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Mechanisms of TfR-mediated transcytosis and sorting in epithelial cells and applications toward drug delivery

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Abstract

Transferrin receptor has been an important protein for many of the advances made in understanding the intricacies of the intramolecular sorting pathways of endocytosed molecules. The unique internalization and recycling functions of transferrin receptor have also made it an attractive choice for drug targeting and delivery of large protein-based therapeutics and toxins. Recent advances in elucidating the role of the intracellular controllers of transferrin recycling and sorting, such as Rab proteins and their effectors, have led to enhancement of transferrin receptor as a drug delivery vehicle. This review focuses on the use of transferrin receptor as an agent for facilitating drug delivery and targeting, and the role that mechanisms of transferrin receptor sorting and transcytosis play in these events.

Keywords: Transferrin-receptor; Transepithelial drug delivery; Transcytosis; Endocytosis

Contents

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1. Introduction

Iron is a fundamental necessity for almost every living organism. The ability of this metal to readily exist in two common redox states leads to its requirement as a co-factor in many fundamental biological processes, including O_2 absorption, ATP generation through oxidative phosphorylation, DNA synthesis, etc. However, the charged and reactive nature of free iron presents a challenge for efficient tissue distribution and cellular uptake. Transferrin (Tf) and its cognate receptor have been used by vertebrate species to overcome these barriers for efficient and regulated intracellular iron delivery. The ubiquitous nature of the transferrin-receptor (TfR) and the fact that it is internalized through endocytic means has made it an essential tool for the general elucidation of sorting and recycling pathways of endocytically derived vesicles in epithelial cells. In addition, the high specificity of endocytic uptake of Tf by TfR has made it a subject of interest for targeted drug delivery via parenteral administration and for delivery of TfR-targeted drug conjugates across epithelial barriers.

This review focuses on the sorting and recycling pathways of TfR in epithelial cell systems. Special focus is given to the methods by which the understanding of these pathways can be exploited to achieve higher efficiency in targeted delivery systems.

2. Transferrin, transferrin-receptor, and associated proteins

2.1. Structure and function of transferrin

Transferrins are a structurally related class of metal-binding glycoproteins of approximately 80 kDa in size whose primary function is the binding and transportation of non-heme iron [1–5]. The transferrins have been classified into three major sub-classes: serum Tf, lactoferrin, and ovotransferrin. Serum Tf is responsible for binding and transporting iron through many of the biological fluids including blood, lymph, cerebrospinal fluid, colostrum, bile, amniotic fluid, and breast milk [6–10]. Lactoferrin is found in breast milk, saliva, and tear secretions; it is thought to function primarily in these environments as an iron chelator, sequestering iron to act as a bacteriostatic agent [11–13]. Lactoferrin also is implicated in modulating leukocyte antibacterial activity and may also be involved with inflammatory responses [14,15]. Unlike serum Tf, lactoferrin does not contribute iron to erythrocytes, and it is cleared by the reticulo-endothelial system [16]. Ovotransferrin is found in reptile and avian oviduct secretions as well as avian egg white, and its function is analogous to that of lactoferrin, purported to primarily serve as a bacteriostat via the sequestration of iron [17–19]. In addition to the three major types of transferrin, melanotransferrin (p97) can also be con-

sidered to be part of the transferrin family. This fourth member of the transferrin family is a structural homologue distinctly different from the others in that it seems to have little to do with the receptor-specific cellular uptake of iron [20,21]. Rather, this protein is predominantly localized to the surface of human melanomas and may assist in rapid cell proliferation via iron scavenging and the prevention of lipid peroxidation [22,23]. Considering that serum transferrin has been used extensively in endocytosis research, and since it is the protein that has been identified as a potential drug carrier candidate, it will be the focus of the remainder of the review; the other three transferrins will not be discussed further.

The serum transferrins from different species share a high degree of homology, typically composed of a single chain polypeptide with approximately 700 amino acids. However, there are varying degrees of carbohydrate content between species [24]. Human transferrin is glycosylated with identically branched heterosaccharide chains linked to the polypeptide by aspariginyl-*N*-acetylglucosamine bonds at two separate residues [25] and one O-linked carbohydrate at Ser51. While the carbohydrate chains of transferrin are seen in all species, they do not affect the ability of transferrin to bind to transferrin-receptor, nor do they affect they ability of the receptor complex to be internalized into the cell [26,27]. In addition, in contrast to other proteins, the desialation of transferrin has little effect on the catabolism rate [28].

Transferrin is composed of two homologous globular domains, known as the N and C lobes, connected by a short linear peptide region, that function in a similar fashion. Both lobes of the transferrin molecule are capable of binding one iron atom with similar affinities. However, one of the necessities for stringent binding of iron to transferrin is the presence of synergistic bicarbonate anions [29–31], which allows Fe^{3+} to tightly coordinate with the amino acids that are suspected of comprising the iron binding pocket of the N and C lobes (i.e., two Tyr, one Lys and one His for each lobe) [32,33]. Lack of the presence of bicarbonate anion also affects the ability of transferrin to properly release Fe^{3+} once it is bound, with bicarbonate-free transferrin demonstrating poor iron release capabilities [34,35]. In addition, transferrin has been suggested as a suitable carrier for medically therapeutic metal ions, since transferrin has the ability to bind to other metal ions in vitro [36–39]. However there is little evidence to suggest that transferrin has significant interactions with metals other than iron under normal in vivo conditions [7].

One of the unique features of transferrin is that the binding of Fe^{3+} alters the shape of the molecule, affecting the binding affinity of transferrin for its receptor. This conformational change seems to occur in a stepwise fashion as each iron molecule is bound [40–42]. The iron-loaded transferrin, or holotransferrin, binds to the receptor with greater affinity than mono-transferrin or apo-transferrin [43,44]. Binding of iron also results in increased stability of the molecule relative to the apo-form, with the iron-loaded molecule exhibiting increased resistance to thermal or proteolytic degradation [45,46].

Transferrin undergoes a conformational change as a result of a trigger-mechanism that is induced by the decrease in pH during the endocytosis cycle. This allows for the release of iron into the endosomal compartment of the cell and the subsequent recycling of the apo-transferrin to the cell surface, where the lowered affinity of the apo-transferrin for the receptor at extracellular pH causes the release of transferrin from the receptor [47,48]. A schematic of the endocytosis and recycling cycle for the transferrin/transferrin-receptor complex is shown in Fig. 1.

The liver is the principle site of transferrin production and catabolism. This can be most dramatically demonstrated in the case of human liver transplant patients, who after receiving a new liver, obtain the electrophoretic profile of the donor's serum transferrin (as determined by the normal inter-individual variability in post-translational modification of Tf) [49,50]. In addition to this example, in vitro studies with rat liver have determined that the rates of incorporation of radiolabeled amino-acids into transferrin-precursors were nearly identical to the total synthesis values obtained by in vivo perfusion studies [51–53]. While the liver is responsible for the large majority of Tf catabolism, a significant amount of Tf is also secreted by the liver into the bile, with estimates for the average normal adult of approximately 100 mg/day passing into the small intestine [54].

