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A novel iron uptake mechanism mediated by GPI-anchored human p97

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The contribution of D.R.Richardson to this study was of equal importance to that of M.L.Kennard

The established process for iron uptake into mammalian cells involves transferrin and its receptor. Here, the role of the glycosyl-phosphatidylinositol (GPI)-linked transferrin homologue, melanotransferrin or p97, was studied using CHO cell lines defective in the transferrin receptor (TR) and transfected with human TR and/or human p97. The presence of p97 doubled the iron uptake, which could be explained by the binding of one atom of iron to one molecule of p97. The internalization of iron was shown to be temperature sensitive and saturated at a media iron concentration of 2.5 μg/ml with a V_{max} of 0.1 pmol Fe/10^{6}cell/min and a K_m of 2.58 μM for p97. Treatment of the cells with either phosphatidylinositol–phospholipase C or monoclonal antibodies against p97 resulted in over a 50% reduction and a 47% increase in the iron uptake respectively. These data identify p97 as a unique cell surface GPI-anchored, iron binding protein involved in the transferrin-independent uptake of iron in mammals.

Keywords: GPI anchor/human p97/iron uptake/transfected CHO cells/transferrin independent

Introduction

A cell's existence is intimately connected with the presence of iron (Fe), too much or too little will result in cell death. Cells from all mammalian tissues have an essential requirement for Fe in order to proceed from G_1 to S phase of the cell cycle (Lederman *et al.*, 1980). Fe-containing enzymes catalyse many key reactions involving energy metabolism, respiration and DNA synthesis (Kühn *et al.*, 1990). In humans, Fe metabolism appears to be highly regulated and Fe is found mainly complexed with the transport protein, transferrin (Tf), or the storage protein, ferritin. Fe is rarely found in the blood plasma in the free state since it is highly toxic (Lauffer, 1992) and Tf serves mainly to mop up free Fe and to shuttle Fe, in a soluble non-toxic form, among the organs of the body. The established mechanism by which cells acquire Fe from Tf involves Tf binding to the transferrin receptor (TR) and Fe being internalized by the mechanism of receptor-mediated endocytosis (RME) (Aisen, 1989; Thorstensen and Romslo, 1990). Since normal levels of serum Tf are high and ~99% of Fe in the plasma is bound to Tf (May *et al.*, 1980), Fe uptake is believed to be regulated by the level of TR expression (Young and Aisen, 1981; Brissot *et al.*, 1985; Thorstensen and Romslo, 1990). Any free Fe generally circulates as low molecular weight complexes such as citrate (Grootveld *et al.*, 1989) and certain amino acids, or in association with other serum proteins such as albumin (May *et al.*, 1980). High levels of free Fe are usually only found in the plasma from dying cells or during iron overload disorders such as haemochromatosis (Smith, 1990), thalassaemia (Modell and Berdoukas, 1984) and atransferrinanaemia (Kaplan *et al.*, 1991).

Based on studies where cells were grown in serum-free, hence Tf-free, media and in cases of iron overload disorders, it has become evident that some cells are able to obtain Fe independently of Tf and the RME pathway. For example, a number of cell types have been shown to express transport systems capable of accumulating Fe from low molecular weight Fe chelates (Brissot *et al.*, 1985; Basset *et al.*, 1986; Wright *et al.*, 1986; Egyed, 1988; Fuchs *et al.*, 1988; Morgan, 1988; Sturrock *et al.*, 1990; Kaplan *et al.*, 1991; Olakanmi *et al.*, 1994). Mutant Chinese hamster ovary (CHO) cells that were unable to release Fe from Tf (Klausner *et al.*, 1984) as well as CHO cells without TR (McGraw *et al.*, 1987; Chan *et al.*, 1992) were both able to take up Fe from Fe salts and grow as well as normal CHO cells. The function of this Tf-independent system appears to be mainly for the removal of non-Tf-bound Fe from the plasma, especially in the case of Fe overload diseases when the plasma Fe concentration exceeds the Fe binding capacity of the plasma Tf. Alternatively, this system could participate in the absorption of Fe by intestinal cells that have no TR on their luminal surfaces (Conrad and Umbreit, 1993) or the redistribution of Fe into tissues that are not accessible to Tf (W.A. Jefferies, M.R.Food, R.Gabathuler, M.L.Kennard, S.Rothenberger, T.Yamada, O.Yasuhara and P.L.McGeer, submitted).

A possible candidate for an Fe binding protein involved in the Tf-independent Fe uptake pathway is melanotransferrin. Melanotransferrin, also known as the human melanoma tumour-associated antigen, p97, was one of the first cell surface markers associated with human skin cancer (Brown *et al.*, 1981a; Hellström *et al.*, 1983). In addition to being detected at various levels in other tumours such as lymphomas (Brown *et al.*, 1981b; Woodbury *et al.*, 1981; Hellström *et al.*, 1983), it has subsequently been found on a wide range of cultured normal cell types including liver cells, intestinal epithelial cells, fetal

intestinal cells, umbilical chord, placenta and sweat gland ducts (Dippold *et al.*, 1980; Brown *et al.*, 1982; Real *et al.*, 1988; Sciot *et al.*, 1989; Alemany *et al.*, 1993). In a recent study (Jefferies *et al.*, submitted), it has been shown that p97 is expressed on normal capillary endothelium of human brain and reactive microglia of Alzheimer's disease patients. Its distribution and cellular location are consistent with p97 having a role in Fe transport into or out of the human brain (Woodbury *et al.*, 1981). Structurally, p97 belongs to the group of Fe binding proteins that include human serum transferrin, human lactoferrin and ovotransferrin from avian egg whites (Baker *et al.*, 1987). p97 shares a 40% sequence identity with human lactoferrin (Baker *et al.*, 1987) and detergent-solubilized p97 is able to bind radioactive Fe added to the media as ^{59}Fe–citrate (Brown *et al.*, 1982). Furthermore, p97, like Tf and the TR, is a sialoglycoprotein and is encoded on chromosome 3 in humans (Plowman *et al.*, 1983). However, in contrast to other molecules in the transferrin family, p97 is the only one so far shown to be connected to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Alemany *et al.*, 1993; Food *et al.*, 1994). Further examination of the p97 sequence in relation to the three-dimensional structures of human lactoferrin (Anderson *et al.*, 1989) and rabbit serum Tf (Bailey *et al.*, 1988) has led to the proposal that it has an intact transferrin type Fe binding site at its N-terminal half but a possible defective site at its C-terminal half (Baker *et al.*, 1987). Baker *et al.* (1992) have shown that p97 has only one binding site, although earlier work by Rose *et al.* (1986) suggested that p97 may have two Fe binding sites. Previous work by Richardson and Baker (1990) identified an Fe binding component on human melanoma cells (SK-MEL-28) which was consistent with p97. However, subsequent studies indicated that this Fe binding component did not appear to donate its Fe to the cell (Richardson and Baker, 1991a), although Fe uptake could be increased by incubation with a monoclonal antibody specific for p97 (Richardson and Baker, 1991b). Based on these studies it was, however, difficult to define a specific role for p97, especially due to the lack of an appropriate negative control and the presence of multiple Fe uptake pathways in these cells (Richardson and Baker, 1992, 1994).

