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Expression of the haemopexin-transport system in cultured mouse hepatoma cells

Links between haemopexin and iron metabolism

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Minimal deviation hepatoma (Hepa) cells, from the mouse hepatoma B7756, synthesize and secrete haemopexin and express both the haemopexin receptor and the membrane haem-binding protein (MHBP) associated with the receptor, making this cell line the first available for detailed study of both haemopexin metabolism and hepatic transport. The 17.5 kDa MHBP was detected in Triton X-100 extracts of Hepa cells by immunoblotting with goat anti-rabbit MHBP. Scatchard-type analysis of haem-^{125}I-haemopexin binding at 4 °C revealed 35000 receptors per cell of high affinity (K_d 17 nM). Haemopexin-mediated haem transport at 37 °C is saturable, having an apparent K_m of 160 nM and a $V_{max.}$ of 7.5 pmol of haem/10^6 cells per h during exponential growth. Haem-transport capacity is highest in the period just before the cells enter their exponential phase of growth and slowest in stationary phase. Interestingly, haem–haemopexin serves as effectively as iron–transferrin as the sole source of iron for cell growth by Hepa cells. Furthermore, depriving Hepa cells of iron by treatment with desferrioxamine (DF) increases the number of cell-surface haemopexin receptors to 65000 per cell and consequently increases haemopexin-mediated haem transport. The effects of DF do not appear to require protein synthesis since they are not prevented by cycloheximide. Treatment of Hepa cells with hydroxyurea, an inhibitor of the iron-requiring enzyme ribonucleotide reductase that is obligatory for DNA synthesis, enhanced haemopexin-mediated haem transport. Thus, these studies provide the first evidence for regulation of haem transport by the iron status of cells and suggest a linkage between haemopexin, iron homeostasis and cell growth.

INTRODUCTION

Haemopexin and haptoglobin are glycoproteins which act to transport haem (Smith & Morgan, 1979) and haemoglobin (Kino *et al.*, 1982), respectively, from the circulation to the liver for degradation of haem and conservation of its iron. Since evidence is accruing that haemopexin and haptoglobin form important links between haem and iron metabolism, the receptor-mediated transport of haem by haemopexin, of haemoglobin by haptoglobin and of iron by transferrin can be considered to be functionally related systems which act together to maintain iron homeostasis by the liver (Fig. 1). The hepatic reclamation of haem by haemopexin plays a significant role in three areas, namely, in aiding 'nutritional immunity' (Kochan, 1973), in conserving iron for re-utilization (shown here) or storage on ferritin (Davies *et al.*, 1979), and in preventing toxic haem effects (Braun *et al.*, 1970; Lips *et al.*, 1978; Liu *et al.*, 1985; Gutteridge & Smith, 1988). Moreover, since haemopexin (Carmel & Gross, 1977; Merriman *et al.*, 1978; Baumann *et al.*, 1984, 1987) and haptoglobin (Owen *et al.*, 1964) are acute-phase plasma proteins in many mammalian species, knowledge of the regulation of their synthesis by the liver is required for understanding this inflammatory response. In addition, although transferrin is not generally considered to be an acute phase protein (Putnam, 1984), transferrin metabolism is altered in response to infection.

Haemopexin is synthesized by the liver (Thorbecke *et al.*, 1973), and haem transport with degradation of haem (Davies *et al.*, 1979) is a function of liver cells (Smith & Morgan, 1979, 1981). The haemopexin receptor has been shown to be located in the liver of rat (Smith & Morgan, 1979, 1981; Tran-Quang *et al.*, 1983), rabbit (Smith & Morgan, 1984) and pig (Majuri & Grasbeck, 1986). Furthermore, several recent reports provide evidence for haemopexin receptors in non-hepatic tissues, including K562 erythroleukaemic (Taketani *et al.*, 1986), HL-60 (Taketani *et al.* 1987*a*) cells and in placenta (Taketani *et al.*, 1987*b*). However, a cell line of physiological relevance for characterizing both haemopexin metabolism and haemopexin-mediated haem transport has been lacking. Hepa cells were chosen for examination here because these cells secrete serum proteins (Ledford & Davis, 1983) and provide a stable, well-defined experimental system for examining the kinetics and regulation of albumin synthesis (Ledford & Davis, 1983, Ledford &

Abbreviations used: Haem, iron–protoporphyrin; mesohaem, iron–mesoporphyrin; MHBP, membrane haem-binding protein; DF, desferrioxamine; PMSF, phenylmethanesulphonyl fluoride; DMEM, Dulbecco's modified Eagle medium; AFC, ammonium ferric citrate; FBS, foetal bovine serum; PBS, phosphate-buffered saline; EMEM, Eagle's minimal essential medium; BSA, bovine serum albumin; PAGE, polyacrylamide-gel electrophoresis.

