted wild-type and mutant transferrin receptors was assessed by total heat-stable alkaline phosphatase activity in the supernatant and was found to be similar in all groups. The rates of secretion were also found to be similar (within 20 %) in all groups when evaluated by measurement of non-heat-treated alkaline phosphatase activity immunoprecipitated by the conformation-sensitive monoclonal anti-transferrin receptor OKT9, indicating that folding, transport and secretion of mutant receptors occurred normally.

Binding of secreted wild-type and mutant receptors was detected by an assay in which human transferrin was coated on to plastic wells, followed by binding of alkaline phosphatase-labelled transferrin receptors. Binding was specific and could be inhibited by nanomolar concentrations of soluble human transferrin (results not shown). Approx. 1000-fold higher concentrations of bovine transferrin were required to achieve the same degree of inhibition, consistent with the very low affinity (K_d = 590 nM) of bovine transferrin for the human receptor [18]. Similar results were obtained using human transferrin covalently attached to CNBr-activated Sepharose beads (results not shown).

Because all receptor constructs had similar rates of secretion, the signal generated by the binding of wild-type human transferrin receptors to human transferrin was defined as 100 %, and the signals generated by mutant receptors expressed as a percentage of this value. After replacement of Arg^{646} by alanine, binding of the receptor to transferrin was no longer detectable in

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HUMAN TFR              635 MGLSLQWLYSARGDFFRATSRLTTDFGNAE 664      M11507
MOUSE TFR              638 MGLSLQWLYSARGDYFRATSRLTTDFHNAE 667      X57349
RAT TFR                    MGLSLQWLYSARGDYFRATSRLTTDFHNAE          M58040
CHINESE HAMSTER TFR    632 MGLSLQWLYSARGDFFRATSRLTTDFHNAE 661      119142
CHICKEN TFR            651 LGLTLDWLFFARGDFQRAVTALRRDIANSD 680      X55348
                           +**+* **+ ****+ **++ *  *+ * +
                           # # # ###     #   #  #   #
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Figure 1 Conservation of RGD sequence in transferrin receptors of all species whose sequence is currently known

Numbering is not given for the rat receptor because the published sequence is incomplete at the N-terminus. GenBank® accession numbers are given to the right. *, Totally conserved between species; +, conservative substitutions; #, conservation of hydrophobicity.

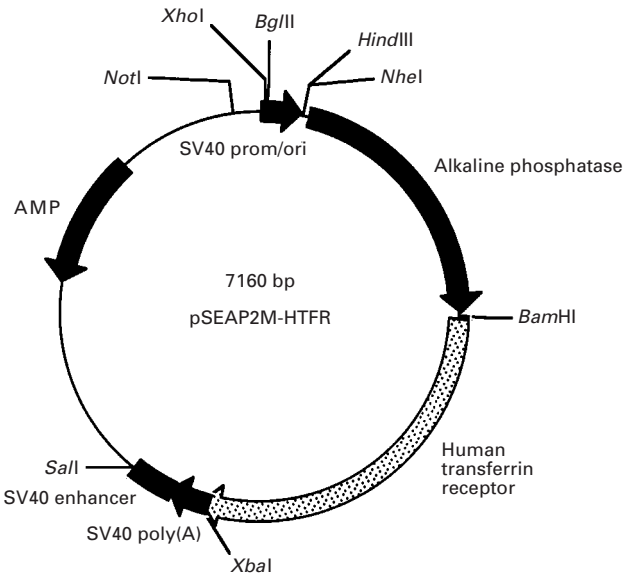

Figure 2 Construction of a vector containing the extracellular domain of the human transferrin receptor fused with a secretory form of human placental alkaline phosphatase

Abbreviations: AMP, ampicillin-resistance gene; prom, promoter; ori, origin; HTFR, human transferrin receptor.

this system, and conservative substitution with lysine decreased binding to 5% of the wild-type value. Replacement of Arg^{646} by histidine also decreased binding to undetectable levels. After replacement of Gly^{647} by alanine (i.e. the addition of a single methyl group), binding was also undetectable. Asp^{648} seemed somewhat less critical, because conservative substitution by glutamic acid allowed 57% of control binding, and replacement of Asp^{648} by alanine still allowed 16% of control binding. Simultaneous replacement of Arg^{646} by lysine and replacement of Glu^{648} by aspartate resulted in no detectable binding.