Here we establish a functional role for p97 and determine its ability to bind and internalize Fe. The uptake of Fe from radioactive Fe–citrate complexes in the complete absence of Tf was investigated by using TR-defective mutant CHO cells transfected with p97. This is the first alternative Fe uptake route to the Tf/TR system identified at the molecular level in mammals. We also suggest that these findings will have considerable impact on our understanding of metal metabolism in humans.

Results

Expression of p97 and TR

The uptake of Fe from Fe–citrate complexes was investigated in the complete absence of Tf and the TR. The cell line used was the mutant CHO cell line TRVb that has defective or no TR (McGraw *et al.*, 1987). These cells were then transfected with human p97 in order to determine the effect of p97 on Fe uptake. Any difference in Fe uptake would be due to the presence of p97. We also

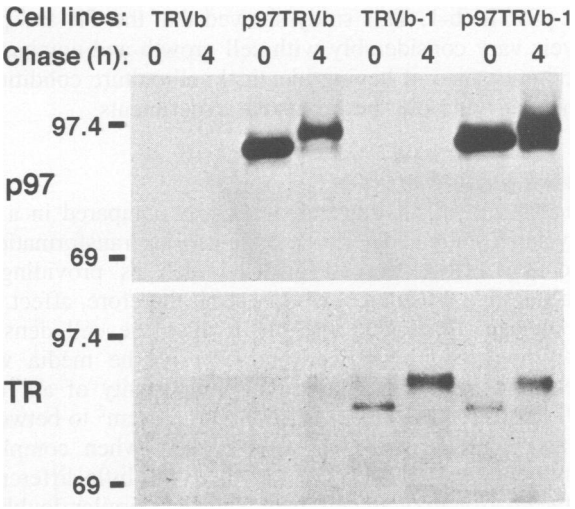

Fig. 1. Expression and transport of p97 and the human TR for TRVb, p97TRVb, TRVb-1 and p97TRVb-1. Cells were labelled with [^{35}S]methionine and chased with excess cold methionine for 4 h. Solubilized antigens were immunoprecipitated with L235 for p97 and OKT9 for the human TR. Molecular weights are indicated on the left.

tested the cell line TRVb-1, which was derived from TRVb and transfected with the human TR (McGraw *et al.*, 1987). These cells were also transfected with human p97 in order to carry out a comparable set of experiments and to observe the effect of the presence of the human TR. The expression of p97 and human TR for the cell lines was compared in a pulse–chase experiment (see Figure 1). Similar levels of p97 were expressed by the p97TRVb and p97TRVb-1 cell lines and similar levels of the human TR were expressed by the TRVb-1 and p97TRVb-1 cell lines. In addition, the higher molecular weight processing of p97 and TR after the 4 h chase indicates that these proteins are transported out of the endoplasmic reticulum at the same rate for each cell line.

The cell surface expression of TR and p97 was monitored by surface staining of EDTA-released cells with fluorescent monoclonal antibodies (mAbs) against the TR and p97 over a 10 day period. Over the first 4 days of exponential growth, the expression of TR for p97TRVb-1 was 80–90% of that for TRVb-1; by the fifth day of growth the TR expression for both cell lines had become identical. TR levels peaked after 3 days, eventually falling and levelling off to one half of the maximum expression by the fifth day. This slight reduction in TR expression during exponential growth for p97TRVb-1 cells may imply that the presence of p97 can affect TR expression. The expression of p97 for p97TRVb and p97TRVb-1 was also similar, and the maximum expression occurred after 4 days during exponential growth. The expression then fell steadily to less than half of the maximum expression by 10 days. This observation was similar to a previous study based on p97-transfected wild-type CHO (CHO-WTB) cells (Kennard *et al.*, 1993). Based on the measurement of the concentration of p97 in the phosphatidylinositol–phospholipase C (PI–PLC) solution used to treat ~10^8 cells, it was possible to determine the number of molecules of p97 per cell. At confluence, the expression of p97 was calculated to be ~$1.2 \pm 0.35 \times 10^6$ molecules of p97/cell for p97TRVb and $1.0 \pm 0.42 \times 10^6$ molecules of p97/cell

for p97TRVb-1. This study showed that the TR and p97 levels vary considerably with cell growth and emphasize the importance of having identical cell culture conditions when carrying out the Fe uptake experiments.

Batch growth studies

The growth of all four cell lines was compared in a 10 day batch study to determine if the various transformations had any effect on cell growth, such as providing a proliferation advantage, which could, therefore, affect the Fe uptake. Over a period of 10 days the cell density, viability and glucose concentration of the media was measured on a daily basis. The cell density of all four cell lines increased from ~1.25×10^4 cells/cm^2 to between 2 and 2.5×10^5 cells/cm^2 after 5 days when complete confluence was reached. Overall, there was little difference between growth of all cell lines. They had similar doubling times of 24 ± 2 h, although the maximum cell density was highest for TRVb and TRVb-1 at ~2.5×10^5 cells/cm^2 versus ~2.0×10^5 cells/cm^2 for p97TRVb and p97TRVb-1. All cell lines remained >98% viable for up to 6 days and had virtually identical glucose uptake rates with the glucose concentration falling from 1.7 mg/ml to ~0.95 mg/ml over the 6 days (i.e. equivalent to ~50 μg glucose/10^6 cells/day at confluence). Overall, the batch growth curves for all cell lines were similar and virtually identical to the batch growth curve of another p97-transfected CHO cell line (CHO-WTBp97) that was used in a previous study (Kennard and Piret, 1994). This is also in agreement with another study that showed there was no difference between the growth of TRVb and the wild-type CHO-WTB cells (Chan *et al.*, 1992). It therefore appears that the presence or absence of TR and p97 does not affect the growth of these CHO cells.