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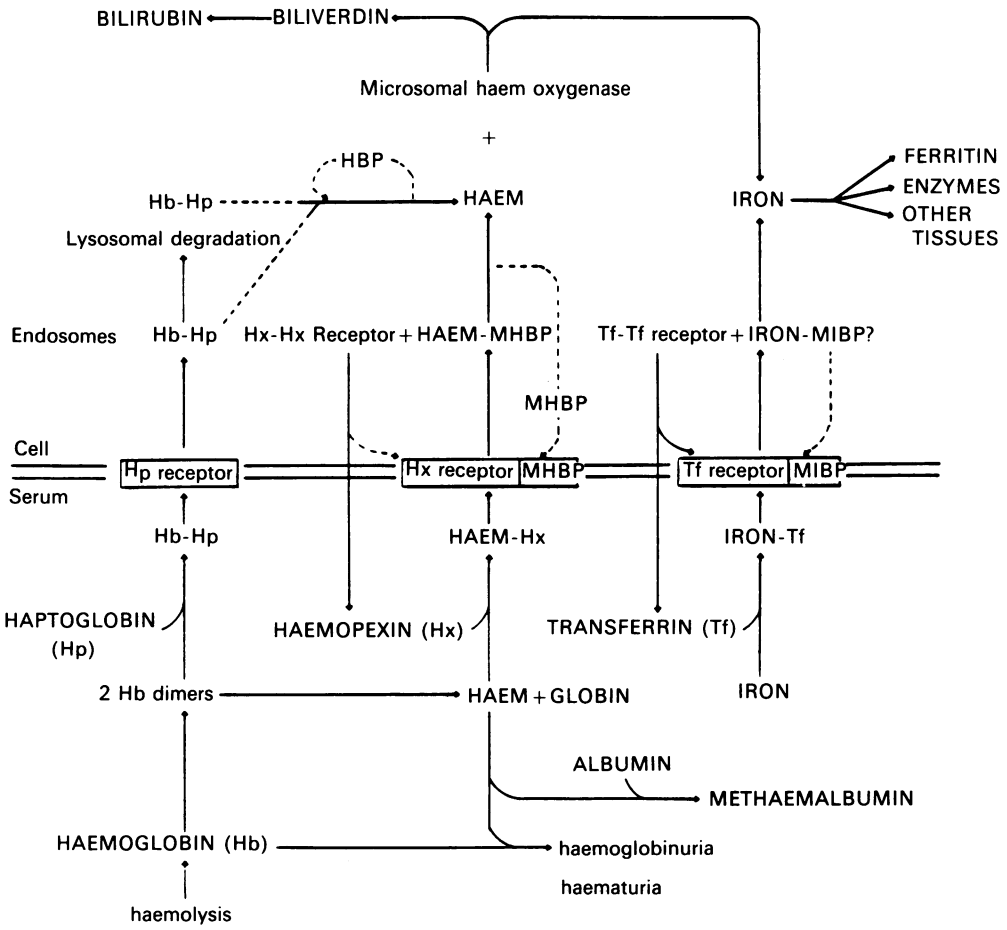

Fig. 1. Physiological role of haemopexin in hepatic haem transport and metabolism

This diagram depicts the basic features of the interactions of haem–haemopexin, haemoglobin–haptoglobin and iron–transferrin with the liver parenchymal cell. Morphological evidence for endocytosis of haemopexin has recently been obtained employing colloidal gold-labelled haem–haemopexin (Hunt *et al.*, 1988; Smith, 1988). It is not known whether haem from haemopexin and haem released from catabolism of haemoglobin–haptoglobin in lysosomes (Higa *et al.*, 1981) join a common pool, or whether there is an equivalent to MHBP in the haptoglobin system, shown here as HBP. Many details are of necessity omitted from this simplified picture, for example, the subcellular organelles which operate in certain of the steps. MIBP denotes the membrane iron-binding protein of Glass *et al.* (1980).

Jacobs, 1985). Furthermore, because they are a highly differentiated mouse hepatoma cell line (originally derived from the hepatoma BW 7756, Bernhard *et al.*, 1973), it seemed likely that they would express the hepatic haemopexin receptor.

Mouse Hepa cells are shown here to provide an informative model system for study of the haemopexin-transport system and for exploration of the relationships between hepatic haem transport, iron metabolism and cell growth.

MATERIALS AND METHODS

Haemopexin was isolated from rabbit serum and its purity (greater than 95%) checked as previously described (Morgan & Smith, 1985; Hrkal & Muller-Eberhard, 1971). Proteins were labelled with ^{125}I (ICN, Irvine, CA, U.S.A.) using Iodobeads (Pierce Chemical Co., Rockford, IL, U.S.A) according to the manufacturer's instructions, and unincorporated ^{125}I was removed by passage over Sephadex G-25 (Sigma Chemical Co., St. Louis, MO, U.S.A.) or exhaustive dialysis.

Mesohaem (iron–mesoporphyrin IX) from Porphyrin Products (Logan, UT, U.S.A.) was used in place of haem (iron–protoporphyrin IX) in this work since mesohaem is more stable than haem, and mesohaem–haemopexin complexes are chemically and biologically equivalent to haem–haemopexin (Smith & Morgan, 1979, 1981, 1984). Mesoporphyrin was also obtained from Porphyrin Products and was labelled with ^{55}Fe (New England Nuclear, Boston, MA, U.S.A.) by refluxing in dimethylformamide and freed of unincorporated iron and metal-free porphyrin by washing with acid (Adler *et al.*, 1970).

Complexes of haemopexin with mesohaem were prepared by mixing one equivalent of tetrapyrrole with one equivalent of protein. Unbound tetrapyrrole was removed by passage over DEAE-cellulose (DE-52; Whatman, Clifton, NJ, U.S.A.; Smith & Morgan, 1979) or by exhaustive dialysis. Concentrations were determined spectrophotometrically using absorption coefficients ($M^{-1} \cdot cm^{-1}$) of 1.1×10^5 at 280 nm for

apo-haemopexin (Seery *et al.*, 1972), of 1.3×10^5 at 405 nm for mesohaem–haemopexin (Morgan & Smith, 1984) and of 1.7×10^5 at 394 nm for mesohaem in dimethyl sulphoxide (Brown & Lantzke, 1969). Absorbance measurements were obtained with a Cary 219 spectrophotometer.

The membrane haem-binding protein (MHBP, formerly termed HBC), of the haemopexin-transport system was isolated and partially purified by ion-exchange chromatography on DEAE-cellulose using published procedures (Smith & Morgan, 1985). Purified MHBP, for use as antigen, was obtained by elution of gel slices after electrophoresis on non-denaturing acrylamide gels and detection of the protein by staining with Coomassie Blue G. Rabbit MHBP used elsewhere in this work, after isolation by DE 52 chromatography, had a specific activity of ~ 2–6 A_{412}/mg of protein and was approximately 60% pure as judged by SDS/polyacrylamide-gel electrophoresis (PAGE) and silver staining. The isolation and characterization of MHBP will be described elsewhere.

Polyclonal antisera to rat haemopexin and to MHBP were raised in goats after intramuscular injection of antigen emulsified in complete Freund's adjuvant (150 μg of protein), followed by a 'boost' injection (75 μg of protein) in incomplete Freund's adjuvant 6 weeks later. Antibodies were generally present in high titre within 7 days of the second injection. Ouchterlony plates were set up (Ouchterlony, 1951) using 3% poly(ethylene glycol) and 0.9% agarose in phosphate-buffered saline (PBS)/azide (3 mm thick). A sample (50 μl) was applied to each well, and double immunodiffusion was carried out at room temperature for 24 h.

