The Macrophage Cell Surface Glyceraldehyde-3-phosphate Dehydrogenase Is a Novel Transferrin Receptor*1,2

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Iron is an essential nutrient for all organisms as a constituent of hemoproteins and iron–sulfur proteins. In addition it is also a critical component of functional groups of several proteins involved in vital housekeeping functions. Cells of the immune system require iron for their normal functions such as proliferation, activation, and maturation of lymphocytes (1–5). Iron is also essential for macrophage-mediated cytotoxicity by the production of highly toxic hydroxyl radicals (6, 7). The mononuclear phagocyte system is composed of monocytes, macrophages, and their precursor cells, which play a vital role in iron metabolism by removing effete erythrocytes and recycling iron. These cells also acquire iron via the receptor-mediated uptake of transferrin and the hemoglobin scavenger receptor (8). Practically all extracellular iron circulating in the plasma is bound to transferrin, an abundant protein with high affinity for iron. Two mammalian transferrin receptors TFR1 3 and TFR2 have so far been characterized. Both these receptors are cell surface transferrin binding protein (22, 23). However, these findings have been contradicted by a more recent study (24), which demonstrated that S. aureus mutants lacking cell surface GAPDH retained the ability to bind transferrin to a level equivalent with that of the parent strain. Furthermore, when GAPDH was overexpressed and purified from the parent strain, it showed no affinity for human transferrin although it retained its enzymatic activity.

With a goal to understand the role of this multifunctional protein in immune cells, we chose macrophages as a model system. In the current study we provide evidence that macrophages express a surface-localized GAPDH that is capable of interacting with the iron transport protein transferrin. In addition, our findings elaborate the trafficking of this GAPDH-transferrin complex to the early endosomes, indicating that cell surface GAPDH functions as a novel transferrin receptor.

EXPERIMENTAL PROCEDURES

Media and Cell Lines—The mouse macrophage cell line J774A.1 and THP-1, a human monocytic cell line were obtained from ATCC and maintained in RPMI 1640 (Hi Media, India) supplemented with 10% fetal calf serum (Invitrogen). For iron depletion experiments, cells were cultured in complete RPMI medium treated with Chelex 100 (Sigma) re-supplemented with other metal ions as described previously (22).

Murine Macrophages—Thiglycollate-elicited macrophages were obtained from 6–8-week-old Balb/c mice using previously established procedures (25) as approved by the institutional

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4 The abbreviations used are: TFR, transferrin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tf, transferrin; NTR, non-transferrin receptor; FACS, fluorescent-activated cell sorting; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; FRET, Förster resonance energy transfer.
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Enzyme Pronase digestion was terminated by the addition of 1 ml of buffer (for negative controls) was added to the tubes. Cells were then incubated at 37 °C for 30 min. Cells were then incubated with 100 μl of FACS buffer supplemented with 5% normal human serum and 5% normal goat serum. Cells were stained with 1.0 μg/tube of a commercial monoclonal anti-GAPDH antibody (Advanced Immunochemicals) alone or in the presence of excess (200 μg) free rabbit muscle GAPDH (Sigma), followed by incubation with Sheep anti-mouse-FITC (Fab2) (Sigma). Cells were analyzed on a FACS Calibur Flow Cytometer.

FACS Analysis—Cells were harvested using a rubber policeman and washed in FACS buffer (20 mM HEPEs pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM KCl, 0.02% NaN3, and 5% fetal calf serum). 2 × 10⁶ cells were used per assay. Blocking was done by incubation on ice for 30 min using 100 μl of FACS buffer supplemented with 5% normal human serum and 5% normal goat serum. Cells were stained with 1.0 μg/tube of a commercial monoclonal anti-GAPDH antibody (Advanced Immunochemicals) alone or in the presence of excess (200 μg) free rabbit muscle GAPDH (Sigma), followed by incubation with Sheep anti-mouse-FITC (Fab2) (Sigma). Cells were analyzed on a FACS Calibur Flow Cytometer.