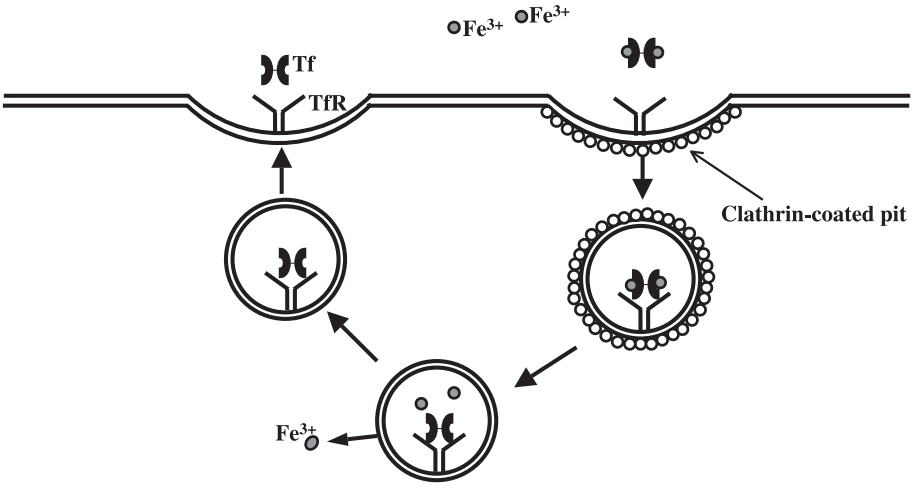

Fig. 1. The classical endocytosis pathway of transferrin-receptor (TfR). Transferrin (Tf) binds two Fe^{3+} atoms per molecule. The iron-loaded Tf is subsequently bound by TfR and the Tf/TfR complex is internalized via clathrin coated pits. The iron is released from Tf in the acidic endosomal environment and Tf/TfR is recycled back to the plasma membrane. Iron-free Tf is then released from TfR to complete the cycle.

2.2. Transferrin-receptor

2.2.1. Structure and function

TfR was first recognized through formation of monoclonal antibodies raised from mice that were immunized against various neoplastic cell lines, with several of the antibodies recognizing antigenic portions of the protein we now know as TfR1 [55–57]. The receptor is a homodimer composed of two identical subunit monomers with an approximate mass of 90 kDa each [58]. The ectodomain of the receptor is composed of three distinct domains, the protease-like, apical, and helical domains (Fig. 2). The helical domains of TfR face each other and interact to form the dimeric receptor [59]. The extracellular portion of the receptor is subject to extensive post-translational modification with three sites of mannose rich *N*-linked glycosylation at residues Asn251, Asn317, and Asn727 and one *O*-linked glycosylation on Thr104 [60]. The monomers are covalently linked by two disulfide bonds at residues Cys 89 and Cys98 [61]. Treatment with trypsin cleaves two 70 kDa fragments that retain the ability to bind to Tf. The protein also has a single transmembrane domain, comprised of residues 68–88 of the polypeptide. Following this, it has a short cytoplasmic tail, of 5 kDa in size, that is the site of phosphorylation and acylation of the receptor [61]. Unlike Tf, the post-translational modifications of TfR seem to be crucial for its biological functionality. Specifically, mutations that eliminate the sites of potential *N*-linked glycosylation severely limits the ability of the receptor to bind to Tf. In addition, mutations to the *O*-linked glycosylation at Thr104 suggest that reduction in this type of carbohydrate linkage may limit the cellular half-life of the receptor [62,63]. The sequence and secondary structural motifs of TfR is shown in Fig. 3.

The receptor has very high affinity for differic-Tf, with binding constants on the order of $2-7 \times 10^{-9}$ M [64]. The exact orientation of Tf binding to TfR is not fully understood, however the primary recognition site appears to reside on the C lobe of Tf, with a sequential synergistic effect between the N and C lobes of Tf that enhances affinity for TfR [65,66]. In addition, site-directed mutagenesis studies have shown that the central helical domains of TfR, formed by the union of the two monomers, are key to the ability of the receptor to recognize Tf [67]. However, inspection of the computational union of Tf and the TfR crystal structures (ectodomain) have also indicated that much favorable interaction occur between the outward facing apical domains of the receptor and Tf [59]. The iron saturation status of Tf also plays a large role in the ability of TfR to recognize Tf, with apo-Tf having very little affinity for TfR at physiological pH 7.4 [43]. However, at pH 5.0 Tf forms a

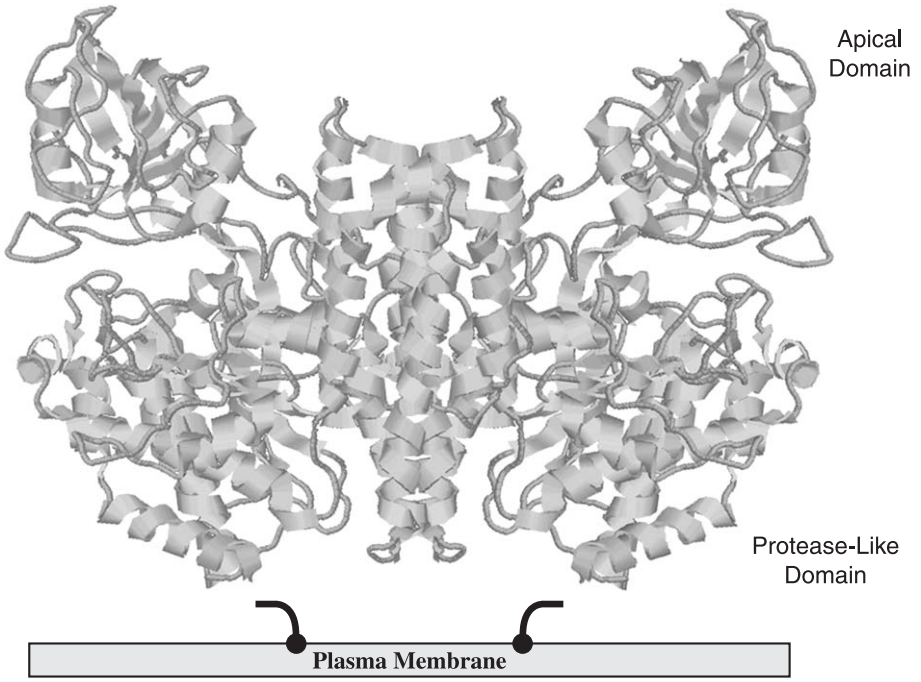

Fig. 2. The X-ray crystal structure of the transferrin receptor ectodomain. The putative dimeric orientation of two transferrin receptor molecules is shown as suggested by Lawrence et al. [59]. The receptor is organized into three distinct domains, composed of the protease-like, apical, and helical domains. The stalk region is also shown with the suggested orientation relative to the protease-like domain and the plasma membrane. The image was rendered from the pdb coordinates of the A subunit of 1CX8 (human TfR, http://www.rcsb.org) [59] using RasMol and Adobe Photoshop.

very stable complex with TfR, which is the underlying basis for the iron exchange and transport capability of Tf [68–70].

Within the past few years a new member of the TfR family was discovered and denoted as transferrin receptor 2 (TfR2). However, unlike TfR1, TfR2 has a much lower affinity for Tf (25 fold lower than TfR1) and it seems to be primarily involved with regulation and maintenance of iron homeostasis in the body, and it might also play a minor role in the delivery of iron to rapidly dividing tissues. The TfR2 gene produces two transcripts of 2.5 and 2.9 kb in length. Analysis of the amino acid sequence suggests that TfR2 is a type II transmembrane glycoprotein, like TfR1, sharing 45% sequence identity and 66% similarity in the extracellular domain [71]. In contrast to TfR1, TfR2 is primarily expressed in the liver [71,72]. It lacks iron-regulatory elements in the coding and noncoding regions of its mRNA, indicating that the expression of TfR2 is not regulated by the iron-responsive protein feedback mechanism, as seen for TfR1. It would appear from this that TfR2 plays a minor role in regulating intracellular iron concentrations, or it perhaps responds to a yet undiscovered intracellular regulatory mechanism [73]. In spite of these differences, TfR2 interacts with Tf in a similar fashion as TfR1. Specifically, TfR2 exhibits changes in binding affinity to Tf as a result of environmental pH and the iron saturation status of Tf. Specifically, TfR2 does not bind very well to apo-Tf at physiological pH, demonstrating a preference for holo-Tf, while an acidic milieu results in preferential binding of the apo-Tf [73]. While there has been some indication that TfR-2 expression levels may be a marker of erythroid precursor cells, as levels of TfR2 mRNA levels in erythroleukemia marrow samples are significantly elevated [74,75], most of the data seem to support a role for TfR2 in modulating iron homeostasis. For example, mutations in TfR2 have been associated with a form of hereditary hemochromatosis that