Monospecific antibodies to rat haemopexin raised in goats showed a single precipitin line with one spur in double-immunodiffusion analysis of mouse serum with rat haemopexin, demonstrating both shared and independent antigenic determinants in these two species of haemopexin (Fig. 2). This antiserum was used in the subsequent experiments to study mouse haemopexin.

Antiserum raised to MHBP reacted only with a 17.5 kDa protein after electrophoresis and immunoblotting of detergent extracts of rabbit liver plasma membranes (A. Smith, unpublished observations). Anti-rabbit MHBP antibodies do not cross-react with rabbit haemopexin. The technique of Towbin *et al.* (1979) for immunoblotting was followed. After separating the samples by SDS/PAGE (Laemmli, 1970), blotting was carried out at 100 V for 2 h on to nitrocellulose (0.2 micron, BioRad) at 4 °C. Goat antisera raised in this laboratory were used at 1:500 dilution in 5% (w/v) Carnation non-fat skim milk powder in PBS for 16 h at 4 °C followed by incubation for 1 h at 25 °C with a 1:2500 dilution of rabbit anti-goat IgG (H+L chains) (affinity-purified, Cappel) covalently coupled to alkaline phosphatase and developed (Johnston & Zabriskie, 1986). Triton X-100 extracts of Hepa cells used in immunoblotting experiments were made by incubating ~ 1×10^7 washed cells with Hepes/NaOH buffer pH 7.7, containing 0.5% Triton X-100 and 2 mM-$CaCl_2$, at 4 °C for 30 min followed by centrifugation at 100000 *g* for 1 h. These cells were routinely cultured as described below.

Immunoaffinity chromatography of cell medium harvested from mouse Hepa cells after metabolic labelling experiments was carried out using goat anti-rat haemopexin IgG covalently linked to CM-BioGel A

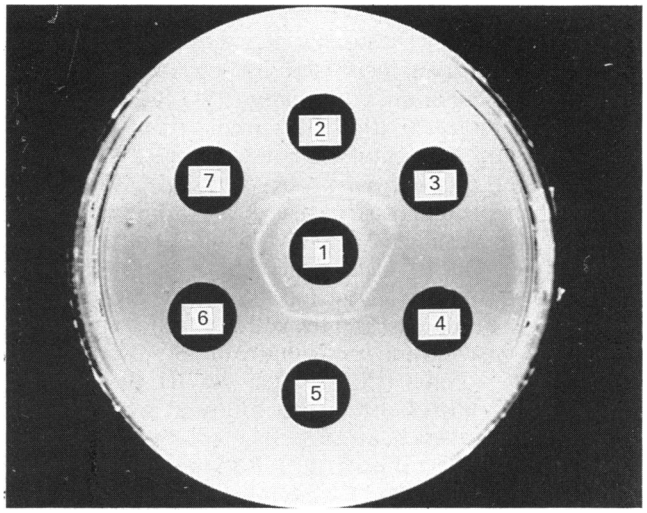

Fig. 2. Double-immunodiffusion analysis of mouse haemopexin produced by mouse Hepa cells

Haemopexin produced by Hepa cells was analysed using monospecific polyclonal antibody to rat haemopexin in Ouchterlony immunodiffusion plates. Well 1, goat anti-rat haemopexin; wells 2 and 5, rat haemopexin; well 3, 0–24 h culture medium (serum free) collected from mouse Hepa cells; wells 4 and 6, normal mouse serum; well 7, 0–24 h culture medium (serum free) from mouse Hepa cells, concentrated 10-fold.

following published procedures (Smith *et al.*, 1985). Immunoprecipitation was carried out on samples of medium after extensive dialysis in the presence of proteinase inhibitors [0.2 mM-phenylmethanesulphonyl fluoride (PMSF) and 2000 k.i.u. of aprotinin/ml] to remove unincorporated radiolabel. Samples (~ 10^7–10^8 c.p.m.) were diluted 1:1 with 2 × buffer D (100 mM-Tris, pH 7.4, containing 2% Triton X-100, 600 mM-NaCl, 0.2% bovine serum albumin (BSA), 0.2 mM-PMSF, 20 μg of leupeptin/ml and 40 k.i.u. of aprotinin/ml) before addition of 1.0 mg of goat anti-rat haemopexin IgG, followed by incubation for 60 min at 4 °C. Protein A–Sepharose (200 μl of 50% suspension) was then added and, after further incubation at 4 °C for 90 min, the sample was centrifuged at 2000 *g* for 2 min. The pelleted immune complex–Protein A–Sepharose was washed extensively in 1 × buffer D (without BSA) and solubilized for analysis by SDS/PAGE on a 4.5–20% (w/v) acrylamide gradient gel.

Cell culture

Minimal deviation hepatoma cells from mouse solid tumour line BW 7756 (Hepa) cells were grown in Dulbecco's modified Eagle medium (DMEM; K.C. Biological, Kansas City, MO, U.S.A.) supplemented with 0.35% glucose, 25 mM-gentamicin and 2% (v/v) foetal bovine serum (FBS) (K.C. Biological) in a humidified atmosphere of 5% CO_2/95% air. Routine subculturing (1:10) was carried out every 4–5 days and cells were fed every 2–3 days. Methionine-deficient DMEM was obtained from Irvine Scientific, Irvine, CA, U.S.A. Gentamicin was included in the growth but not the experimental media.

For metabolic labelling of haemopexin the growth

medium was removed, the cells were washed, treated with experimental methionine-deficient DMEM for 1 h at 37 °C, and then incubated for up to 4 h in fresh experimental medium containing ^{35}S-TRAN (50 μCi/ml; specific radioactivity 993 Ci/mmol from ICN).