Enzyme Pronase Sensitivity of Cell Surface GAPDH—To determine if the surface-expressed GAPDH was sensitive to the proteolytic enzyme Pronase, J774 cells were washed three times with neutral buffer (20 mM HEPEs pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM KCl). Cells were then resuspended at a concentration of 2 × 10⁶ cells/100 μl per tube. Subsequently either 100 μl of 4 mg/ml of Pronase (Roche Applied Science) or 100 μl of buffer (for negative controls) was added to the tubes. Cells were then incubated at 37 °C for 30 min in a water bath. Pronase digestion was terminated by the addition of 1 ml of chilled FACS buffer to each tube, followed by three washes at 4 °C. To check for the recovery of cell surface GAPDH expression after Pronase treatment, an aliquot of these cells was resuspended in fresh medium at 37 °C for 30 min. Cells were then blocked and stained for GAPDH expression by FACS analysis as described above.

Western Blot Analysis of GAPDH—Cells were grown to confluence and harvested for membrane preparation. After three washes in neutral buffer, the pellet was frozen overnight at −20 °C. Membrane and cytosolic protein fractions were prepared as described previously (26). All samples were resolved using 10% SDS-PAGE. Rabbit muscle GAPDH (Sigma) was loaded along with the samples as a standard molecular mass marker (37 kDa), because it is almost identical (≈ 96% homology) to human and mouse GAPDH.

In addition, proteins from conditioned medium were precipitated using chilled acetone and analyzed similarly. Protein samples were transblotted onto nitrocellulose membrane and then probed with monoclonal anti-GAPDH antibody, for 1 h at room temperature, followed by incubation with rabbit anti-mouse-HRP (Sigma). Blots were developed with TMB H2O2 (Molecular Probes) and 5 μg/ml transferrin-TRITC (Molecular Probes). Cells were washed and fixed with 1% paraformaldehyde and imaged using a Zeiss LSM 510 META Laser Confocal Microscope using a ×63 oil immersion objective and an aperture of 1 Airy unit.

In Vitro Interaction of GAPDH-Transferrin by ELISA—Poly-styrene wells were coated overnight at 4 °C using 350 pmol/well of rabbit muscle GAPDH in PBS and blocked using 5% BSA (Pierce) for 24 h at 4 °C. Wells were then incubated with 2.5 nM of human holo-transferrin (Sigma), in PBST containing 0.5% BSA for 6 h at 4 °C. After extensive washing bound transferrin was detected by incubation with rabbit anti-transferrin antibody (Polysciences) followed by goat anti-rabbit-HRP (Sigma). TMB H2O2, for ELISA (Bangalore Genei, India) was used to develop the reaction. The absorbance was measured at 450 nm. As a positive control for transferrin a set of wells had been coated with transferrin alone, which was detected using rabbit anti-transferrin antibody. Controls were also set up in the assay to determine the nonspecific interaction of (i) anti-transferrin antibody with GAPDH, (ii) anti-transferrin antibody with BSA, (iii) transferrin with BSA, (iv) secondary antibody with GAPDH, and (v) secondary antibody with BSA. Each set consisted of eight wells in replicates.

In Vitro Interaction of GAPDH-Transferrin by Dot Blot—Dot blot assay was carried out as described previously (22). Briefly, rabbit muscle GAPDH in PBS was spotted on nitrocellulose membrane (350 pmol/spot). Membranes were washed with PBST and blocked using 5% BSA for 24 h at 4 °C. Blots were

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then incubated with 6.25 nM human holo-transferrin in PBST containing 0.5% BSA for 6 h at 4 °C. Bound transferrin was detected by incubation with rabbit anti-transferrin antibody followed by incubation with goat anti-rabbit-HRP. Blots were developed using TMB H2O2 for localization of HRP.

Co-immunoprecipitation of GAPDH-Transferrin from Cell Membranes—J774 cells were harvested from confluent cultures and washed three times with FACS buffer. 2 × 10^7 cells per tube were incubated with 12.5 nM human transferrin for 1 h on ice. Cells were washed and processed for preparation of the membrane protein fraction, as for the Western blot analysis. Co-immunoprecipitation was done from this fraction using anti-transferrin antibody followed by capture with Protein A Magnabeads (Polysciences) per the manufacturer’s instructions. The reverse experiment was also performed, wherein the membrane protein fraction was incubated with monoclonal anti-GAPDH antibody followed by capture with goat anti-mouse Magnabeads (Polysciences). Beads were boiled in sample buffer, and eluted proteins were analyzed by Western blot as described previously. Blots were probed with monoclonal anti-GAPDH antibody or anti-transferrin antibody, respectively.