Iron uptake from Fe–citrate

Figure 2 shows the Fe uptake from Fe–citrate for all four cell lines for incubation times up to 4 h. Figure 2A clearly shows that for cells transfected with p97 (i.e. p97TRVb and p97TRVb-1) the internalized Fe uptake is greater than double that found for cells without p97 (i.e. TRVb and TRVb-1). The level of internalized Fe increased linearly with no apparent induction period, which was observed by Olakanmi *et al.* (1994) who studied the uptake of Fe from low molecular weight chelates by human monocyte-derived macrophages. The internalization was virtually identical for p97TRVb and p97TRVb-1 at 20 pmol/10^6 cell after 4 h, whereas it was 8 and 10 pmol/10^6 cell for TRVb and TRVb-1 respectively. Figure 2B shows that the membrane-bound Fe for all cell lines plateau off after ~60 min of incubation at a level of 1.6 pmol/10^6 cell for TRVb and TRVb-1 and at 2.2 pmol/10^6 cell for p97TRVb and p97TRVb-1, which is less than one fifth to one tenth the internalized Fe after 4 h. It is important to note that the membrane-bound Fe after 4 h is greater for cells expressing p97 and this is probably due to Fe bound to p97 at the cell surface. All cell lines, therefore, appear to internalize Fe from the Fe–citrate, even the TRVb cells which have neither TR or human p97. Presumably TRVb is internalizing its Fe either by Fe non-specifically binding to membrane proteins followed by endocytosis or pinocytosis or by a specific membrane-bound channel or carrier

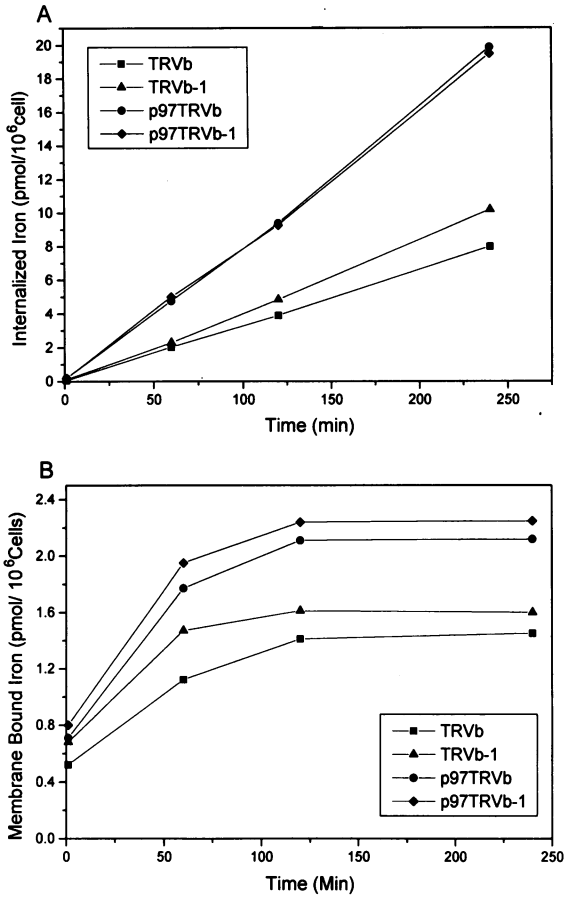

Fig. 2. Fe uptake as a function of time at 37°C for TRVb, TRVb-1, p97TRVb and p97TRVb-1. Cells incubated for up to 4 h in the presence of Fe–citrate complexes at an Fe concentration of 2.5 μM. After Fe incubation, the cells were treated with pronase at 4°C for 30 min to separate the internalized Fe (**A**) from the membrane-bound Fe (**B**). Results are the mean of three determinations.

(Egyed, 1988; Morgan, 1988). It is even possible that this internalization may indicate the presence of a hamster p97.

Effect of temperature

The uptake of Fe at 4°C was compared with that at 37°C for TRVb and p97TRVb. As seen in Figure 3, the level of internalized Fe is reduced 20-fold at 4°C. The internalized Fe uptake for p97TRVb is over double that of TRVb at both 37 and 4°C. The level of membrane-bound Fe appears to be relatively unaffected by temperature (Figure 3b), although it took longer at 4°C to reach the saturation level. At 4°C, the amount of internalized Fe is reduced to one quarter the level of membrane-bound Fe. This strong dependence on temperature of Fe uptake indicates that the internalization process is most likely mediated by an active transport mechanism. Even at 4°C, the presence of p97 has increased the level of internalized and membrane-bound Fe. Similar results were obtained for TRVb-1 and p97TRVb-1 (data not shown).

Effect of pronase on levels of p97

In a previous study (Food *et al.*, 1994) it was shown that p97 was relatively insensitive to pronase treatment. It was, therefore, possible that the difference between internalized Fe uptake levels could just be due to Fe bound to surface p97 that had not been removed by the pronase treatment.