For binding and uptake studies the cells were maintained in the exponential phase of growth. Binding of ^{125}I-labelled protein was measured by adding 1 ml aliquots of Hepes-buffered DMEM, pH 7.2, containing the protein to (1–2) $\times$ 10^6 cells in one well of a six-well tissue-culture dish. At the indicated time, the medium was aspirated from the well and the cells washed with 3 $\times$ 2 ml of cold 10 mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl (PBS). Then NaOH (0.1 M, 2 ml) was added to dissolve the cells followed by 0.23 ml of 50% acetic acid to neutralize. The solution (2 ml) was removed and ^{125}I counted in a Beckman 7500 gamma spectrometer. Uptake of ^{55}Fe-labelled mesohaem was measured in a similar manner except that 2 ml aliquots were mixed with 13 ml of liquid-scintillation fluid (Betablend, Westchem Scientific, San Diego, CA, U.S.A.) and cell-associated ^{55}Fe measured in a Beckman 9800 scintillation counter. In each experiment three wells were used for each determination. In these binding and haem uptake assays the standard deviation generally ranged from 3 to 10% of the mean. Each experiment was repeated at least twice. Specific binding or specific uptake is defined as the difference in binding or uptake in the presence and absence of excess unlabelled mesohaem–haemopexin (generally 100-fold excess) added to the cells before the labelled complex. Additional details are given in the legends to the Figures and Tables. Specific binding of mesohaem–^{125}I-haemopexin (50 nM) to the Hepa cells, at pH 7.2, was greater than 80% of the total bound radiolabelled complex as demonstrated by competitive inhibition with 5 μM unlabelled complex. Cell counts were made on at least two wells before each experiment by releasing the cells with trypsin and counting the cells in a haemocytometer after dilution in Trypan Blue. Protein was determined on aliquots of the neutralized cell extracts using the Pierce BCA protein assay system with BSA as a standard.

For the series of cell-growth experiments employing haem–haemopexin as the sole source of iron, cells in stationary phase were detached by trypsin treatment, diluted and plated out at 0.5 $\times$ 10^6 cells per well in six-well plates. After allowing cells to attach during a further 6 h incubation, the medium was then changed to either control or experimental medium and cell counts were taken 24, 48 and 72 h later. The experimental medium either consisted of DMEM containing 0.1 mg of iron/l (GIBCO) and supplemented with concentrations of serum greater than 2% or Eagle's minimal essential medium (EMEM) (GIBCO, which has no added inorganic iron salts) containing serum or supplements (see below and in the Table legends). All growth factors were obtained from Collaborative Research Incorporated, Bedford, MA, U.S.A. The effects of 5 mg of insulin/l, 5 μg of selenium/l (sodium salt of selenous acid), diferric transferrin (equivalent to 0.01–2.5 mg of iron/l), and linoleic acid bound to BSA (5.35 mg of BSA/l) were examined. This source of albumin was found to be contaminated by bovine haemopexin after analysis by Western immunoblotting using either anti-human haemopexin or anti-rabbit haemopexin polyclonal antisera, which cross-react with bovine haemopexin (a generous gift from Dr. D. R. Babin, Creighton University, Omaha, NE, U.S.A.). Because of this contamination and because preliminary experiments established that linoleic acid was not an obligatory growth factor for these cells over the 3-day period examined, this compound was not employed in subsequent experiments. Protohaem–haemopexin, the naturally-occurring form of haem, was employed as the source of haem iron at a range of concentrations (0.75–5.0 μM) calculated to be equivalent to 0.04–2.5 mg of iron/l. This range of diferric transferrin and haem–haemopexin concentrations were chosen for investigation because: firstly, normal circulating levels of transferrin are about 33 μM, of which 30% is saturated with iron (equivalent to $\sim$ 1 mg of iron/l); secondly, serum iron levels are 1–1.5 mg of iron/l; and thirdly, concentrations of diferric transferrin efficacious for cell growth *in vitro* range from 0.5 to 100 mg of transferrin/l of medium, depending upon cell type (Barnes & Sato, 1980*a*,*b*).

RESULTS AND DISCUSSION

Haemopexin synthesis, receptor binding and haem uptake in hepatoma cells

A model system to study hepatic haemopexin metabolism and haemopexin-mediated haem transport must have at least the following features: synthesis and secretion of haemopexin; binding of haemopexin by its specific receptor; and MHBP-mediated transport of haem. To establish that Hepa cells provide a suitable model system, we first examined haemopexin synthesis, receptor binding and haem uptake by these cells. Both the synthesis and secretion of haemopexin was demonstrated using metabolic labelling with [^{35}S]methionine and characterization of the secreted protein by immunoprecipitation followed by analysis on SDS/PAGE. Medium removed from cultured cells after 2 and 4 h incubation with [^{35}S]methionine (as [^{35}S]TRAN) was passed over an anti-rat haemopexin immunoaffinity column (Fig. 3*a*). The haemopexin at 4 h represented 3.5% of the total secreted radiolabelled acid-precipitable protein. In addition, the ^{35}S-labelled protein precipitated by the immune sera from the medium was found to migrate similarly to purified rat haemopexin on PAGE (Fig. 3*b*). These results established that the ^{35}S-labelled protein isolated by the anti-rat haemopexin is mouse haemopexin secreted by these Hepa cells.

Specific binding of mesohaem–^{125}I-haemopexin (50 nM radiolabelled complex in the presence and absence of 5 μM unlabelled complex, 15 min at 37 °C, at the pH maximum of 7.2) during exponential growth constituted 86% of total binding and was 0.6 pmol/mg of protein or 0.095 pmol/10^6 cells. Consistent with earlier studies (Smith & Morgan, 1981) showing decreased affinity of the haemopexin receptor for the apoprotein, specific binding of apohaemopexin under the same conditions constituted only 61% of the total, and was 0.16 pmol/mg of protein or 0.036 pmol/10^6 cells. Scatchard-type analysis of equilibrium binding at 4 °C revealed that Hepa cells in their exponential phase of growth have 35000 high-affinity receptors per cell for haem–haemopexin (K_d 17 nM; Fig. 4). This is a higher affinity than previously estimated for rat liver *in vivo* (K_d 700 nM; Smith & Morgan, 1979), but similar to that reported for freshly-isolated hepatocytes (K_d 50 nM at pH 7.4; Smith & Morgan, 1981), for rat hepatic plasma membranes