FRET Analysis—J774 cells were maintained and processed as for colocalization studies described above. Cells were immunostained with monoclonal anti-GAPDH followed by anti-mouse-Alexa-633 (Molecular Probes) and Transferrin-FITC (Sigma). As a control, anti-IA^d (BD Biosciences) was used in place of anti-GAPDH. Förster resonance energy transfer (FRET) experiments were carried out using the acceptor photobleaching method (27).

Analysis of Protein-Protein Interactions using Resonant Mirror Technology—Binary Interaction Analysis: association and dissociation between transferrin and GAPDH were followed in real time by Resonant Mirror-based detection using IAsys Plus system (Cambridge, UK). Experimental procedure was essentially as described previously (28, 29). Streptavidin was captured on biotin cuvettes. This was followed by the attachment of biotinylated transferrin to the streptavidin, which was then allowed to interact with varying concentrations of GAPDH. Data were analyzed using the FASTfit software, supplied by the manufacturer.

Cell Surface GAPDH-Transferrin Is Internalized to Early Endosome in Live Cells—J774 and THP-1 cells were cultured in Lab Tek Chambered coverglass. Cells were washed with FACS buffer, blocked, and stained for transferrin and GAPDH as described previously for colocalization experiments. To initiate endocytosis cells were rinsed three times with culture medium and maintained at 37 °C for 5 min, using a heating block along with the addition of pre-warmed medium. Endocytosis was arrested by rapidly bringing down the temperature to 4 °C, by replacing the existing medium with chilled buffer. Cells were washed and immediately fixed using 1% paraformaldehyde and 1% glutaraldehyde. Imaging was done using a Zeiss LSM 510 META Laser Confocal Microscope as described previously.

Immunoprecipitation of Biotinylated GAPDH from Early Endosome Confirms Its Cell Surface Origin—J774 cells were harvested and washed three times with neutral buffer. Surface proteins were biotinylated by incubation with sulfo-SS-NHS biotin (Pierce) per the manufacturer’s instructions. Cells were washed with neutral buffer and transferrin was added at a final concentration of 12.5 nm for 2 h on ice. Excess transferrin was removed by washing and endocytosis was allowed to proceed as described above. The early endosomal fraction was prepared based on previously described methods (30, 31). Briefly, cells were washed and resuspended in homogenization buffer (250 mM sucrose, 20 mM HEPES, pH 7.2) containing protease inhibitors (10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml antipain) and 0.5 mM EDTA. The cells were then homogenized by 15 passages through a 27-gauge needle fitted on a 1-ml plastic syringe. The homogenate was centrifuged at 800 × g, 10 min at 4 °C, the postnuclear supernatant (PNS) obtained was rapidly frozen using liquid nitrogen and stored at −70 °C. To prepare the enriched endosomal fraction, 3.0 ml of the PNS was quickly thawed and loaded onto a discontinuous sucrose gradient formed by layering 1 ml of 54%, 4 ml of 40%, and 4 ml of 30% sucrose (w/v) in homogenization buffer. After centrifugation at 100,000 × g, 1 h at 4 °C, the early endosomal fraction was isolated from the 30% interface of the gradient. The endosomes were pelleted at 50,000 × g for 15 min at 4 °C, and proteins were extracted as described previously (26). The presence of transferrin and GAPDH in the extract was confirmed by Western blot analysis. Subsequently for immunoprecipitation experiments the protein fraction was incubated with monoclonal anti-GAPDH and anti-mouse Magnabeads, overnight at 4 °C on a rotamixer. The beads were then washed and boiled in sample buffer in the absence of reducing agents. Eluted proteins were analyzed for the presence of biotinylated GAPDH by Western blot analysis using streptavidin-HRP (Bangalore Genei, India) as a probe.