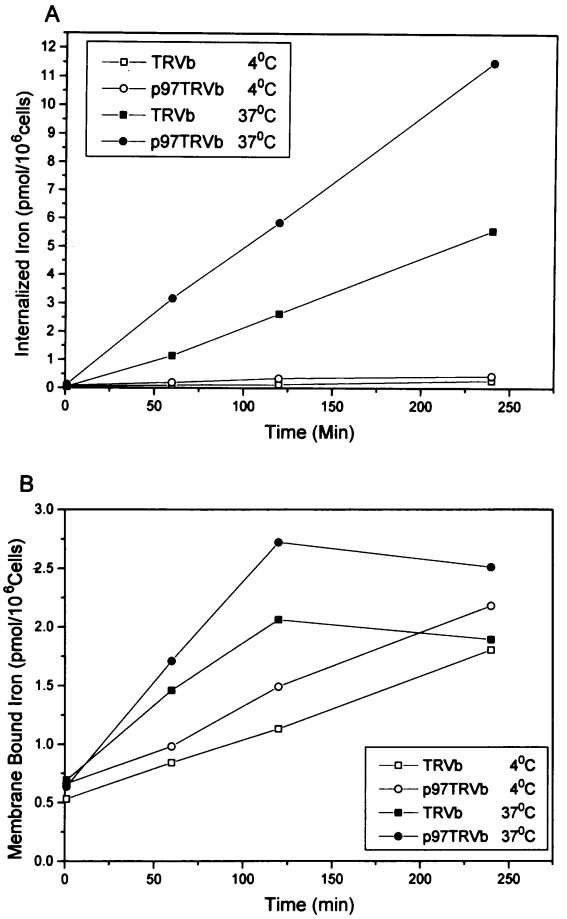

Fig. 3. Fe uptake as a function of time at 37 and 4°C for TRVb and p97TRVb. Cells incubated for up to 4 h in the presence of Fe–citrate complexes at an Fe concentration of 2.5 μM. After Fe incubation, the cells were treated with pronase at 4°C for 30 min to separate the internalized Fe (**A**) from the membrane-bound Fe (**B**). Results are the mean of three determinations.

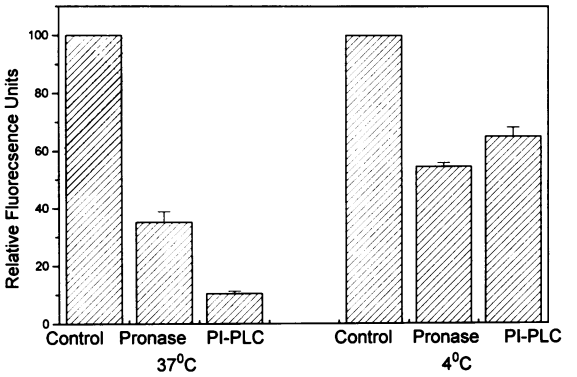

Fig. 4. Effect of PI–PLC (300 mU/ml) and pronase (1 mg/ml) on the removal of surface levels of p97 from TRVb and p97TRVb. Cells were incubated for 30 min at 37 and 4°C. Cell surface levels of p97 were determined using fluorescence concentration analysis. Results are the mean of two experiments.

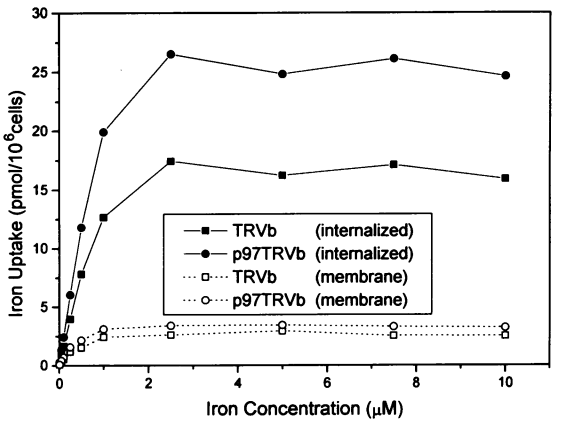

Fig. 5. Fe uptake as a function of medium Fe concentration by TRVb and p97TRVb after 4 h at 37°C. Results are the mean of three determinations.

However, from Figure 2B it is clear that pronase treatment of cells released markedly more Fe from cells transfected with p97 than from those not transfected with p97. If it is assumed that p97 is completely pronase insensitive and that there are ~1×10^6 molecules of p97 per cell surface, then the maximum amount of Fe that could be bound to the surface p97 is ~1.7 pmol/10^6 cell, assuming that one molecule of p97 binds one atom of Fe (Baker *et al.*, 1992). Even if it assumed that two atoms of Fe are able to bind per molecule of p97, this level is still not sufficient to explain the difference of 10 pmol/10^6 cell between the Fe internalized by TRVb and p97TRVb after 4 h (see Figure 2A). Furthermore, if the p97 is totally resistant to pronase, then the membrane-bound p97 should be the same for both TRVb and p97TRVb. This is clearly not the case, as seen in Figure 2B. The membrane-bound Fe was always between 0.6 and 0.9 pmol/10^6 cell greater for p97TRVb than for TRVb after 4 h. These levels are less than the amount that could be bound by 1×10^6 molecules of p97 and indicate that the pronase treatment only partially removes the p97. Based on these considerations, the effect of pronase on p97 removal was studied. Figure 4 compares the effect of pronase and PI–PLC on p97 removal from p97TRVb (similar results were obtained for p97TRVb-1) at 4 and 37°C. These results clearly show that p97 is partially sensitive to pronase and that ~45% of p97 is removed by the pronase at 4°C. For comparison, the PI–PLC is more effective at 37°C and is able to remove >90% of the p97. Therefore, it can be concluded that the difference between the membrane-bound levels of Fe is due to the 45% removal of surface p97 with bound Fe. If we assume that there are 1×10^6 molecules of p97 per cell and that one molecule of p97 binds only one atom of Fe, and the pronase treatment removes only 45% of the p97, then the calculated difference between the membrane-bound levels of Fe for TRVb and p97TRVb is 0.75 pmol/10^6 cell, which is within the observed range of 0.6–0.9 pmol/10^6 cell (Figure 2B). Furthermore, the amount of Fe bound to pronase-resistant p97 is only ~1.0 pmol/10^6 cell and accounts for only 10% of the difference in internalized Fe after 4 h.