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  1 LYWDDLKRKL SEKLDSTDFT STIKLLNENS YVPREAGSQK DENLALYVEN
      HHHHHHHH HHHHHT  HH HHHHHTTTGG GSS  TTSHH HHHHHHHHHH

 51 EFREFKLSKV WRDQHFVKIQ VKDSAQNSVI IVDKNGRLVY LVENPGGYVA
    HHHHTT S    EEEEEEEEE EE SS  EEE EE SSS  S    B  SS BT

101 YSKAATVTGK LVHANFGTKK DFEDLYTPVN GSIVIVRAGK ITFAEKVANA
    T   EEEEE  EEE TTTTTT TTT SSS  T TSEEEEES S S HHHHHHHH

151 ESLNAIGVLI YMDQTKFPIV NAELSFFGHA HLGTGDPYTP GFPSFNHTQF
    HHTT   EEE E  TTTS  S  SS  B B    SSSS TT S SS    SSSS

201 PPSRSSGLPN IPVQTISRAA AEKLFGNMEG DCPSDWKTDS TCRMVTSESK
       S SS  S S EEEE HHH HHHHHHTB S    SSSSS T T  SEE TT

251 NVKLTVSNVL KEIKILNIFG VIKGFVEPDH YVVVGAQRDA WGPGAAKSGV
    EEEEEE EEE EEEEEEEE   EE  SSBTT  EEEEE      SS  TTTTHH

301 GTALLLKLAQ MFSDMVLKDG FQPSRSIIFA SWSAGDFGSV GATEWLEGYL
    HHHHHHHHHH HHHHHHHTT     SB EEEE E  SGGGTTH HHHHHHGGGT

351 SSLHLKAFTY INLDKAVLGT SNFKVSASPL LYTLIEKTMQ NVKHPVTGQF
    TTHHHHB EE EE TT  S S S  EEEE GG GHHHHHHHHT T B SSSSSB

401 LYQDSNWASK VEKLTLDNAA FPFLAYSGIP AVSFCFCEDT DYPYLGTTMD
    SS TT SS    B   TTTHH HHHHHTS    EEEEE   SS   SSTTBTT

451 TYKELIERIP ELNKVARAAA EVAGQFVIKL THDVELNLDY EEYNSQLLSF
     HHHHHTTTT THHHHHHHHH HHHHHHHHHH HHSSS    S SHHHHHHHHH

501 VRDLNQYRAD IKEMGLSLQW LYSARGDFFR ATSRLTTDFG NAEKTDRFVM
    HHHHHTTHHH HHHSS   HH HHHHHHHHHH HHHHHTHHHH HTTTT HHHH

551 KKLNDRVMRV EYHFLSPYVS PKESPFRHVF WGSGSHTLPA LLENLKLRKQ
    HHHHHHHHHH TGGGBTTTTT TTT S  BTT T  STTTHHH HHHHHHHHHT

601 NNGAFNETLF RNQLALATWT IQGAANALSG DVWDIDNEF
     SS S  HHHH HHHHHHHHHH HHHHHHS S SSS
```

Fig. 3. The sequence and secondary structure of human transferrin receptor. Each residue in the sequence is reported as a single letter code. Secondary structure is calculated and described according to an implementation of the method of Kabsch and Sander (1983) *Biopolymers* **22**, 2577–2637. The assignments are: H = helix; B = residue in isolated beta bridge; E = extended beta strand; G = 310 helix; I = pi helix; T = hydrogen bonded turn; S = bend. From the Protein Data Bank, http://www.rcsb.org.

is not associated with forms of hemochromatosis that arise from mutations in the hemochromatosis protein (HFE) [76–79], suggesting role for TfR2 in excessive iron uptake. In addition, TfR2 has also been implicated in regulating expression of hepcidin, a modulator of dietary iron uptake [80].

2.2.2. Regulation of expression and tissue distribution

TfR1 expression is regulated at the post-transcriptional level by interactions between iron-regulatory proteins (IRP) and iron-response elements (IRE). The IRE are portions of the 3′-untranslated portion of TfR mRNA that form secondary structural features in the shape of five hairpins [81,82]. These hairpins are required for the iron-dependent regulation of TfR expression. The IRE of TfR mRNA are recognized and bound by the IRP, thus controlling the extent of TfR mRNA translation and stability [83]. Under iron deprivation conditions, IRP bind to the IRE increasing the stability of the mRNA and thus up-regulating the expression of TfR [84–86]. There are two distinct IRP that interact with TfR IRE, known as IRP1 and IRP2. IRP1 and IRP2 respond to variations in iron concentration via differing mechanisms [83,87].

IRP1 has been considered to be a bifunctional enzyme, functioning in a similar fashion to aconitase when cellular iron levels are plentiful, with no RNA binding ability, and subsequently switching to a predominantly RNA binding protein functionality when cellular iron levels are low [88–92]. IRP1 also shows dual functionality with regard to regulation of intracellular iron concentrations. For example, not

only does it bind the IRE of TfR to up-regulate TfR production, but evidence suggests that in iron-rich conditions it also binds the 5′-region of ferritin mRNA to inhibit the complete translation of the ferritin protein[93–96]. The resultant down-regulation of ferritin would conceivably result in the complementary liberation of free iron from intracellular storage.

In contrast, IRP2 synthesis is directly affected by intracellular iron levels, with regulation occurring at the ubquitination level [97–99]. For example, when iron stores are plentiful, IRP2 rapidly undergoes ubiquitination and is degraded in the cellular proteosome machinery [97]. In conditions where intracellular iron levels are low, IRP2 is up-regulated by the renewed production of non-proteosome directed protein [100,101]. While there are differences in the regulation of IRP1 and IRP2 in response to iron levels, their functional significance in relation to TfR is not necessarily mutually exclusive [102]. While constitutive expression of mutant inactive IRP1 in vitro may adversely affect normal iron-metabolism [103], in vivo data with mice that are IRP1 deficient demonstrate no abnormalities in iron utilization, implying that when intracellular IRP1 is lacking, IRP2 may be sufficient to successfully regulate TfR expression [104].

The regulation of TfR2 is not well understood at the cellular level. For example, in contrast to TfR1, chelation of iron does not up-regulate the expression of TfR2 [105]. Indeed, in several instances TfR2 behaves in a manner opposite to TfR1. For example, in liver development, TfR2 is up-regulated, while TfR1 is down-regulated; conversely, during erythrocytic differentiation of murine erythroleukemia cells, expression of TfR1 steadily increases, while TfR2 expression is down-regulated [75].

TfR is expressed in many tissue types in the body, particularly those areas that feature a normally high turnover of cells. The level of receptors is particularly high in erythrocyte precursors, placenta, and the liver [106–110]. Of particular note is the presence of TfR in appreciable amounts on in the capillaries of the brain [111], epithelium of the small intestine [112,113], type II pneumocytes [114], and neoplastic carcinomas [115]. Knowledge of the TfR present in these tissues had led to the focus of targeting of TfR for the delivery of therapeutic molecules.

2.3. Proteins associated with transferrin and transferrin-receptor

2.3.1. HFE

Hereditary hemochromatosis is a genetic disorder that affects approximately 1 in 200 Caucasians. It is a disease characterized by excess dietary iron absorption and iron deposition in several tissues. Clinical consequences include hepatic failure, hepatocellular carcinoma, diabetes, cardiac failure, impotence, and arthritis. Although an increase in plasma iron levels may be evident early in life, in most cases these clinical symptoms do not present themselves until age 40–50, as iron-levels are slowly but steadily increased throughout the life of the individual [116,117]. The symptoms of hereditary hemochromatosis are thought to derive from a defect in the hemochromatosis gene which results in a decrease in the amount of functional gene product, known as HFE, a protein closely related to the major histocompatibility complex class I molecules [118]. HFE is preferentially expressed in duodenal crypt cells and Kupffer cells of the liver, predominantly localized to the perinuclear region of the cell [119,120]. The HFE protein is a 343-residue type I transmembrane glycoprotein that contains a membrane bound heavy chain (like MHC class I proteins) with three extra-cellular domains [121]. However, unlike the MHC class proteins, two of the helices in the extra-cellular domain are spaced much closer together, eliminating what would normally be the peptide-binding groove for the MHC class I proteins. This difference reflects the fact that HFE is important for modulating Tf-mediated iron uptake rather than peptide binding [122,123]. The majority of hemochromatosis patients have a defect in a single codon of the gene that results in the mutation of Cys282 to Tyr282, the result of which is the abolishment of a crucial disulfide bond in HFE [124,125]. Shortly after the discovery of the HFE gene, it was shown that HFE closely interacts with the transferrin receptor [121,122,126,127]. In addition, confirmation of HFE's role in hemochromatosis was provided by gene knockout studies in mice where the HFE knockout mice demonstrated elevated transferrin saturation and increased iron storage in hepatocytes by 10 weeks of age [128]. HFE binds to TfR1 with a high affinity at physiological pH of 7.4, similar in magnitude to Tf, and it competes with Tf for binding to TfR, reducing