Analysis of Purified Endosomes using Immuno-EM Demonstrates Internalization of the Surface GAPDH and Transferrin—J774 cells were incubated with transferrin followed by immunolabeling with monoclonal anti-GAPDH and rabbit anti-transferrin antibody at 4 °C for 1 h to allow cell surface binding. Subsequently, cells were washed and endocytosis was allowed to proceed as described above. This was done to allow internalization of surface GAPDH and transferrin along with bound primary antibodies. Early endosomes were then purified and resuspended in a minimal volume of homogenization buffer. Purified endosomes were adsorbed onto carbon coated nickel grids and processed for immunolabeling using the negative staining technique. Blocking was carried out for 1 h using 2% casein with 1% fish skin gelatin in neutral buffer. To detect internalized GAPDH and transferrin, samples were immunostained using goat anti-mouse 20-nm gold conjugate (Sigma) along with goat anti-rabbit 6-nm gold conjugate (Polysciences) as described previously (32).

RESULTS

GAPDH Is Expressed on the Surface of Macrophage Membrane—The mouse macrophage cell line J774 and the human monocytic cell line THP-1 were stained with monoclonal anti-GAPDH antibody. FACS analysis demonstrated that both these cell lines express GAPDH on the membrane surface. The specificity of this staining was validated by the observation that free rabbit muscle GAPDH inhibits the binding of anti-GAPDH to cells (Fig. 1, A and B). The cell surface
GAPDH was found to be sensitive to digestion by Pronase, a nonspecific proteolytic enzyme (Fig. 1E). After 30 min of incubation in fresh medium the GAPDH on the cell surface begins to show signs of recovery.

To confirm that GAPDH is associated with the membrane of these cells, we prepared membrane protein preparations, cytosol fractions, and conditioned medium from both J774 and THP-1 cell lines. We subjected these samples to Western blot analysis using rabbit muscle GAPDH as a positive control. The functional enzyme is known to be a homotetramer composed of 37-kDa subunits; it was found that the molecular weight of membrane GAPDH was identical to the cytosolic form as well as to standard rabbit muscle GAPDH. In addition, we also detected this protein in conditioned medium (Fig. 1, C and D). The presence of GAPDH on the outer surface of intact J774 cell membranes was further confirmed by immunolabeling followed by transmission and scanning electron microscopy (Fig. 1, F and G). Unlike the two cell lines tested, thioglycollate-elicited mouse peritoneal macrophages did not show any significant surface expression of GAPDH on FACS analysis (Fig. 2B).
Surface GAPDH Expression Is Modulated by Availability of Iron in the Culture Medium—To establish whether this surface expression is regulated by the presence of iron in the culture medium, mouse peritoneal macrophages, and J774 cells were cultured for 48 h under conditions of iron depletion and analyzed for surface GAPDH expression by flow cytometry. The expression of surface GAPDH on J774 cells was found to be significantly enhanced upon culture in iron-depleted medium (Fig. 2A). More significantly, mouse peritoneal macrophages that initially did not show any surface GAPDH by FACS analysis, were now found to express this molecule on the membrane surface (Fig. 2B). No increase in surface GAPDH expression was observed in control experiments where cells were cultured in Chelex-treated medium re-supplemented with iron (data not shown).

The Surface-expressed GAPDH Is Enzymatically Active—To determine whether surface GAPDH is present in its enzymatically functional form, we assayed intact J774 cells for GAPDH specific enzyme activity. It was found that the GAPDH present was an enzymatically active molecule that was capable of converting NAD to NADH in the presence of its specific substrate glyceraldehyde 3-phosphate (Fig. 2C). This enzyme activity was also seen to increase substantially upon culturing the cells in iron-depleted media for 48 h (Fig. 2C). We also assayed mouse peritoneal macrophages under identical conditions. Although no GAPDH expression was detected on the surface of mouse peritoneal macrophages by FACS staining, the cell surface enzyme assay demonstrated a minimal level of GAPDH expression (Fig. 2D). In addition an increase in surface enzyme activity was observed upon culture of these cells in iron-depleted media comparable to that of J774 cells under similar culture conditions (Fig. 2, C and D). The observed increase in cell surface enzyme activity in both the cell lines and peritoneal macrophages correlates well with the observed increase in GAPDH surface staining as determined by FACS analysis.

In Vitro Interaction of GAPDH with Transferrin—The observation that cell surface GAPDH levels are sensitive to iron in the culture medium indicates that it may be playing a role in iron
GAPDH and transferrin interact under in vitro conditions (Fig. 3A). Transferrin capture by GAPDH was also detected in the dot blot assay (Fig. 3B).