Effect of iron concentration on iron uptake

To determine if the internalization of Fe is saturatable, the concentration of the Fe in the medium was varied from 0.01 to 10 μM. Fe uptake was measured after a 4 h incubation period. Figure 5 again shows the difference in Fe uptake between TRVb and p97TRVb. The figure also shows that the process saturates at around 2.5 μM, which corresponds to a rate of ~4.4×10^4 atoms Fe/cell/min for TRVb and 6.7×10^4 atoms Fe/cell/min for p97TRVb. Since the internalized Fe uptake from Fe–citrate followed

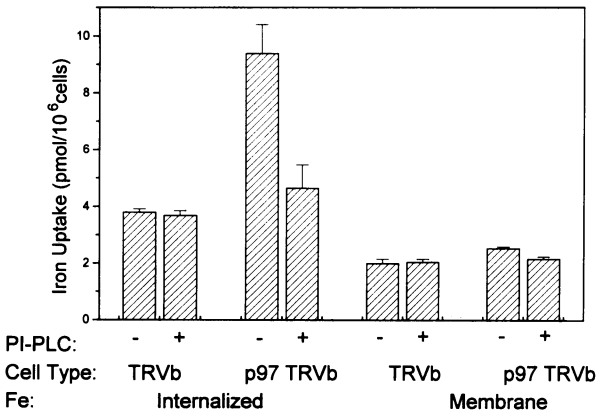

Fig. 6. Effect of pre-treating TRVb and p97TRVb with PI–PLC (300 mU/ml) for 45 min at 37°C on Fe uptake. Cells were incubated for 2 h in the presence of Fe–citrate complexes at an Fe concentration of 2.5 μM. Results are the mean of three experiments.

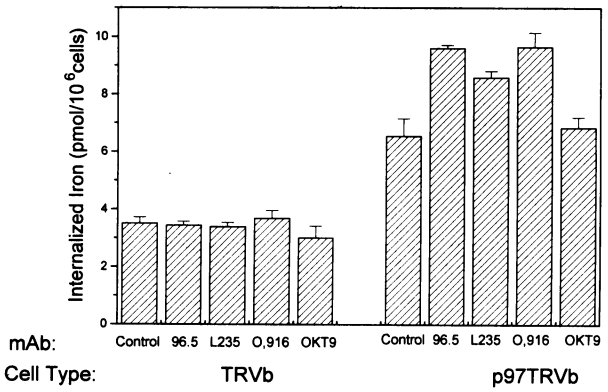

Fig. 7. Effect of pre-incubating TRVb and p97TRVb with mAbs against p97 (30 μg/ml) for 2 h at 37°C on Fe uptake. Cells were incubated for 2 h in the presence of Fe–citrate complexes at an Fe concentration of 2.5 μM plus the same concentration of mAb. Results are the mean of three determinations.

Michaelis–Menten type kinetics (Figure 5), it was possible to derive V_{max} and K_m parameters for Fe uptake using the Eadie–Hofstee plot. The V_{max} for TRVb was found to be ~0.13 pmol Fe/10^6 cell/min (0.79×10^5 atoms of Fe/cell/min) and ~0.24 pmol Fe/10^6 cell/min (1.45×10^5 atoms of Fe/cell/min) for p97TRVb. The corresponding values for K_m were 1.74 and 2.01 μM. These values compare well with other Tf-independent Fe uptake studies (Morgan, 1988; Sturrock *et al.*, 1990). By subtracting the Fe uptake for p97TRVb from that for TRVb it was possible to determine the specific Fe uptake due to p97, with a corresponding V_{max} of 0.1 pmol Fe/10^6 cell/min (0.61×10^5 atoms of Fe/cell/min) and a K_m of 2.58 μM.

Based on the knowledge that the difference between the membrane-bound Fe levels was due to 45% removal of p97, it was possible to extrapolate the data from Figure 5 to construct a Scatchard plot. This plot revealed that, at saturation, ~1.25×10^6 atoms of Fe are bound per cell to the surface p97. Since there are ~1.0×10^6 surface molecules of p97/cell then each molecule of p97 is only able to bind one atom of Fe.

Effect of PI–PLC on iron uptake

To confirm that p97 was playing a role in the Fe uptake, cells were treated with PI–PLC prior to the Fe uptake experiments. Figure 6 compares the Fe uptake from Fe–citrate after 2 h for cells pre-incubated with and without PI–PLC. The PI–PLC treatment had little effect on internalized and membrane-bound Fe for the TRVb cell line, which does not express human p97. In contrast, Fe internalized by the p97TRVb cells was reduced by >50%, and is almost reduced to the level found for TRVb. Futhermore, there is also a slight reduction of the membrane-bound Fe due to removal of the surface p97. Similar results were obtained for TRVb-1 and p97TRVb-1. These results show that removal of surface p97 reduces the amount of internalized Fe uptake from Fe–citrate and again confirms a role for p97 in Fe uptake from low molecular weight Fe chelates.

Effect of mAbs on iron uptake

This experiment was carried out to see if mAbs specific to p97 could block the binding of Fe or, as observed in the case of Fe uptake by SK-MEL-28 cells (Richardson and Baker, 1991b), actually increase the internalization of Fe. The mAb OKT9 was used as a control since it is specific to the human TR. Incubation with the mAbs specific to p97 had little effect on the internalization of Fe cells lacking human p97 (i.e. TRVb). However, in the case of cells transfected with p97 (i.e. p97TRVb), after a 2 h incubation the internalization was increased by 47% for mAb96.5, 31% for mAbL235 and 47% for mAb0,916 (see Figure 7). OKT9 had little effect on either of the cell lines. The mAbs also had relatively little effect on membrane-bound Fe. The significant increase in the internalization of Fe by p97TRVb suggests that the binding of the mAbs and the modulation of p97 results in an increase in the rate of internalization of the mAb–p97–Fe complex. This result is consistent with the results of Richardson and Baker (1991b). Other workers have also demonstrated that p97 is able to internalize mAb96.5 conjugated to anti-cancer agents (Casellas *et al.*, 1982; Rowland *et al.*, 1985; Wang *et al.*, 1987).