the affinity of the receptor for Tf in a noncompetitive manner [123,129]. Unlike apo-Tf, HFE has very low binding affinity for TfR at pH 6.0, which is representative of the environment that would be seen in the early part of the endosomal pathway [121]. HFE is closely related to the TfR-specific iron uptake pathway [130–132], and it co-localizes with TfR rich intracellular compartments [127] and with TfR on the basolateral surface of enterocyte precursor cells [120]. It has been shown that HFE binding to TfR is required for HFE to be transported to TfR positive endosomes and for the regulation of iron homeostasis in cultured cells, but binding is not required for HFE to be transported to the basolateral membrane [133]. While HFE has been shown to avidly bind to TfR1, it has very little affinity for TfR2 [134]. HFE, through its interactions with Tf and TfR, is implicated in sensing and regulating iron-levels in the body, particularly at the level of iron uptake through the duodenal enterocyte. It is possible that HFE is not the sole mechanism by which the iron-body stores are regulated, as HFE deficient mice are still capable of regulating uptake of iron from the diet to some extent [128,135], but HFE plays a vital role in regulating iron-stores. The exact mechanism of HFE and Tf/TfR interactions that lead to iron uptake regulation remains to be discovered.

2.3.2. Cubilin and megalin

Cubilin and megalin are large cell-surface receptor proteins that are the mediators of endocytic uptake for a wide array of ligands. These two proteins, depending upon the tissue, are closely associated but structurally very different. Both receptors are expressed primarily on the apical surfaces of polarized epithelial cells. Megalin and cubilin bind distinct ligands with variable affinities. In some cases, the two proteins act independently of one another, while in other situations the two receptors are co-expressed and act in concert with one another as a dual-receptor complex. The cubilin/megalin receptor system is of interest to this review because recent evidence has suggested that this receptor system is a major pathway for the uptake of transferrin from the apical membranes of polarized epithelia.

2.3.2.1. Structural features of cubilin and megalin. Cubilin is a 3600 amino acid protein with a non-glycosylated molecular weight of approximately 400 kDa. Post-translational modification of the protein results in the mature protein with a molecular weight of 460 kDa [136,137]. The complete cDNA sequence has been elucidated for human [138], canine [139], and rat [137]; it shows a high degree of homology between the species. Analysis of the sequences revealed that cubilin is composed of an initial region of roughly 110 amino acids followed by eight epidermal growth factor (EGF)-like domains followed by 27 CUB (complement subcomponents C1r/C1s, EGF-related sea urchin protein and bone morphogenic protein-1) domains [140]. While cubilin is believed to be bound to the plasma membrane of the cell at its amino terminus [141], no transmembrane segments have been identified in the protein, and it does not have a glycosylphosphatidylinositol (GPI)-anchor. Helical plotting of the amino-terminal region of cubilin has suggested that an amphipathic helix is formed by the protein that is similar to the lipid binding regions of apolipoproteins [141]. It has been suggested that cubilin is anchored to the plasma membrane by the insertion of the hydrophobic residues of the conserved helices into the lipid bilayer, as is postulated for other proteins [142–146]. In addition, cubilin has a potential palmitoylation site at the amino terminal region which could further assist in anchoring the protein to the membrane [147,148].

Megalin is a 4600 amino acid type I transmembrane receptor protein with a non-glycosylated molecular weight of roughly 517 kDa. Post-translational modification results in the mature protein with a molecular weight near 600 kDa. Megalin belongs to the low-density lipoprotein receptor family [149]. The complete cDNA sequences have been elucidated in rat [150] and human [151]; there is 77% identity between the species. The ectodomain of megalin consists of four lipid binding regions composed of cysteine-rich complement-type/LDLR class A repeats. These are separated by 17 EGF-like repeats and eight cysteine-poor spacer regions, which contain YWTD motifs and are involved in the pH-sensitive release of ligands in endosomal compartments [152]. The short carboxy-terminal cytoplasmic tail of megalin mediates clustering in clathrin coated pits and it contains certain motifs that have been implicated in the genesis of signal transduction [153,154].

2.3.2.2. Expression and interaction with Tf. Megalin and cubilin are co-expressed at the plasma mem-

brane and throughout the endosomal system of epithelial cells. In particular, they are expressed in several absorptive epithelia, including the placental cytotrophoblast [155,156], visceral yolk sac [157,158], renal proximal tubule [157,159–161], and the small intestine [162,163]. In the kidney proximal tubule and visceral yolk sac, the two receptors have been colocalized at the brush border membrane, coated pits, endocytic vesicles and dense apical tubules, which comprise the membrane-recycling machinery in these cells [157,164,165]. In addition, studies have also suggested that megalin and cubilin have some limited presence in lysosomal compartments.

Cubilin has recently been shown to be a receptor for transferrin. In an elegant study by Kozyraki et al., the endocytosis of transferrin by cubilin was shown to be accomplished in a megalin-dependant fashion [166]. This hypothesis is supported by the finding that lysosomes of human, dog, and mouse renal proximal tubules strongly accumulate Tf, whereas no Tf is detectable in the endosomal/lysosomal system of the renal tubule epithelium of dogs with deficient surface expression of cubilin. Elevated levels of Tf are seen in the urine of these dogs. Mice with deficient expression of megalin also excrete excessive levels of Tf in the urine. In addition, it was demonstrated that in megalin-deficient murine kidney-proximal-tubule epithelial cells, transferrin is localized to the lumenal plasma membrane, but not in endocytic vesicles, indicating defective internalization of the cubilin/megalin complex. Cubilin may be responsible for a major portion of the catabolism of Tf in the body. After the liver, the kidney is the site of the majority of Tf catabolism. In addition, the cubilin-mediated endocytosis and lysosomal-routing of Tf in the kidney may have an indirect down-regulating effect on the intestinal uptake of iron via the maintenance of plasma iron levels.

3. The recycling of transferrin receptors

Transferrin receptors have been studied extensively in filter-grown Madin–Darby Canine Kidney (MDCK) cells, a well characterized model system to study vesicular transport in polarized epithelial cells [167]. In MDCK cells, endocytosis occurs from both apical and basolateral plasma membrane [168,169] and the basolateral and apical endosomes appear to be interconnected [170–172]. Odorizzi et al. [172] reported the colocalization of the apically and basolaterally internalized transferrin receptors in the endosomal tubules in the cytoplasm of MDCK cells. The internalized transferrin receptors were transported to the basolateral membrane in a signal dependent manner [172]. However, transferrin recycling in MDCK cells has been shown to occur from both the basolateral early endosomes and the endosomes located close to the centrioles in the cells [173,174]. Sheff et al. [174] described two transferrin recycling pathways with different kinetics in MDCK cells. In their analysis, they used a model that was defined by a series of first order rate constants for the transfer of the ligands, such as transferrin, between different compartments. The majority of transferrin (65%) was found to be recycled from the early endosomes within 10 minutes after internalization [174]. The slow phase of transferrin recycling occurs from the recycling compartment [174]. A recent investigation of transferrin recycling in CHO cells in which perinuclear RE was removed microsurgically, showed the existence of distinct recycling endosome populations in the cytoplasm [175]. The endosomes appeared to correspond to the early endosomes (EEA1-positive), and the recycling endosomes (EEA1-negative), which recruited Rab11 with time [175].

3.1. Rab proteins involved in transferrin trafficking