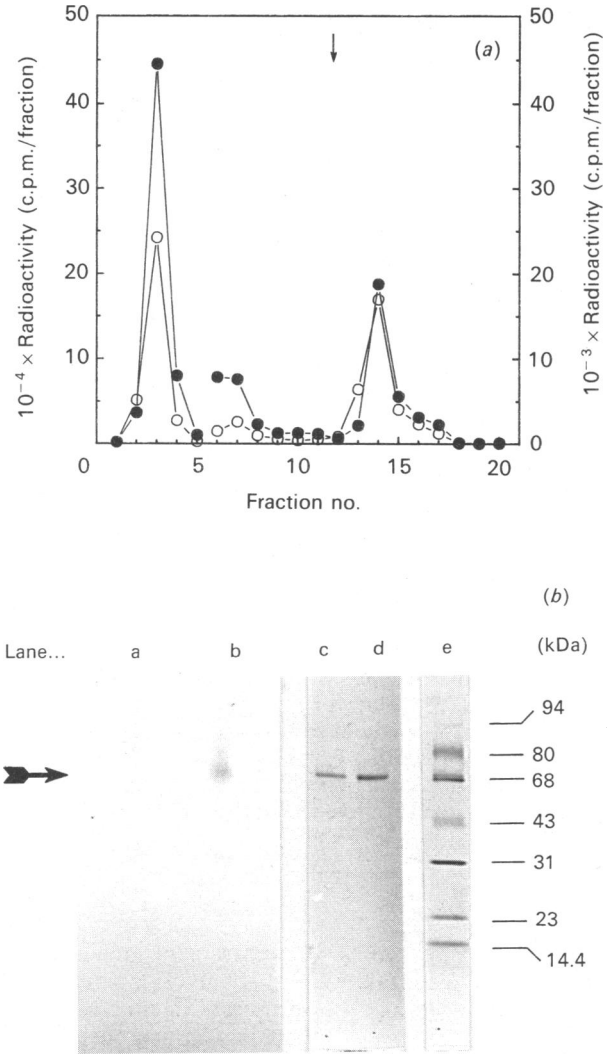

Fig. 3. Isolation of metabolically-labelled mouse haemopexin secreted by Hepa cells using immunoaffinity chromatography and immunoprecipitation

(*a*) Elution profile of medium isolated from mouse Hepa cells after 2 (○) and 4 (●) h of metabolic labelling with ^{35}S on a goat anti-rat haemopexin immunoaffinity column. Specifically-bound material is eluted with 2 M-sodium thiocyanate, indicated by the arrow. Note that the scale changes after fraction number 5. (*b*) Analysis of immunoprecipitated material from the 4 h-medium sample after SDS/PAGE on 4.5–20% (w/v) acrylamide-gradient gels, followed by autoradiography. The migration of pre-stained molecular-mass standards run simultaneously are marked (lane e). Also shown is 5 (lane c) and 10 μg (lane d) of rat haemopexin after SDS/PAGE and stained with Coomassie R250. Lanes a and b show the material precipitated with pre-immune and immune IgG to rat haemopexin, respectively.

(K_d 32 nM at pH 7.2; Tran-Quang *et al.*, 1983) and for K562 cells (5 nM, pH not reported; Taketani *et al.*, 1986).

A MHBP, formerly termed HBC (Smith & Morgan, 1985), appears to play a pivotal role in haemopexin-mediated haem transport (Smith & Morgan, 1985). The 17.5 kDa haem-binding subunit (A. Smith, unpublished

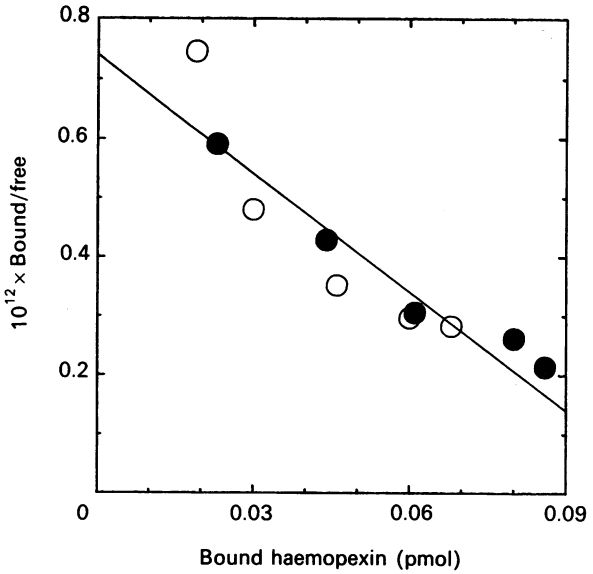

Fig. 4. Scatchard analysis of mesohaem–haemopexin binding to mouse Hepa cells

Mesohaem–^{125}I-haemopexin (0.01–1.0 μM) was incubated with cells at 4 °C for 90 min. The cells were then rinsed and harvested as usual and the amount of bound haemopexin determined. This produced a curvilinear Scatchard plot from which the data for high-affinity sites are shown (correlation coefficient of 0.982). ●, control cells (1.75×10^6 cells/well); ○, DF-treated cells (0.95×10^6 cells/well).

work) was detected in Triton X-100 detergent extracts of Hepa cells using SDS/PAGE followed by immunoblotting (Fig. 5). Because the mouse protein migrated with a slightly higher apparent M_r than rabbit MHBP, the mixing experiment in Fig. 5, track 2 was carried out. Since only one band of enhanced intensity is seen, the large amount of other proteins present in the cell extract in track 3 appears to have retarded the migration of the mouse MHBP. Since the antisera was raised against MHBP isolated from rabbit liver membranes, these results also demonstrate that rabbit and mouse MHBP contain conserved epitopes.

Haemopexin-mediated haem transport in mouse Hepa cells was demonstrated using ^{55}Fe-mesohaem–haemopexin complexes. When these cells were in their exponential phase of growth, haem uptake at 37 °C was linear for up to 4 h (see below) and saturable (Fig. 6). Haem uptake occurred with an apparent K_m of 160 nM and with a $V_{max.}$ of 7.5 pmol of haem/10^6 cells per h (equivalent to 75000 molecules of haem/cell per min) during exponential growth (Fig. 6).