Iron uptake from iron bound to Tf

To investigate the uptake of Fe from Fe bound to Tf, all four cell lines were incubated with Fe–Tf. Figure 8A shows that uptake of internalized Fe for TRVb-1 and p97TRVb-1 was linear with time up to 4 h and virtually identical with an internalization rate of ~5.0×10^5 atoms Fe/cell/min, which was similar to studies involving the human melanoma cell line, SK-MEL-28 (Richardson and Baker, 1991b). Uptake for TRVb and p97TRVb, which have no TR, was reduced to less than a tenth and was similar to observations by McGraw *et al.* (1987) who compared the TRVb and TRVb-1 cell lines. The membrane-bound Fe levels (Figure 8B) were highest for TRVb-1 and p97TRVb-1 and 0.56 pmol Fe/10^6 cell greater than for TRVb and p97TRVb. This difference can be accounted for by the amount of Fe–Tf bound to the 1.5×10^6 TR/cell (McGraw *et al.*, 1987). These results show that the human TR is functional in transfected cells (McGraw *et al.*, 1987), as well as indicating that cells without the TR are still able to internalize a low level of Fe–Tf. It is likely that, in the case of TRVb and p97TRVb, Fe–Tf is binding non-specifically to the cell membrane and being internalized by endocytosis or pinocytosis. The internalized Fe for p97TRVb is almost three times that of TRVb (4.1

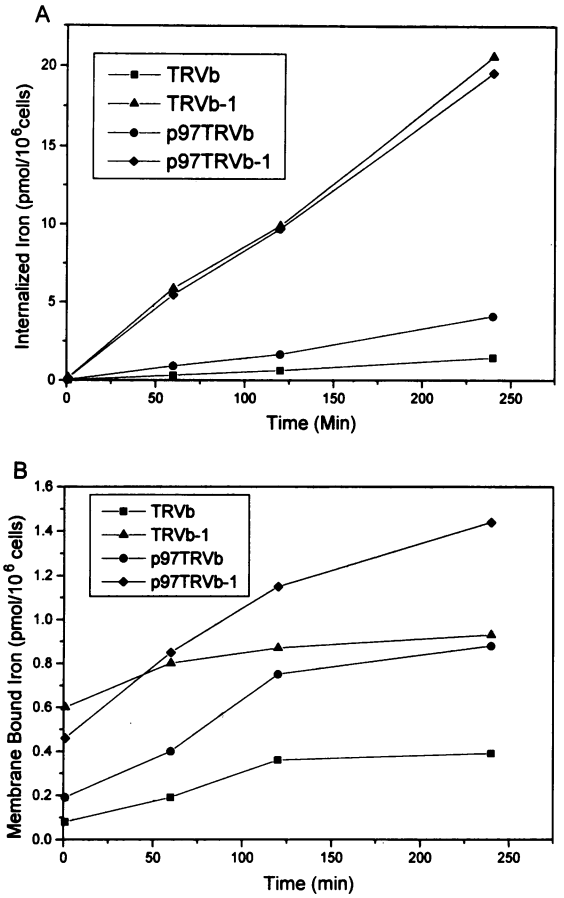

Fig. 8. Fe uptake as a function of time at 37°C for TRVb, TRVb-1, p97TRVb and p97TRVb-1. Cells were incubated for up to 4 h in the presence of Fe–Tf at an Fe concentration of 2.5 μM. After Fe incubation, the cells were treated with pronase at 4°C for 30 min to separate the internalized Fe (**A**) from the membrane-bound Fe (**B**). Results are the mean of three determinations.

versus 1.42 pmol Fe/10^6 cell) after 4 h and may indicate that the Fe from the non-specifically bound Fe–Tf is transferred to p97 at the surface and is then internalized. There is also a considerable difference in the amount of Fe bound to the membrane (0.88 versus 0.39 pmol Fe/10^6 cell), although it is not entirely clear what is causing this.

Discussion

Iron is an essential requirement for mammalian cell growth and in this study we have convincingly shown that p97 is able to bind and internalize Fe into cells from Fe–citrate but not from Fe–Tf. p97 is, therefore, identified as the first membrane-bound protein that internalizes Fe in the absence of Tf and the TR. This pathway is able to internalize Fe at a rate equivalent to Fe uptake from Tf via the RME pathway. The internalized Fe was separated from the membrane-bound iron by pronase treatment, which was shown to be able to remove 45% of surface p97 under the experimental conditions employed. The difference between membrane-bound Fe for cells with and without p97 could be completely accounted for by the removal of the 45% of surface-bound p97 by the pronase treatment, assuming as shown that one molecule of p97 bound one atom of Fe. The membrane-bound Fe saturated after ~60 min for all four cell lines, whereas the internalized Fe increased linearly with time over 4 h. The presence of p97 on the cells resulted in over a doubling of the measured internalized Fe uptake. This effect was clearly due to increased internalization of Fe and not the presence of pronase-resistant p97 on the cell surface, since Fe bound to this pronase-resistant p97 was less than 10% of the difference in internalized Fe after 4 h. Furthermore, the internalized Fe was very sensitive to temperature, with a 10-fold reduction in internalized Fe at 4°C, whereas the membrane-bound Fe did not change, although it took longer at 4°C to reach the saturated level of bound Fe at 37°C. This, together with the fact that the internalization of Fe was saturatable at an Fe concentration of 2.5 μM, suggests an energy-dependent carrier-mediated process rather than passive diffusion or simple fluid phase pinocytosis. The fact that PI–PLC treatment of the cells prior to Fe uptake experiments resulted in a >50% reduction in internalized Fe by p97-transfected cells and the fact that mAbs against p97 caused modulation of the Fe uptake, confirmed the role of p97 in Fe uptake. Considering the observed difference between membrane-bound Fe for cells with and without p97 and that only 45% of p97 is removed by the pronase treatment, it appears that one molecule of p97 is able to bind one atom of Fe, which supports the conclusions of other studies (Baker *et al.*, 1987). Previous studies have demonstrated that GPI-anchored proteins can be internalized and recycled back to the cell surface (van den Bosch *et al.*, 1988; Tausk *et al.*, 1989; Lemansky *et al.*, 1990; Keller *et al.*, 1992). The binding of Fe to p97 and the internalization of Fe is consistent with a GPI-anchored protein being involved in the binding and internalization of a ligand. To date, only one other example has been described, this being the folate receptor or folate binding protein that is able to internalize folate (Rothberg *et al.*, 1990; Anderson *et al.*, 1992). Further studies are necessary to confirm that p97 is actually internalized.