Although we have not substituted each amino acid in the RGD triad with all of the other 19 amino acids, our data suggest that a basic residue at position 646 is required for transferrin binding. Arginine is strongly preferred over lysine, and substitution by histidine abolished detectable binding. Even the simplest and smallest possible modification of Gly^{647} by addition of a methyl group, converting it to alanine, abolished binding, suggesting that glycine is essential at position 647. Position 648 seems to require an acidic residue.

The interpretation of site-directed mutagenesis is complicated by the possibility that even point mutations could destabilize the whole protein or have distant effects. This issue was addressed by setting up a 'capture' assay using Protein G-coated plates and two different monoclonal antibodies that had been shown to recognize conformational epitopes on the human transferrin receptor (OKT9 and N-L009; see the Experimental section). The signals observed for the wild-type receptor and all mutants were typically within 20% of each other when tested on the same antibody. Since most monoclonal antibodies recognize conformational epitopes [19] and the antibodies had been selected because they only reacted with the non-reduced receptor, we conclude that none of the mutations caused major conformational changes in the receptor.

In addition, many of the changes that had major effects on binding to transferrin were minor and conservative, such as the addition of a single methyl group in the case of changing glycine to alanine, or the change of a positively charged amino group of lysine to the positively charged guanidino group of arginine. Even if one of these substitutions caused unexpected deleterious effects, it seems unlikely that all of them would have such a marked effect on binding of transferrin by such indirect mechanisms.

Further evidence against a large global change in conformation induced by these mutations is that in each case the secreted protein was quantitatively precipitable by the conformation-sensitive monoclonal antibody OKT-9, and that the rates of secretion of the variants were not markedly different from the wild-type receptor. Malfolded proteins are usually retained in the endoplasmic reticulum bound to chaperonins, where they are degraded [20].

Previous studies using chicken–human receptor chimaeras have been able to localize the transferrin-binding site of the receptor to the C-terminal 192 amino acids (amino acids 569–760; [10]), which contain the RGD sequence. The RGD tripeptide in the transferrin receptor is in the centre of this region (amino acids 646–648) and is encoded in exon 18, which encompasses amino acids 634–680 [11]. To our knowledge our data show, for the first time, that single point mutations in the transferrin receptor affect binding of transferrin.

Structural data are now available for a number of functional RGD sequences, including the RGD region of fibronectin [21], disintegrins [14] and leech anticoagulant proteins [15]. In each case, the RGD tripeptide lies close to the tip of a loop that protrudes from the body of the protein. The requirement for conservation of the glycine residue seems to reflect the need for conformational freedom for the formation of a reverse turn for the loop to fold back upon itself. We note that positions 635, 637, 639, 641–643 on the amino side of the RGD, and 649, 653, 656

and 660 on the carboxy flanking regions of the RGD sequence contain highly conserved hydrophobic amino acids (Figure 1) which might be buried together and stabilize such a loop.

Our data implicate the RGD region in binding of transferrin, but, by analogy with the integrin systems, specificity of binding probably involves a considerably larger area. The presence of a functionally significant RGD sequence in the transferrin receptor might signify a distant evolutionary relationship between proteins involved in cellular adhesion and those involved in iron uptake. However, a BLAST search of the GenBank® database using exon 18 did not reveal any significant similarities other than the transferrin receptors themselves. An alternative possibility is that RGD represents a versatile motif that can be used in many different ways, and that the observed similarities represent convergent evolution.

Structural data are also available for transferrin [22], and although the transferrin receptor has been crystallized [23], its structure has not yet been solved. Recently, the structure of the haemochromatosis gene product, HFE, has been shown to resemble a class I major histocompatibility antigen and to bind to the transferrin receptor and decrease its affinity for transferrin [24,25]. Determination of the structure of the transferrin receptor–transferrin–HFE trimolecular complex is now feasible and may clarify many issues concerning the uptake of iron by cells.

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