Relationship between haem transport and cell growth

The results presented above demonstrated that Hepa cells are a good model system for studying haemopexin metabolism and haemopexin-mediated haem transport. The first questions addressed with this system concerned the relationship between haem transport and growth. Interestingly, haem transport varies with the growth phase of the Hepa cells, being highest at 5–8 h after seeding (results not shown), just before cells enter their exponential growth phase (Fig. 7). Under these conditions the cells have a doubling time of 11 h.

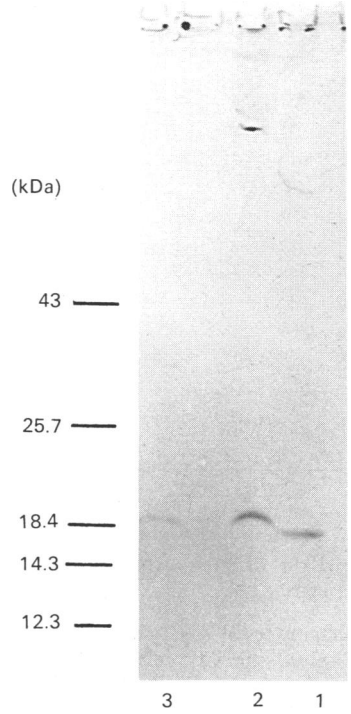

Fig. 5. Expression of the MHBP of the haemopexin-transport system in mouse Hepa cells demonstrated by immunoblotting

Samples were subjected to electrophoresis under reducing, denaturing conditions on a 4.5–20% (w/v) polyacrylamide-gradient gel, followed by transfer to nitrocellulose and immunoblotting with goat anti-rabbit MHBP. This polyclonal antibody was raised against the 17.5 kDa haem-binding subunit of MHBP, isolated from rabbit liver plasma membranes using ion-exchange chromatography as described in the Methods section (track 1, 2 μg of total protein). Track 3 shows the cross-reaction of the antibody with a protein of similar size from Triton X-100 extracts (200 μg of total protein) of mouse Hepa cells. Track 2 contains a mixture of the samples in tracks 1 and 3. The mobilities of pre-stained molecular-mass standards present in the same gel are also shown.

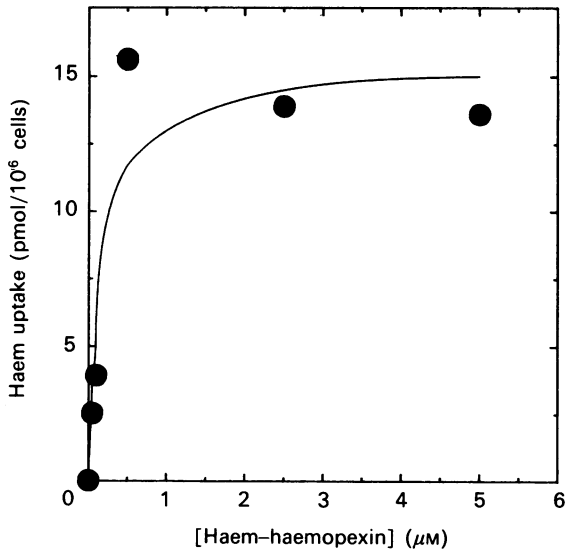

Fig. 6. Haemopexin-mediated haem uptake by mouse Hepa cells

Mouse Hepa cells ($\sim 1.5 \times 10^6$ cells) were incubated in Hepes-buffered medium at 37 °C with [^{55}Fe]haem–haemopexin (0.05–5.0 μM) for 2 h. Haem uptake, measured as described in the Methods section, has an apparent K_m of 160 nM and occurs with a $V_{max.}$ of 7.5 pmol/10^6 cells per h. The data were fitted to a hyperbolic binding function using a non-linear statistical curve-fitting routine; the theoretical best fit line is shown as a solid line.

Evidence for a role for haemopexin in linking haem and iron metabolism

We next addressed whether the haem iron released intracellularly by haem oxygenase from haem transported by haem–haemopexin enters the same low-molecular-mass intracellular iron pool as does iron derived from iron transferrin. Hepa cells were cultured in defined EMEM with haem–haemopexin as the sole source of iron. The results in Table 1 clearly demonstrate that cell growth is maintained as effectively by haem–haemopexin as by diferric–transferrin in the presence of insulin and selenium. No additive or synergistic effects were observed when both transferrin and haemopexin were present. These findings clearly show a role for haemopexin in linking haem and iron metabolism.

That the support of growth by haem–haemopexin may be due to haem itself, rather than the iron moiety, seems unlikely for the following reasons. The incorporation of intact haem into hepatic apoproteins, including cytochrome *P*-450 isoenzymes, is an extremely small fraction of the injected haem taken up by the liver (Correia *et al.*, 1979). At the same time the amount of haem catabolized by the liver is extensive, and it is well established that haem oxygenase releases iron from haem. Importantly, haem taken into the hepatocyte by the haemopexin receptor is rapidly degraded with iron being detectable on ferritin within 10 min after intravenous injection of haem–haemopexin (Davies *et al.*, 1979). Moreover, haem oxygenase is readily induced by its substrate haem (Tenhunen *et al.*, 1970) and by haem–haemopexin in Hepa cells (J. Alam & A. Smith, unpublished observations). Finally, the majority of cells in culture require transferrin as an obligatory growth factor due to their requirement for iron which is transported into cells by transferrin. Lack of iron or of transferrin results in decreased cell growth and division.