An interesting observation relating to the co-expression of the TR and p97 in transfected CHO cells is that the expression of p97 appears to have little effect on the level of TR expression after 4 days of growth, even though the iron uptake has doubled. It has been established previously that TR expression is decreased when cells are replete with Fe. However, in this study, cells were cultured in Ham's F12, which contains 0.168 μg Fe/ml. This media, supplemented with 10% serum, contains considerably higher concentrations of iron than that used in studies on the effect of Fe on TR expression (Ward *et al.*, 1982, 1984; Louache *et al.*, 1984). Therefore, it is likely that the level of TR expression by CHO cells cultured in Ham's F12 is already at a reduced level and the presence of p97 has little effect on TR expression. We have recently demonstrated that TR expression in both TRVb and p97TRVb-1 cell lines can be increased by culturing the cells in RPMI medium with reduced levels of Fe (data not shown). Another possibility is that p97 delivers Fe to a separate pool that does not affect TR expression. These points are presently being addressed experimentally in order to further understand the regulation of TR and the internalization of Fe by p97.

Surprisingly, this study also shows that the presence or absence of the TR and p97 has little effect on cell growth and proliferation. TRVb cells are able to grow as well as other CHO cell lines and internalize substantial amounts

of Fe in the presence of non-Tf-bound Fe. It seems unlikely that these cells are internalizing Fe by a non-specific pathway such as fluid phase pinocytosis, since Fe internalization by TRVb is also temperature sensitive and saturatable. Does this route represent Fe uptake mediated by another Fe binding membrane protein or is this uptake due to hamster p97? It is possible that many of the Tf-independent Fe uptake pathways observed by other workers (Brissot *et al.*, 1985; Basset *et al.*, 1986; Wright *et al.*, 1986; Egyed, 1988) may in fact be mediated by low levels of p97.

As mentioned earlier, blood plasma usually contains very little free Fe and, therefore, the p97 Fe uptake route may not function in the normal recirculation of Fe within the body. It may, however, play a role during Fe overload conditions, acting as an Fe scavenger to reduce toxic levels of Fe. This pathway does not appear to be tightly regulated since in iron overload disorders it is the cells with apparently elevated levels of p97 that are most likely to be damaged due to excessive Fe uptake (Grace and Powell, 1974; Sciot *et al.*, 1989). It is even possible that p97 is an ancestral metal transport system whose function has been superseded by the joint function of serum transferrin and the Tf receptor. Since p97 is found expressed at high levels on tumour cells, especially melanoma cells, it may be suggested that these rapidly proliferating cells are using p97 as a primary pathway to increase the Fe uptake. Under physiological circumstances, where almost all Fe is bound to serum Tf, it is not clear how these cells are able to obtain the excess Fe. However, it is known that metastasing tumour cells secrete various enzymes that act to lyse neighbouring normal cells and that p97 may be used to scavenge the released Fe. Alternatively, more simply, p97 is used to scavenge free Fe and to reduce the toxic effect of Fe released during normal cell death. It has also been noted by some researchers that the Tf-independent Fe uptake pathway is inhibited by a number of transition metals (Wright *et al.*, 1986; Morgan, 1988; Kaplan *et al.*, 1991; Seligman *et al.*, 1991; Olakanmi *et al.*, 1994). Furthermore, based on a molecular model of p97, it appears that p97 has zinc binding properties (Garratt and Jhoti, 1992). This raises the question of whether the Tf-independent or p97-mediated Fe uptake pathway actually provides an alternate pathway for other metals to enter the cell non-competitively with Fe, which would enter the cell via the RME pathway. Only when the level of free Fe is relatively high will p97 begin to bind and internalize Fe. Finally, it is also speculated that p97 may function in the trancytosis of Fe across the blood–brain barrier. This is based on our recent finding that p97 is found expressed in the capillary of the brain endothelium and that p97 and the TR appear co-localized in the brain (Jefferies *et al.*, submitted). The fact that the localization of Tf does not coincide with its receptor has led to the proposal that p97 may act as the Fe transport protein within the brain.

In conclusion, we have identified at the molecular level the components of an alternative pathway to the RME of Tf and the TR for Fe uptake, and have clearly identified p97 as being the Fe binding protein mediating this pathway. This process provides an example of internalization and recycling of a GPI-anchored protein that is able to bind and release a ligand, and may act as a model to define the steps in the GPI and Fe uptake pathways, which are currently under debate.

Materials and methods

Cells and culture

The CHO cell line, TRVb, which expressed no TR, was obtained from Dr F.Maxfield (New York University, NY) (McGraw *et al.*, 1987). This cell line was co-transfected with the human p97 expression vector pSV2p97a and the geneticin (G418) resistance vector (Food *et al.*, 1994). The p97 vector, which contained the entire coding region of p97 cDNA driven by the SV40 early promoter, was obtained from Dr G.Plowman (Bristol-Meyers Squibb, Seattle). Transfected cells were selected with 800 μg/ml G418 (Life Technologies, Inc.) and were analysed and sorted by fluorescence-activated flow cytometry (FACS) and finally subcloned by limiting dilution (Food *et al.*, 1994) and subsequently called p97TRVb. The CHO cell line TRVb-1, transfected with the human TR (150 000 surface receptors/cell) was obtained from Dr F.Maxfield (McGraw *et al.*, 1987). This clone was transfected with the p97 in the expression vector pNUT obtained from Dr R.Palmiter (Howard Hughes Medical Institute, University of Washington). Cells were selected with 500 μM methotrexate (Sigma, St. Louis, MO), sorted by FACS, subcloned by limiting dilution (Food *et al.*, 1994) and called p97TRVb-1. All four cell lines were maintained in Ham's F12 medium (Gibco, Burlington, Ontario), supplemented with 10% fetal calf serum (Gibco), 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco) at 37°C in a 5% CO_2 humidified atmosphere.

In preparation for the Fe uptake experiments, the cells were subcultured from confluent 75 cm^2 T-flasks using 1 mM EDTA (Gibco) in phosphate-buffered saline (PBS) and transferred after pelletization and resuspension to 3.4 cm^2 Petri dishes at ~1×10^5 cells/cm^2 in 1.5 ml of media. After 48 h, the cells were checked for confluence and only totally confluent plates were subsequently used for the Fe uptake assay. Sample plates were examined to determine the cell density using a haemocytometer and trypan blue exclusion.