**Transferrin Colocalizes with Cell Surface GAPDH**—To verify whether this interaction is significant with respect to surface GAPDH on intact cells, colocalization of GAPDH and transferrin was visualized by confocal microscopy. We carried out double immunofluorescence staining of intact J774 cells using monoclonal anti-GAPDH and anti-mouse-Alexa-488 in combination with transferrin-TRITC. Confocal microscopy clearly demonstrated that GAPDH and transferrin co-localize on the surface of these cells (Fig. 3D). Similar results were also obtained with THP-1 cells.

**Co-immunoprecipitation Assays**—To substantiate the fact that GAPDH and transferrin not only co-localize but also interact on the membrane surface we performed co-immunoprecipitation experiments. Intact cells were incubated with transferrin under conditions that prevent endocytosis. The membrane protein fraction was prepared and co-immunoprecipitation was done using anti-transferrin antibody bound to protein A-coupled beads. Interacting proteins were eluted and subjected to Western blot analysis, using a monoclonal anti-GAPDH antibody as a probe. It was found that GAPDH does indeed interact with transferrin in vivo (Fig. 3C). Identical results were obtained in a reverse experiment where the interacting proteins were co-immunoprecipitated using monoclonal anti-GAPDH antibody bound to anti-mouse Ig beads and transferrin was detected in the eluted protein fraction (supplementary information, Fig. S1).

**FRET Analysis for GAPDH-Transferrin Interaction**—A further confirmation that GAPDH and transferrin interact on the cell surface was provided by acceptor photobleaching based FRET analysis (27) of double fluorescence-labeled J774 cells (using monoclonal anti-GAPDH followed by anti-mouse-Alexa-633 and Transferrin-FITC). Upon bleaching the anti-GAPDH-Alexa-633 fluorescence, a significant increase was evident in the transferrin-FITC signal, thereby indicating the presence of FRET between the two fluorophores (Fig. 4A). In a control experi-
Co-immunoprecipitation Assays Confirm That Cell Surface GAPDH Is Internalized to the Early Endosome—To further confirm that cell surface GAPDH is internalized into the endosomes, J774 cells were surface biotinylated and allowed to interact with transferrin. After internalization for 5 min at 37°C, the early endosomal fraction of these cells was purified. Subsequently, the protein fraction from these endosomes was purified and GAPDH was immunoprecipitated using monoclonal anti-GAPDH. The eluted proteins were analyzed for the presence of biotinylated-GAPDH by gel electrophoresis followed by Western blot analysis using a streptavidin-HRP conjugate. The immunoprecipitation of biotinylated surface GAPDH, purified from the endosomal fraction, confirmed that it is cell surface GAPDH that is internalized to the endosomes after a 5-min incubation at 37°C (Fig. 5B).

Immuno-EM Further Demonstrates Internalization of the Surface GAPDH and Transferrin to the Early Endosomes—J774 cells were incubated with transferrin at 4°C to allow surface binding. This was followed by immunolabeling with monoclonal anti-GAPDH and rabbit anti-transferrin at 4°C. To allow internalization to the early endosomes, cells were incubated for 5 min at 37°C subsequently the early endosomal fraction from these cells was prepared. To detect the presence of surface GAPDH and transferrin, endosomes were stained with goat anti-mouse 20-nm gold conjugate along with goat anti-rabbit 6-nm gold conjugate. Immunolabeled endosomal preparations were subsequently processed for negative staining by TEM. The presence of both GAPDH and transferrin was confirmed within the early endosomes (Fig. 5A). Similar results were also seen in experiments with THP-1 cells (supplementary information, Fig. S2).

DISCUSSION

Iron is an essential component of many diverse cellular processes, it is also an important component of several proteins and enzymes necessary for the normal functioning of the cells. The reticuloendothelial system plays a central role in iron metabolism by recycling iron from senescent red blood cells and for the storage of excess iron. Iron is delivered to most tissues via receptor-mediated endocytosis of the plasma iron-binding protein transferrin. Isolated human monocytes are reported to express transferrin receptors and acquire iron from transferrin.
Transferrin binding has also been reported in various macrophages from mice and humans (8). Our results provide direct evidence for the novel localization of the glycolytic enzyme, GAPDH on macrophage cell surface and describe its role as an additional transferrin receptor.