Treatment of several different types of transformed cells with the cell-permeable iron-chelator desferrioxamine (DF) increased their number of surface transferrin receptors (Bridges & Cudkowicz, 1984; Louache *et al.*, 1984; Ward *et al.*, 1984) due to an increase in receptor synthesis following increased transcription of its mRNA (Mattia *et al.*, 1984; Rao *et al.*, 1986). Since haem added to the medium prevents this induction of transferrin receptor mRNA (Roualt *et al.*, 1985), Hepa cells were deprived of iron to determine whether the haemopexin-transport system of the cells would be similarly affected. Treatment of Hepa cells with DF increases both haemopexin-mediated haem uptake (Fig. 8) and the specific binding of haem–haemopexin complexes per cell (Table 2). The enhancement of both haem uptake and haemopexin binding can be observed within

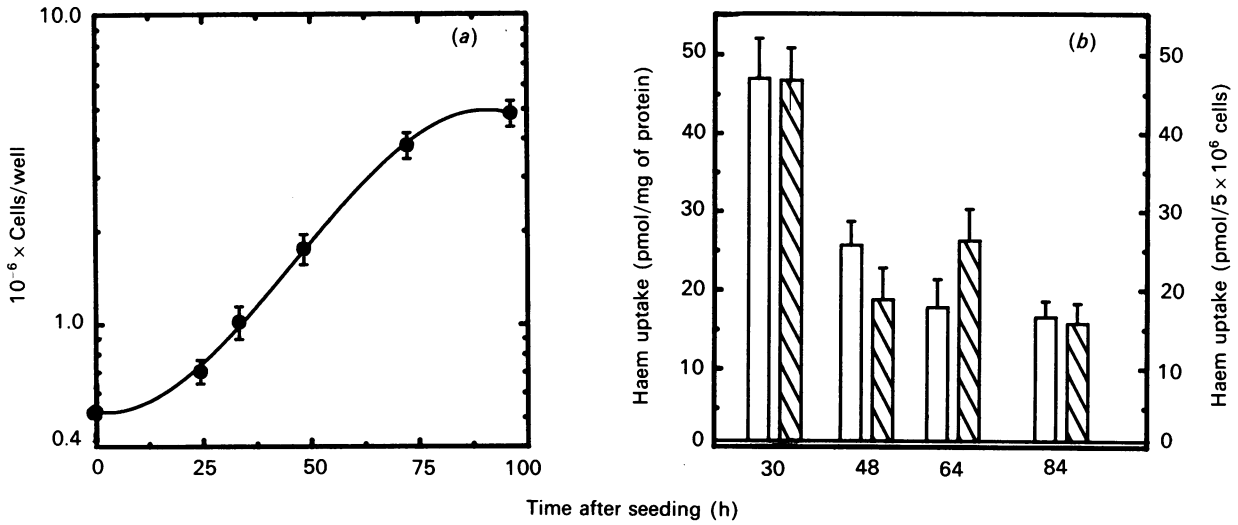

Fig. 7. Haemopexin-mediated haem uptake varies with cell growth in Hepa cells

(*a*) A typical growth curve is shown in which Hepa cells have a doubling time of ~ 11 h. Cell counts (mean ± 2 S.D.) were determined from four separate wells. (*b*) Haem uptake was measured for 2 h at 37 °C using 500 nM-[^{55}Fe]mesohaem–haemopexin at 30, 48, 64 and 84 h after seeding. Results are expressed as pmol of haem per mg of cell protein (hatched bars) or pmol of haem per 5×10^6 cells (open bars).

Table 1. Maintenance of growth of Hepa cells by various physiological iron sources

Hepa cells were passaged normally in DMEM containing 2% (v/v) FBS and allowed to reach stationary phase before trypsinization and plating out (at 0.5×10^6 cells/well) in fresh growth medium. After attachment for 6 h the growth medium was replaced with either control medium (DMEM with 2% FBS) or experimental medium (DMEM with 10% serum or EMEM with or without supplements including haemhaemopexin (HMPX). EMEM was supplemented with insulin (5 mg/l) and the sodium salt of selenous acid (5 μg/l). Addition of linoleic acid–BSA (5.35 mg/l) in the presence of diferric transferrin (Fe_2Tf) and selenium did not affect growth rates and was therefore not included in this series of experiments. Cell growth was measured by counting the number of viable cells in a haemocytometer. The average cell counts from duplicate wells are reported at 24, 48 and 72 h after seeding with control values being 0.78×10^6, 1.59×10^6, and 3.0×10^6 cells per well. The results are from a representative experiment repeated at least three times.

Growth conditions	Fe (μg/ml)	Cell growth (% of control) Day 1	Day 2	Day 3
DMEM + FBS (2%)	> 2	100	100	100
+ FBS (10%)	> 2	98	120	124
EMEM	0	70	51	46
EMEM + HPX (0.75 μM)	0.04	86	84	94
+ HHPX (2.5 μM)	0.1	91	90	96
+ HHPX (5.0 μM)	0.25	94	109	101
EMEM + Fe_2Tf (0.9 μM)	0.1	91	102	92
+ Fe_2Tf (2.3 μM)	0.25	91	113	103
EMEM + Fe_2Tf + HHPX	0.2	95	114	113

7 h after addition of 50–75 μM-DF. Scatchard-type analysis revealed no change in affinity of the receptor after DF treatment (apparent $K_d \sim 11$ nM), but the number of surface receptors increased from 35000 to 65000 per cell. Incubation of DF with ferric ammonium citrate (AFC, Fig. 8) or medium containing 2% (v/v) FBS (results not shown) before addition to cells prevented the increase in haem uptake. Incubation with up to 3.5 μg of Fe/ml decreased haemopexin-mediated haem

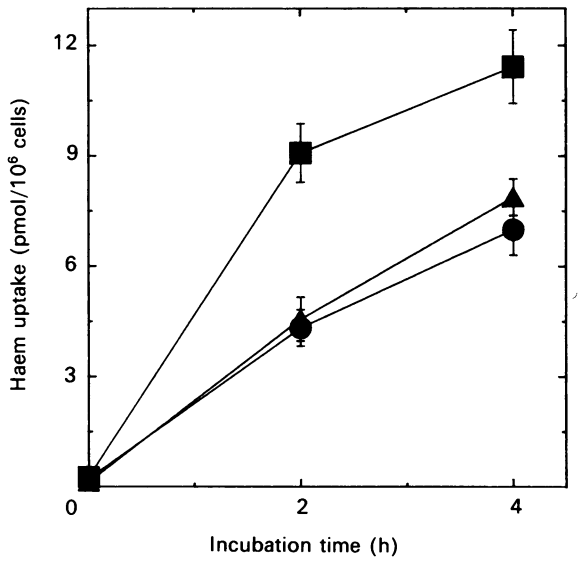

Fig. 8. Effect of desferrioxamine on haemopexin-mediated haem uptake

DF (final concentration 25 μM) was added to Hepa cells 24 h before measuring haemopexin-mediated haem uptake using 500 nM-[^{55}Fe]mesohaem–haemopexin (■). Data from control cells are shown as (●). Pre-incubation of DF (42 μg/ml), theoretically capable of chelating 3.5 μg of iron/ml, with ferric ammonium citrate (at 20 μg/ml, equivalent to 3.5 μg of iron/ml) for 30 min before addition to the cells prevented the DF-induced increase in haemopexin-mediated haem transport (▲). The results are presented as the mean ± 4 S.D.