Pulse–chase experiments, immunoprecipitation and SDS–PAGE

Cells were incubated in MEM medium without methionine for 1 h. The methionine-depleted cells were then labelled with MEM containing 250 μCi/ml of [^{35}S]methioninie for 30 min and chased with normal MEM containing an excess of cold methionine. Labelled cells were lysed and immunoprecipitated with mAbs against p97 and the human TR, as described previously (Gabathuler *et al.*, 1994). SDS–PAGE analysis was finally carried as described previously (Kvist *et al.*, 1982).

Batch growth studies

All four cell lines were seeded at ~$1–2\times10^4$ cells/cm^2 in 10 ml of medium in 9 cm diameter Petri dishes. Over a period of 14 days, parallel cultures were examined at daily intervals to determine cell density, glucose consumption and viability (Kennard and Piret, 1994). p97 and TR expression were also monitored using a cellular immunofluorescent assay based on fluorescence concentration analysis (IDEXX, Westbrook, ME) (Jefferies *et al.*, 1994).

Iron uptake assay

The role of p97 in Fe uptake from Fe–citrate was investigated with established techniques used to examine Fe uptake from small molecular weight Fe complexes (Richardson and Baker, 1991b). Prior to the Fe uptake experiments, it was an essential prerequisite in these studies to ensure that the incubation media was free of Tf. Sussman *et al.* (1985) have shown that bovine Tf is able to bind Fe and donate it to CHO cells via the TR. To ensure complete depletion of bovine Tf from the cells (Richardson and Baker, 1991b), three separate 30–45 min incubation periods at 37°C in MEM (Gibco) were used to wash the confluent plates. After this washing procedure, the medium was replaced with 1 ml of MEM containing ^{59}Fe–citrate (molar ratio of Fe:citrate = 1:100 and Fe concentration of 2.5 μM), 1% NEAA, 20 mM HEPES (pH 7.4) and 5 mg/ml BSA. It should be noted that a 1:100 molar ratio of ^{59}Fe (Dupont, NEN products, Boston, MA) to citrate was used to prevent hydrolytic polymerization of Fe, which occurs at pH 7.4 (Spiro *et al.*, 1967a,b). This labelling solution was then incubated with the cells for up to 4 h at 37 or 4°C. At the end of the incubation period, the plates were placed on ice, the medium decanted and the cells washed four times with ice-cold balanced salt solution (BSS). The amount of ^{59}Fe

internalized was measured by incubation with the general protease, pronase (Boehringer Mannheim, Laval, Quebec) at 1 mg/ml in BSS, for 30 min at 4°C. The cells were then removed from the plate in the pronase solution using a Teflon spatula, transferred to ice-cold microcentrifuge tubes and centrifuged at 14 000 r.p.m. for 1 min in an Eppendorf microcentrifuge to separate internalized radioactivity in the pellet from formerly membrane-bound radioactivity in the supernatant. The radioactivity of the cell pellet and supernatant was measured separately in a gamma scintillation counter, and the Fe uptake in all experiments expressed as pmol Fe/10^6 cells. In some experiments, Fe uptake was studied at a range of ^{59}Fe–citrate concentrations (0.01–10 μM) to investigate saturatability of the Fe uptake process.

Iron uptake from iron bound to Tf

Human apo-Tf was prepared and labelled with ^{59}Fe as described previously (Richardson and Baker, 1990), by saturating the apo-Tf with ^{59}Fe using ferric nitriloacetate. After washing the cells to remove bovine Tf, the cells were incubated with ^{59}Fe–Tf at an Fe concentration of 2.5 μM in MEM containing NEAA, HEPES and BSA for 2 h at 37°C. The cells were then treated as before.

Effect of PI–PLC treatment on iron uptake

p97 was removed from the cell surface prior to the Fe uptake experiments using PI–PLC, which is a bacterial enzyme that has been shown to specifically remove GPI-anchored p97 from the proteins from the cell surface (Kennard *et al.*, 1993; Food *et al.*, 1994). Cell plates that had been washed once with MEM were pre-incubated with 300 mU of PI–PLC (Kennard *et al.*, 1993) in MEM for 45 min at 37°C. After incubation, the cells were washed twice in MEM for 30 min at 37°C to ensure complete removal of PI–PLC and bovine Tf. The cells were then incubated in medium containing ^{59}Fe–citrate and treated as before.

Effect of monoclonal antibodies on iron uptake

Cell plates were washed to remove bovine Tf, as described previously, and then pre-incubated with the following mAbs against p97: 96.5 (Dr J.Brown, Oncogene, Seattle); L235 (ATCC); and 0,961 (The Wistar institute, Philadelphia). OKT9 (ATCC), which is against the human TR, was used as a control. The cells were incubated for 2 h with the mAbs at 30 μg/ml in MEM plus NEAA, HEPES and BSA. After the pre-incubation period, the medium was replaced with medium containing ^{59}Fe–citrate plus the same concentration of mAb and incubated for 2 h. The cells were then treated as before.

Effect of pronase and PI–PLC digestion on surface p97

Plates of washed p97TRVb and p97TRVb-1 were incubated with 1.5 ml of: BSS; BSS plus 300 mU/ml PI–PLC; and BSS plus 1 mg/ml pronase for 30 min at 37 and 4°C. Cells were removed with 1 mM EDTA and assayed for surface levels of p97 using fluorescence concentration analysis (Jefferies *et al.*, submitted).

Determination of p97 molecules/cell

Approximately 10^8 cells were removed from T-flask cultures using 1 mM EDTA, counted, resuspended in 1 ml of 300 mU/ml of PI–PLC in PBS and incubated for 1 h (Kennard *et al.*, 1993). This removes >98% of p97 from the cell surface, as determined by FACS (Kennard *et al.*, 1993) and fluorescence concentration analysis (Jefferies *et al.*, submitted). The PI–PLC solution was recovered and the concentration of p97 determined by a rapid immunofluorescent assay (Kennard *et al.*, 1993) using fluorescence concentration analysis. Based on the total cell number and total p97 removed, the number of molecules per cell could then be determined.

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