Several proteins that have more than one function have been identified in both prokaryotic and eukaryotic cells. Such proteins are referred to as moonlighting or multifunctional proteins. Some of these proteins have multiple functions within the cell, whereas others perform different functions depending on the cell type, oligomeric state or cellular concentration of a ligand, substrate or cofactor. Many of these proteins are ubiquitous enzymes that are essential components of the glycolytic or TCA pathway. The most well characterized example is that of the cytosolic glycolytic enzyme, phosphoglucose isomerase. It is also secreted by cells and has at least four additional roles. Other such examples include pyruvate kinase, α-enolase, aconitase, lactate dehydrogenase, and GAPDH (33–35).

Although GAPDH is predominantly present in the cytoplasm where it functions as a metabolic enzyme, several recent studies have demonstrated that it exhibits multiple functions within the cell, unrelated to its role in glycolysis (19). It is of interest to note that in bacteria GAPDH belongs to a novel class of anchorless cell wall-associated surface proteins. Several reports indicate that bacterial GAPDH binds plasmin, fibronectin, laminin, and also cleaves complement C5a (36, 37). However, to date only a single report describes the presence of GAPDH on the surface of mammalian cells. Mammalian cell surface GAPDH is thought to play a role in invasion of epithelial cells by the pathogen Porphyromonas gingivalis (38). The receptor for GAPDH has been recently identified as SerB653, a member of the haloacid dehalogenase (HAD) family phosphatase (39).

In this report we show for the first time that an enzymatically active form of GAPDH is expressed on the cell surface of both human and murine macrophage cell lines (Fig. 1) as well as mouse peritoneal macrophages (Fig. 2). Cell surface expression of GAPDH was found to be sensitive to the enzyme Pronase, with a slight recovery observed after 30 min of incubation in fresh medium (Fig. 1E). Culture supernatants of J774 and THP-1 cell lines also demonstrated the presence of GAPDH (Fig. 1, C and D) indicating that this protein is secreted. This finding is corroborated by a recent report that identifies GAPDH in the conditioned medium of mammalian cell lines such as COS-7, HEK293, MCF-7, HepG2, PC-12, and Neuro-2a cells. In COS-7 cells, the extracellular GAPDH inhibited cell spreading without influencing cell growth (40). Several well characterized multifunctional glycolytic enzymes are known to be secreted from cells despite the lack of any secretion signals. It has been proposed that these cytosolic proteins may be secreted by utilizing non-classical secretory pathways (41, 42).

Our studies indicate that cell surface GAPDH was expressed on both the cell lines tested however, mouse peritoneal macrophages showed a very minimal level of surface GAPDH. Interestingly, we found that upon depletion of iron from the culture medium, cell surface expression of GAPDH was significantly enhanced in both J774 and THP-1 cells. Furthermore, mouse peritoneal macrophages showed an increase in expression, comparable to that observed in the cell lines (Fig. 2). Our findings are also supported by previous reports that describe a 2.3-fold increase of liver GAPDH mRNA in iron-deficient rats. This overexpression of GAPDH mRNA has been attributed to a post-transcriptional effect of iron depletion on the liver mRNA and is believed to be related to an increase in message stability (43). Because surface GAPDH expression is modulated by iron, we also examined its potential as a protein involved in transferrin uptake. In vitro binding assays proved that GAPDH was capable of binding transferrin (Fig. 3). The physiological significance of the GAPDH-transferrin interaction was validated using in vivo assays that showed colocalization, co-immunoprecipitation (Fig. 3) and interaction by FRET analysis (Fig. 4).

Currently, two receptors for transferrin have been identified, these are referred to as TIR1 and TIR2. These receptors are membrane glycoproteins. Each receptor is a homodimer composed of two identical transmembrane units each of ~80 kDa. The receptor subunits are joined by two disulfide bonds, and each unit consists of a large extracellular C terminus, a transmembrane region and an intracellular N-terminal domain. TIR 2 shares 45% homology with TIR 1 (44). It is highly expressed in the liver and normal erythroid precursor cells (45). The role of transferrin in iron uptake is well characterized (46). Interestingly, though GAPDH interacts with transferrin, it bears no homology to the two previously identified transferrin receptors. Additionally, GAPDH does not possess any known signal sequences, membrane-anchoring motifs or hydrophobic membrane-spanning regions.