Rab proteins belong to a family of monomeric GTPases with molecular masses of 20–40 kDa that are involved in different intracellular processes including regulation of vesicular trafficking pathways [176]. The small GTPases Rab4, Rab5, and Rab11 have been studied extensively, and transferrin in the endocytotic route has been found in structures containing Rab5, Rab4 and Rab11 (Table 1). There are three endosomal populations. These are characterized by structures which contain only Rab5, both Rab5 and Rab4, and both Rab4 and Rab11, respectively [177]. Rab5, which is localized primarily to the early endosomes, can be detected in low abundance in Rab11-positive recycling endosomes [178,179]. Transferrin colocalizes with Rab5 in clathrin coated vesicles and within Rab5-positive endosomes [177,179]. It was reported that overexpression of the dominant positive mutant of

Table 1
Rab proteins involved in intracellular processing of transferrin receptor

Rab proteins	Effectors and interacting proteins	Localization	Function
Rab4	Rab Coupling Protein (RCP) [201]	Early and recycling endosomes [185,186]	Regulation of Tf transport between early and recycling endosomes [190]
Rab5	EEA [181,205–211], PI3(K)s hVPS34 and p85α-p110β [218]	Early endosomes, clathrin coated vesicles [177–179]	Regulation of fusion between early endosomes [182]
Rab11	Rip11, RCP, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3, Rab11-FIP4 [200–203]	Recycling endosomes [180,195,196], TGN [197,198]	Regulation of transferrin recycling from recycling endosomes [180]

Rab5, Rab5(Q79L) inhibits the exit of transferrin from the Rab5-positive endosomes to the Rab11-positive recycling endosomes [180]. On the other hand, the rates of transferrin-mediated transcytosis and recycling were not affected by the overexpression of Rab5(Q79L) in Hela cells. This suggests that the GTP hydrolysis by Rab5 is not rate-limiting for the endocytosis of TfR [181], and transferrin receptors that are accumulated in the early endosomes may be recycled back to the plasma membrane by the fast recycling pathway. After the discovery of Rab5 isoforms, Rab5b and Rab5c, the Rab5 protein was renamed Rab5a [182,183]. These three isoforms share all the structural features required for the regulation of the endocytosis. Both Rab5b and Rab5c increased the rate of transferrin endocytosis in vivo [182]. They were also shown to stimulate the early endosome fusion in vitro [182]. However, the Rab5 isoforms are phosphorylated by different kinases in vitro [184].

Rab4 is associated with early endosomes [185] and recycling endosomes, but not with plasma membrane [186]. It appears to be less abundant on recycling endosomes compared to early endosomes [174]. In addition, it is phosphorylated by p34cdc2 kinase [187]. In MDCK cells cotransfected with human transferrin receptor and either Rab4 or the dominant positive mutant of Rab4(Q67L), transferrin receptors have been found to redistribute from the basolateral surface to the apical surface [188]. An enhancement of the apically directed transcytosis of transferrin has also been observed in the transfected MDCK cells and Rab4 [188]. Expression of the dominant negative mutant of Rab4(S22N) or of the chimeric NHRab4cbvn, in which the carboxyl-terminal Cys-Gly-Cys motif of Rab4 was replaced with the transmembrane domain of cellubrevin, did not show any effect on transferrin transcytosis in MDCK cells [188,189]. These results suggest that not only binding to GTP, but also the routing between membrane and cytosol phases, are important for the function of Rab4. However, the role of Rab4 in the transferrin recycling is not completely understood. Chavrier et al. [190] suggested that Rab4 is involved in transferrin transport between early and recycling endosomes.

The presence of Rab11-positive membranes which are enriched in actin, annexin II and t-SNARE syntaxin 13 has been reported [179]. This is consistent with the role of actin filaments in transferrin recycling [191]. The polarized recycling of transferrin in MDCK cells was inhibited by latrunculin B, which depolymerizes the actin filaments [192]. In Caco-2 cells latrunculin A, a latrunculin B analog, caused 20% missorting of pre-internalized transferrin to the apical side [193].

Rab11 has been detected in several different cell types, including epithelial cells [194]. The expression of Rab11 in the apical vesicular population in epithelial cells was reported by Goldenring et al. [194]. Other studies also reported the presence of Rab11 in the pericentriolar recycling endosome [180,195,196] and trans-Golgi network [197,198]. Rab11 is also involved in the recycling of internalized transferrin [180]. Expression of a dominant negative mutant of Rab11 inhibited the slow phase of transferrin recycling [180]. The constitutively inactive mutant of Rab11 (Rab11S25N) was shown to block the transferrin recycling at both 16 and 37 °C, while the wild type Rab11 and the dominant positive mutant form of Rab11 (Rab11Q70L) inhibited the transferrin recycling only at 37 °C [196]. Therefore, the activation of Rab11 by GTP appears to be required for the exit of transferrin from early endosomes towards either the recycling endosome or the plasma membrane [196].

The Rab11 subfamily consists of two isoforms, Rab11a and Rab11b, with a homology of about 89%.

Rab11b has been shown to colocalize with internalized transferrin in the pericentriolar recycling endosome and is essential for recycling of transferrin to the plasma membrane [199]. Rab25, a small GTPase closely related to Rab11, colocalizes with Rab11 in MDCK cells [167]. The rate of IgA transcytosis and of apical recycling of internalized ligands was decreased upon the overexpression of Rab25 in MDCK cells [167]. The basolateral recycling of internalized transferrin was not affected by overexpression of Rab25, suggesting that Rab25 may play a role in apically directed trafficking [167].

3.2. The Rab11 interacting proteins

A number of rab11 interacting proteins including Rip11 [200], Rab Coupling Protein (RCP) [201], Rab11-Family Interacting Proteins, Rab11-FIP1, Rab11-FIP2, Rab11-FIP3 [202], and Rab11-FIP4 [203] have been identified. The interactions between Rab11 and Rab11-FIPs primarily involve the C-terminal domains of the FIPs, which includes the Rab binding domain (RBD) [201–204].

RCP interacts with both Rab4 and Rab11. It is likely to be involved in transferrin recycling [201]. Rab11-FIP2 and Rab11-FIP3 have been localized to the rab11-positive compartment in MDCK and HeLa cells [202]. Rab11-FIP2 appeared to be involved in recycling of transferrin [204]. Rab11-FIP4 colocalizes with both Rab11 and transferrin in HeLa cells [203]. It interacts with Rab11 in a GTP-dependent manner [203]. The expression of a mutant form of Rab11-FIP4 (rab11-FIP4(C-ter)) did not show any effect on transferrin recycling in HeLa cells but dispersed the Rab11-positive compartment toward the peripheral region of the cell [203].

3.3. Rab5 effectors, EEA1 and PI(3)K, involved in early endosome fusion

Early endosomal antigen 1 (EEA1), a hydrophilic 162 kD protein localized to the early endosomal membrane, plays a critical role in the fusion of early endosomes [181,205,206]. It has also been found as a homodimer in the cytosolic pool [206,207]. EEA1 functions as a tethering protein for two Rab5 positive membranes [208] which may facilitate the pairing of SNARE proteins [209] and subsequently membrane fusion [207,210]. It contains two binding sites for Rab5 at the N and C termini [208]. Association of EEA1 with the endosomal membrane appears not to be dependent on its interaction with Rab5 [211]. However, the interaction between Rab5 and EEA1 may regulate early endosome fusion [211]. According to Stenmark et al., the endosomal localization of EEA1 requires the presence of a FYVE domain [212], a cysteine-rich domain at the C-terminal of EEA1 protein which binds two zinc ions [205,212] and has a binding site for PI(3)P [213–216]. The interaction between the FYVE domain and PI(3)P is not only specific [217], but also necessary and sufficient for the binding of EEA1 to the endosomal membranes [211]. PI(3)P on the early endosomes or endocytic vesicles could be generated by the recruitment of PI(3)kinase hVPS34 by Rab5-GTP [218], which limits the recruitment of EEA1 to a specific region of the membrane [205,208]. In addition, the interaction between hVPS34 and Rab5 may be mediated by hVPS34-associated protein p150 [218].

So far, two distinct PI(3)Ks, hVPS34 and p85α-p110β, have been found to bound to Rab5 [218]. Class-III PI(3)K hVPS34 is the only PI(3)K required for early endosome fusion, and PI(3)P is the most important 3′-phosphatidyllinositides in the fusion process [218]. The class-I PI(3)K, p85α-p110β, which produces phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, may be involved in the formation of CCVs at the plasma membrane [218]. The involvement of p85α-p110β in transferrin recycling has also been demonstrated. Activation of class-I PI(3)K by insulin receptor enhanced the rate of transferrin recycling while antibodies against the p110α subunit of PI(3)K inhibited transferrin recycling [219,220].