Though the transferrin–transferrin receptor pathway is the major mechanism for iron uptake, there are indications that alternative non-transferrin receptor (NTR)-mediated iron uptake mechanisms contribute to the iron requirement vital for the survival of cells. Evidence for the presence of this additional pathway is provided by the isolation of several cell lines that lack transferrin receptors but survive and display growth comparable to parental cell lines (47, 48). In addition, even in the absence of transferrin receptors, the CHO–TRVb cell line demonstrates a basal level of transferrin binding as well as uptake of iron from this transferrin (11, 15). In a separate study, homozygous TIR knock-out mice (Tfr−/−) embryos survived until E 8.5–12.5, and a few did not reveal any symptoms of tissue edema or necrosis characteristic to these embryos (18). The most compelling evidence for the presence of a low affinity, NTR-mediated transferrin uptake mechanism, has been provided by previous studies using the hepatoma cell line HuH7 transfected with TIR antisense RNA expression vectors (16, 17). It has been reported that transferrin taken up by this mechanism is internalized into these cells releasing its iron before being recycled to the extracellular medium. In addition it was observed that the proteolytic enzyme Pronase reduced the uptake of transferrin, suggesting that this NTR-mediated process involved the binding of transferrin to plasma membrane proteins. However, no specific surface receptor molecule has as yet been described in this process.

In context to these reports (16, 17), we have observed that surface GAPDH is sensitive to Pronase digestion. Furthermore, to determine the significance of the GAPDH-transferrin interaction we determined the equilibrium dissociation constant (Kd) using surface plasmon resonance. While transferrin binds...
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to TFR1 with an equilibrium dissociation constant ($K_D$) of 1 nM, TFR2 binds with a 25-fold lower affinity ($K_D = 27$ nM) (12). In comparison, the equilibrium dissociation constant ($K_D$) of GAPDH with transferrin was found to be 120 nM. This indicates that although TFR1 may be the major pathway of transferrin mediated iron uptake, cell surface GAPDH could function as a lower affinity receptor for transferrin. We also used three different approaches, confocal microscopy, co-immunoprecipitation of biotinylated surface GAPDH and localization of GAPDH and transferrin by immuno-EM to demonstrate that this complex traffics to the endosome at 37 °C (Fig. 5). Because transferrin is a marker for the early endosome it can be inferred that the GAPDH-transferrin complex is trafficking to this compartment as observed with other transferrin receptors. With this finding we report for the first time that GAPDH is localized within the early endosome and is involved in the uptake of transferrin.

The observed expression of transferrin receptor levels described in previous reports also offers significant information. It is well documented that cell lines and rapidly proliferating cells have a greater requirement of iron and therefore a higher expression of transferrin receptors (49—51). In addition, expression of transferrin receptors on monocytes is known to increase upon their maturation to macrophages (8). This is in accordance with our findings regarding the expression of cell surface GAPDH. The two cell lines showed elevated expression of GAPDH as compared with primary cells (mouse peritoneal macrophages). However, upon iron depletion, all the three cell types showed comparable levels of cell surface GAPDH expression. Taken together, our study provides strong evidence that cell surface GAPDH is the low affinity, Pronase-sensitive NTR mechanism involved in transferrin uptake.

Expression of GAPDH on the surface of epithelial cells has been reported (38) in addition we have also observed a similar expression on numerous mammalian cell lines. Our findings describe the presence of a novel uptake mechanism for the iron transport protein transferrin in mammalian macrophages, wherein these cells utilize the ubiquitous moonlighting protein GAPDH as a receptor. This mechanism provides an elegant method by which this abundant cellular protein is relocated to the membrane for this additional role. We therefore propose that mammalian cell surface GAPDH represents a primitive mechanism for the uptake of iron transport proteins that has been conserved in cells. Because GAPDH is a ubiquitous protein, the broader implications of this finding are that in addition to macrophages, this may be an alternative mechanism for iron acquisition in other mammalian cells and tissues.

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