The involvement of PI(3)Ks in transferrin transcytosis has also been studied by using PI(3)K inhibitors, such as wortmannin and LY294002. Wortmannin is a noncompetitive, irreversible inhibitor of mammalian PI(3)Ks at low concentration (100 nM), while the inhibitory effect of LY294002 is more reversible and specific for PI(3)Ks [221–223]. The rate of the transferrin recycling was reduced by Wortmannin and LY294002 [224–227].

According to van Dam et al. [228] transferrin receptors may be recycled back to the plasma membrane via two different independent recycling path-

ways that require the activities of PI(3)K and dynamin. Transferrin receptor recycling from perinuclear recycling endosomes was inhibited upon the overexpression of a dominant negative dynamin-1 mutant (dynts), which accumulates clathrin-coated buds on endosomes [229]. An accumulation of endocytosed transferrin in the early endosomes was observed in the presence of LY294002 indicating that PI(3)K activity is required for the transferrin exit from early endosomes [228]. No interference between LY294002 treatment and transferrin transport between either early endosome and recycling endosome or recycling endosome and the plasma membrane was reported, suggesting that LY294002 may inhibit the transferrin recycling from the early endosomes to the plasma membrane [228]. The combination of LY294002 and BFA, which appeared to inhibit the assembly of clathrin coats on recycling endosomes, had a synergistic inhibitory effect on transferrin recycling [228]. We also have found that the apical to basolateral transcytosis of transferrin in MDCK cells was greatly enhanced by treatment with LY294002 (unpublished results).

4. Utilization of TfR for drug delivery

TfR targeted therapy has emerged as an interesting drug-delivery tool with dual functionality. For example, targeting of TfR can lead to delivery of therapeutic agents into tissues of choice or across epithelial barriers of choice. The seemingly contradictory effects can be achieved by focusing the targeting strategies toward different aspects of TfR-related biology. This section focuses on recent advances in TfR-facilitated drug delivery.

4.1. TfR-based targeting for anti-cancer therapeutics

One of the most well explored avenues for TfR-based drug targeting strategies is the use of anti-cancer based therapeutics conjugated to Tf (or TfR recognizing antibody) to preferentially direct the drug to TfR-rich cancer cells. High levels of TfR expression have been demonstrated in many tumors [108,230–234] and importantly, studies have also shown that the TfR is expressed more abundantly in malignant tissues than their normal counterparts [108,115,233,235]. TfR is also more abundantly expressed in rapidly dividing cells than quiescent cells [236,237] because of its pivotal role in iron uptake and the absolute requirement for iron in rapid cell proliferation. Therefore, the TfR expressed on cancer cells has been seen as a suitable target for the delivery of therapeutics by receptor-mediated endocytosis.

Many anti-cancer agents have been considered for conjugation to Tf by varying methods, including direct chemical linkage [238,239], liposomal packaging of toxin and linkage to Tf [240,241], conjugation of DNA/polylysine complexes to Tf [242–245], and conjugation of liposome/DNA complexes to Tf [246–252].

A great variety of cytotoxic agents have been conjugated to Tf and investigated as potential anti-cancer therapeutics. Some of the more notable examples have been methotrexate [238,240], doxorubicin [238,239], cisplatin [253], ricin A [254–257], daunorubicin [241,258], and toxin CRM107 [259,260]. Conjugation of these toxins to Tf has the dual benefits of reducing toxicity in undesired tissues and increasing the targeting efficiency to the cancerous cells. Of special note is that conjugation to Tf significantly enhances the effectiveness of these agents in many multi-drug resistant cell lines. For example, Tf-doxorubicin was 5–10 times more effective than doxorubicin-control in killing doxorubicin-resistant cell lines, whereas in doxorubicin-sensitive cell lines, the conjugate was only four to five times more effective than control [261]. The Tf-based enhancement effect becomes even more dramatic as one considers the very highly resistant KB cell line. In this case, the Tf-doxorubicin conjugate exhibited an IC_{50} value as low as 0.025 μM, while the un-conjugated doxorubicin demonstrated no cytotoxic effects with concentrations as high as 1 μM [262]. The exact mechanism by which Tf conjugation allows doxorubicin to effectively bypass the drug-resistanance machinery of the resistant cell lines is not fully understood; however, it is conceivable that internalization of the Tf-doxorubicin conjugate allows for sequestration into the endocytic pathway, away from drug efflux proteins (such as PGP and/or MDR), which normally reside at the plasma membrane.

A variation on the theme of using toxins conjugated to Tf has been investigated to increase efficiency of uptake at the cellular surface. This approach relies on

the formulation of the toxin in liposomal delivery vehicles to further enhance the efficiency of cellular uptake. For example, methotrexate that has been formulated in a liposome complex and conjugated to Tf demonstrated a higher efficiency of cellular uptake and cytotoxicity compared to free methotrexate and methotrexate-liposome control [240]. In a similar fashion, when daunorubicin-containing liposomes are conjugated to OX26 ant-rat TfR antibody, they show an increased delivery and uptake in the CNS relative to the non-OX26 containing liposomes [241].

An alternate approach toward treating cancerous tissues relies upon gene therapy to correct errors in genes, such as the tumor repressor gene p53, rather than killing the cell. Tf-conjugation has been investigated as means to overcome some of the limitations of cationic liposome-based p53 gene delivery vehicles, such as poor stability in serum, binding to serum proteins, and lack of specific targeting [263–267]. Tissue culture experiments have revealed that the Tf conjugate significantly enhanced the transfection efficiency of the p53 gene in cancerous cell lines compared to the control DNA-liposome [265]. This enhancement was seen even in the presence of high concentrations of serum. In addition, experiments with nude mice containing subcutaneous DU-145 tumors revealed that administration of Tf-liposome conjugate was sufficient for high levels of p53 gene expression in the tumor. This contrasts with the non-Tf bearing p53-liposome which did not demonstrate any p53 gene expression in the tumor [267]. This strategy has even developed to the point where beneficial effects are seen in human patients. For example, when a cationic liposome (carrying p53 DNA) was investigated as a Tf conjugate, it demonstrated high efficiency in p53 gene delivery to human head and neck, prostate cancers, as well as long-term therapeutic benefits. For example, when Tf-liposome therapy was combined with conventional radiation therapy, complete tumor regression was observed in human prostate cancer and the treatment group showed no signs of relapse up to six months later [266]. Using similar plasma membrane permeability-enhancing logic as the DNA/liposome complexes, reports have also focused on DNA/polycation complexes conjugated to Tf to increase uptake. Comparable benefits in enhanced uptake have been observed for the polylysine and polyethylenimine containing complexes [242,245,268–273]. Further developments in Tf-based targeting of anti-cancer drugs will most likely play important roles in anti-cancer therapy in the future.

4.2. TfR-mediated transepithelial protein-drug delivery

Modern biotechnology has produced many potent and beneficial drugs, yet most of the products suffer from limited choices regarding potential routes of administration. The large sizes and charges of protein drugs prevents their passage across epithelial barriers. TfR has emerged as a potential mediator to enable the transport of these large molecules across epithelial barriers.

4.2.1. Anti-TfR antibody drug conjugates for trans-epithelial drug delivery

The capillaries of the brain have been well known to have relatively high levels of TfR, and since the blood brain barrier (BBB) effectively excludes many therapeutic drugs, including almost all peptide and protein-based therapeutics, targeting of the BBB's TfR for drug delivery has been seen as an attractive strategy. However, utilizing a Tf-based drug conjugation approach is seen as undesirable for trans-BBB delivery because under physiologic conditions the TfR of the BBB are postulated be nearly saturated with Tf, due to the relatively high amounts of Tf in serum [274]. A viable alternative to target BBB TfR has been shown to be antibodies that specifically recognize TfR, with or without the presence of bound Tf [275–278]. In particular, OX26, a monoclonal antibody for rat TfR that has been the subject of most of the research in this area, demonstrated an ability to preferentially target the TfR of the BBB [111]. OX-26 is postulated to not interfere with the normal functioning of iron uptake via TfR, since the antibody binds an extra-cellular region that is distinct from the binding pocket for Tf [111].

Upon binding to TfR at the BBB, OX26 exhibits superior transport abilities into the CNS. Studies performed with isolated bovine brain capillaries demonstrated that nearly 50% of bound radiolabeled OX26 was taken up via endocytosis during a 2-h incubation period [279]. In addition, carotid artery perfusion studies indicate that more than 65% of the

administered OX26 passed through the BBB and into the extracellular space. This value was significantly higher than for the control proteins, bovine serum albumin and IgG, thus implying that transcytosis via TfR significantly enhanced the transport of OX26 across the BBB. Intravenous bolus administration of OX26 also resulted in a steady accumulation of radiolabeled OX26 in the brain during a 5-h time period, while radiolabeled OX26 was rapidly cleared from serum after injection. This suggests that OX26 rapidly binds TfR at the BBB and is subsequently endocytosed across the BBB in a more gradual fashion [279].

The next logical course of action was to determine if OX26 could enable the efficient CNS uptake of therapeutics that would normally exhibit poor CNS-bioavailability. The strategy most commonly reported in the literature involved the creation of OX26-therapeutic conjugates based on strepavidin/biotin linker technology. The avidin/biotin linkage between OX26 and the therapeutic entity has been created using chemical based and genetic-cloning based strategies. Several promising OX26 conjugates have been created, using a vasoactive intestinal peptide analog [280], brain-derived neurotrophic factor (BDNF) [281–283], and basic fibroblast growth factor (bFGF) as the therapeutic cargo [284,285]. Depending upon the pharmacokinetic properties desired, the conjugates have been prepared with avidin-modified OX26 biotin conjugated to the drug directly or via a PEB 2000 spacer. When BDNF-PEG2000-SA-OX26 was administered to rats for 1 week after an induced episode of transient forebrain eschemia, a neuroprotective effect was observed. Specifically, the neuronal density in the hippocampus of the BDNF-PEG2000-SA-OX26 treatment group was maintained, while the group receiving pure BDNF exhibited a 68% decrease compared to control [286]. In addition, BDNF-OX26 conjugate demonstrated effectiveness in ameliorating the effects of acute stroke. For example, when adult rats were subjected to 24 h of middle cerebral artery occlusion, the infarct volume was found to decrease in a BDNF-OX26 dose dependent fashion. Reduction in stroke volume was significant even if administration of BDNF-OX26 was delayed for 1–2 h after the occlusion event [281,282,287,288].

bFGF is an attractive drug candidate for OX26 conjugation because it retains receptor-binding affinity and has increased brain uptake following conjugation to OX26-SA. Studies also have demonstrated that bFGF-OX26 conjugate protects cortical cell cultures against hypoxia/reoxygenation insult in a dose-dependent manner in vitro [285]. In addition, in vivo studies showed that a single intravenous injection of bFGF-OX26, equivalent to a dose of 25 μg/kg bFGF, produces an 80% reduction in infarct volume in the brains of rats subjected to permanent occlusion of the middle cerebral artery. This result paralleled with a significant improvement of neurologic deficit. The neuroprotective action was also time-dependent, with a 67% reduction in stroke volume if bFGF-OX26 was administered 60 min after arterial occlusion, whereas no significant reduction was observed if treatment was delayed by 2 h [284,285].

In addition to the delivery of large therapeutic molecules across the BBB, TfR based transcytosis has also been explored for targeted gene delivery to the CNS. For example, high levels of reporter gene expression have been observed when plasmids encoding for luciferase or β-galactosidase are enclosed in liposomes that are conjugated to OX26 via a PEG2000-thio-ether linkage were administered intravenously to rats [289–291].

4.2.2. Enhancement of TfR-mediated drug delivery

In addition to the use of Tf-drug conjugates to target therapeutics to select tissues from the blood stream, Tf based conjugates have also been considered for the systemic non-invasive delivery of therapeutics. Several absorptive epithelia have been shown to express TfR, such as the small intestine [112] and type II pneumocytes [114], providing a potential target to enable transepithelial delivery of large bioactive molecules that would normally have negligible bio-availability when administered orally. One of the problems associated with this approach is the fact that most of the surface TfR in differentiated epithelial cells lies at the basolateral, or serosal, membrane [292]. This would tend to preclude the ability to achieve apical-to-basolateral transcytosis of Tf-drug conjugates. While there is indication that transepithelial transport may still be feasible (especially in areas where large numbers of semi-differentiated cells may occur, such as the crypt of Lieberkuhnin of the small intestine), enhancers of Tf-conjugate transepithelial transport have been examined as a means to overcome the problem of basolateral-localized TfR polarity.

4.2.2.1. Lysosomotrophic amines. Lysosomotrophic amines (such as ammonium chloride and chloroquine) have been considered as a means to enhance Tf-drug conjugate transepithelial transport. Most of these agents increase intraendosomal and intralysosomal pH, with a concomitantly enhanced accumulation of radiolabeled Tf inside the cell. However, these agents also seem to cause a non-specific enhancement of ^{125}Tf transcytosis. For example when MDCK monolayers were treated with NH_4Cl (20 mM), a seven-fold increase in basolateral-to-apical transcytosis was observed. The increase in transcytosis was thought to be derived from the overloading of labeled ^{125}Tf inside the cell [293]. The serious drawback to these agents is the nonspecific alteration of membrane permeability and integrity.

4.2.2.2. Lipophilic carboxylic ionophores. Carboxylic ionophores, such as monensin and nigericin, exhibit effects similar to lysosomotrophic amines in altering the pH of endosomal and lysosomal compartments [294]. Monensin, is a Na^+-ionophore that can elicit a variety of effects in mammalian cells, including alterations of vascuolization and vesicle sorting in the Golgi and trans-Golgi networks. Treatment of MDCK monolayers with 6 μM monensin resulted in a 24-fold increase in TfR-mediated transcytosis. The large increase in TfR-mediated transcytosis appeared to originate from a different mode of action than lysosomotrophic amines. This was presumed to result from the effects of monensin on trans-Golgi sorting, since TfR is routed to the TGN as a part of the normal course of its intracellular sorting [295,296]. The development of monensin as a useful therapeutic agent has been hampered by the highly lipophilic nature of the drug, which is reflected in its poor stability and potentially short serum half-life.

4.2.2.3. Brefeldin-A. Another transcytosis enhancing reagent that has been shown to have an effect in modulating the trans-Golgi network is the fungal metabolite, brefeldin-A (BFA). BFA has been extensively used in the study of intracellular trafficking events [297]. It has dramatic effects on the structure and function of the Golgi complex and the endosomal sorting pathways [298–300]. For example, BFA causes the rapid rearrangement of the Golgi, through microtubule dependent processes, causing Golgi-specific markers to redistribute in a retrograde fashion back to the endoplasmic reticulum [301,302]. In addition, the trans-Golgi network tubulates in the presence of BFA, forming a fused trans-Golgi/endosomal network that appears to exclude vesicles that are lysosomal in nature [298,303]. While the lysosomes do not appear to fuse with the trans-Golgi/endosomal tubulates, they do appear to form an extensive microtubule dependent network in the presence of BFA [298]. In addition to these effects, BFA also blocks the anterograde transport of sorted proteins from the endoplasmic reticulum [301,302]. Unlike the reagents discussed above, BFA does not cause a change in intra-lysosomal or intra-endosomal pH [298].

In polarized epithelial cells, BFA treatment significantly up-regulates the basolateral-to-apical transcytosis of internalized TfR and bulk plasma membrane [304]. In spite of these observations, the Golgi of some cell lines seem to be resistant to the effects of BFA. For example, the Golgi of MDCK cells largely resists the effects of BFA [305]. Nonetheless, BFA causes significant alteration of trans-Golgi and endosomal function in MDCK cells [305]. The fact that BFA causes significant enhancement of TfR-specific transcytosis in this cell line would indicate that this enhancement depends upon sorting events at the TGN/endosome level.

BFA significantly alters the intracellular sorting of internalized proteins in polarized epithelial cells. For example, BFA treatment results in the missorting of basolateral LDL receptors [306], reduces the efficiency of polymeric immunoglobin receptors to the cell surface [307]. In addition, the effects of BFA on protein sorting appear to be much more sensitive at the apical surface, as the concentration of BFA required to see effects at the apical surface is much lower than for the basolateral surface [308,309]. This is significant when considering BFA as an enhancer of TfR-mediated drug delivery. The relative preferential alteration of events at the apical surface would imply the ability to achieve net enhancement of TfR-mediated transcytosis in the apical-to-basolateral direction. This effect has been demonstrated in two highly polarized cell systems, MDCK cells [292] and primary cultured rat type II pneumocytes [310]. In both of these systems, BFA treatment results in the specific enhancement of apical-to-basolateral

transport of conjugates of Tf-insulin and Tf-GCSF (filgrastim) [292,310]. The rates of apical-to-basolateral transport of the conjugates are significantly higher in the presence of BFA than for comparable molar concentrations of the native protein. The enhancement of transport appears to result from the transient missorting of basolateral TfR to the apical surfaces of the cells, from whence they are rapidly internalized and redirected back to the basolateral membrane, releasing the Tf-protein-drug conjugate into the basolateral fluid. This is evidenced by results which demonstrate that BFA-treatment does not increase the static level of TfR at the apical surface, as measured by ^{125}I-Tf binding at 4 °C, but rather increases the rate of TfR internalization, as measured by ^{59}Fe-Tf uptake. While BFA does affect the trans-epithelial electrical resistance (TEER) of the monolayers, the effect is transient and TEER returns to normal levels once the BFA is removed [292,310].

4.3. Modification of Rab-dependent sorting pathways for enhancement of TfR-mediated gastrointestinal transepithelial transport

While investigations into BFA-induced enhancement of TfR-mediated transport have demonstrated a significant elevation in Tf-protein-drug conjugate transport in polarized epithelia, the fact that BFA is a fungal metabolite makes it undesirable in some respects. For example, BFA affects many properties of intracellular organelles, even those that might be unrelated to TfR-based sorting or transcytosis events. The non-specific side effects of BFA prompted efforts to discover agents that would have the potential to be more specific in their mode of enhancement of TfR-mediated transcytosis. As discussed in Section 2, several small GTPases of the Rab family have been shown to be involved in the endocytosis and transcytosis of TfR. Therefore one can pose the hypothesis that chemical entities that inhibit or alter the actions of key Rabs, or their downstream effectors, could possibly control the sorting and recycling events of TfR to elicit a specific enhancement of TfR-transcytosis.

Recent reports have focused on the ability of AG-10 (aka tyrphostin-8 [T-8], or 4-hydroxybenzylidenemalononitrile), a known GTPase inhibitor, to increase the transport of an insulin-Tf conjugate across Caco-2 monolayers (in the apical-to-basolateral direction) and enhance the oral absorption of the conjugate in streptozotocin-induced diabetic rats across the GI epithelium [311,312]. In Caco-2 monolayers, treatment with AG-10 induced a 20-fold enhancement of TfR-mediated transport of insulin (via the insulin-Tf conjugate) compared to control, while similar treatment with BFA only resulted in a three-fold enhancement of insulin transport [312]. This enhancement of transport was determined to be dependent upon TfR-mediated processes, since an insulin–albumin conjugate demonstrated no transport across the monolayers, and treatment with AG-10 had no effect on its transport properties [312]. The results from the tissue-culture experiments have been further validated by in vivo uptake studies. In diabetic rats, the orally administered insulin-Tf conjugate demonstrated an extended duration of action, with hypoglycemic effects observed 11 h after administration [113]. In addition, AG-10 exhibited the ability to enhance the hypoglycemic effect of orally administered insulin-Tf conjugate, relative to BFA-treated and enhancer-free insulin-Tf treatment groups. The effects of AG-10 were also dose dependent, indicating the observed results resulted from of the effects of AG-10 [312].

In an effort to further increase the specificity of enhancement of TfR-mediated transcytosis, downstream effectors of Rab proteins have been considered as potential targets for inhibition. One of the downstream effectors of Rab proteins are the phosphatidylinositol 3-kinases (PI3K). As noted earlier, PI3K's are required for cellular membrane trafficking and they have been implicated in the recycling of TfR. For example, the PI3K inhibitors, LY294002 and wortmannin, have been shown to reduce the rate of Tf recycling [224–227,313,314], whereas activation of PI3K activity is known to accelerate the rate of TfR recycling [315,316]. One can hypothesize that inhibition of recycling of basolaterally derived TfR may result in an increase of TfR traffic to the apical membrane, thus increasing the potential for enhancement of TfR-mediated transcytosis. Recent results have suggested that LY294002 is capable of enhancing TfR-mediated transcytosis in a manner that is similar to AG-10 and different from the mode of action of BFA. For example, LY294002 specifically

enhanced TfR-mediated transcytosis of ^{125}I-Tf and ^{125}I-Tf-GCSF conjugate in Caco-2 monolayers, yet demonstrated less of an effect in MDCK monolayers (unpublished results). This contrasts with BFA, which has much greater enhancement ability in MDCK monolayers compared to Caco-2 [292,312]. Caco-2 and MDCK are known to have distinct TfR-sorting pathways, i.e. predominately direct TfR sorting in MDCK cells, and both indirect and direct sorting in Caco-2 [312]. This suggests that AG-10 and LY294002 affect one type of sorting mechanism, while BFA affects another. This hypothesis has been supported by a recent report, which demonstrated that endocytosed TfR are recycled via distinct dynamin and PI3K dependent pathways, where BFA and LY294002 exhibited distinct yet synergistic inhibitory effects [228].

5. Conclusion

TfR has developed as a potential ligand to enable drug targeting and delivery of therapeutic agents that would normally suffer from poor pharmacokinetic characteristics. TfR-directed targeting has enabled the efficient delivery of therapeutic agents to sites of interest, including the central nervous system and malignant tissues. In addition, by utilizing knowledge of the intracellular sorting and recycling pathways of TfR, including Rab and PI(3)K mediated processes, one can maximize the transepithelial delivery of peptide-based therapeutics. Depending upon the desired result, apparently paradoxical effects can be achieved. For example, TfR-based strategies can achieve accumulation of the carried-drug within targeted tissues or delivery of the therapeutic entity across tissues of interest. Further understanding of the intra-cellular events that govern the destiny of internalized TfR will result in ever increasing interest in TfR as a pharmaceutically relevant molecule.

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