Table 2. Comparison of the effects of altering cellular iron levels on haemopexin binding and haem uptake

Normal growth medium (containing 0.1 μg of iron/ml) was supplemented with either 8.5 or 20 μg of ammonium ferric citrate/ml, ~ 16% by wt. iron (AFC, equivalent to 0.8 and 3.5 μg of iron/ml) or with DF for 24 h while cells were in log phase of growth. DF, at 25 μM, can chelate 2.1 μg of ferric iron per ml (100 parts by wt. of DF binds 8.5 parts by wt. of ferric iron, Ciba–Geigy Corporation). Binding of mesohaem–^{125}I-haemopexin and haem uptake from [^{55}Fe]mesohaem–haemopexin were measured after incubation with a saturating amount of radiolabelled complex at 4 °C for 90 min or at 37 °C for 2 h, respectively. The results shown are from at least two experiments, three wells per experiment, except for the DF treated cells in which data from three experiments for binding and six for uptake are given.

Treatment	Concentration	Haem–haemopexin bound in pmol/10^6 cells (% control)	Haem uptake in pmol/10^6 cells (% control)
None		100±6	100±3
DF	25 μM	176±55	194±67
AFC	8.5 μg/ml	111±7	79±5
	20 μg/ml	107±12	73±13

Table 3. Effect of desferrioxamine concentration on haemopexin-mediated haem uptake

Growth medium was supplemented with DF 36 h after seeding and haem uptake measured (500 nM-[^{55}Fe]-mesohaem–haemopexin for 90 min) 17 h later in the absence of DF. Results are expressed as means±S.D. for three wells from one representative experiment in a series of three.

Treatment	Haem uptake in pmol/10^6 cells (% control)	10^6 × Cells/well
None	100±8	2.70
DF (10 μM)	159±6	1.70
DF (15 μM)	181±16	1.55
DF (25 μM)	241±10	1.25

uptake in Hepa cells (Table 2). Under the experimental conditions employed, there was no apparent decrease in the number of surface receptors, possibly due to a slower turnover of the haemopexin receptor compared with that of the transferrin receptor (A. Smith, unpublished work). It should be noted that in the transferrin system iron uptake does not correlate directly with the number of surface receptors and endocytosis of transport protein (Schulman *et al.*, 1981; Thorstensen & Romslo, 1984).

The effects of DF are dose-dependent with higher concentrations of DF producing higher levels of haem uptake (Table 3). However, 17 h after DF addition cell division is significantly inhibited without obvious toxic effects, but haemopexin-mediated haem uptake remains higher in these treated cells than in controls. The increase

Table 4. Effect of hydroxyurea on haemopexin-mediated haem uptake

Cells were treated for 16 h with hydroxyurea before measuring haem uptake over 90 min at 37 °C in the absence of hydroxyurea. Results are given as mean±S.D. for triplicate wells from a representative experiment repeated twice. Control values were 24.0±4 pmol/mg of protein per h and 5.6±0.3 pmol/10^6 cells per h.

Hydroxyurea concentration (10^6 × M)	Haem uptake: pmol/mg of protein (% control)	Haem uptake: pmol/10^6 cells (% control)	10^6 × cells/well
None	100±15	100±5	2.35
0.01	130±8	133±14	2.1
0.1	115±1	102±1	2.1
1	132±6	125±8	2.25
10	171±8	206±8	0.81
100	231±15	168±33	0.69
1000	189±19	200±28	0.62

in haemopexin-mediated haem transport does not require protein synthesis *de novo* (results not shown), since the increase in transport capacity is not diminished by treating Hepa cells with concentrations of cycloheximide known to inhibit protein synthesis in these cells (Ledford & Jacobs, 1985).

Links between haemopexin-mediated haem transport, cell growth and iron metabolism

Since hydroxyurea is an inhibitor of ribonucleotide reductase (Atkin *et al.*, 1973), an iron-requiring enzyme obligatory for DNA synthesis associated with cell growth (Moore, 1969), the effects of this agent on haemopexin-mediated haem transport were examined. At doses up to 1 μM hydroxyurea had only minimal effects on cell growth, but significantly increased haemopexin-mediated haem transport (Table 4). When cell growth was inhibited at higher levels of hydroxyurea cell protein levels were 89±15% of controls in two separate experiments and cell viability was not altered. Nevertheless, haemopexin-mediated haem transport remained twice that of control cells whether expressed per mg of protein or per 10^6 cells. These results reinforce the concept that haem transport and metabolism are linked to cell growth and raise the possibility that one iron pool involved in the regulation of expression of haemopexin-mediated haem transport may be ribonucleotide reductase.

In conclusion, Hepa cells express all the components of haemopexin-mediated haem transport and are a useful model for studying the mechanism of action and regulation of this system. One response of liver cells to severe iron deprivation is an attempt to salvage both iron and haem iron for survival. While it is known that a significant amount of haem delivered to the liver by haemopexin is degraded and the haem iron stored on ferritin (Davies *et al.*, 1979), the haem or iron released from it potentially has several different intracellular fates (Fig. 1). Cell growth can be maintained with haem–haemopexin as the sole source of iron, which strongly supports the concept that some haem iron enters the

same pool as that derived from transferrin (Fig. 1). Furthermore, at least one enzyme intimately involved in cell growth, namely the iron-requiring ribonucleotide reductase, influences regulation of the haemopexin-transport system. Conditions such as dietary or pathological iron status, infection or inflammation are known to influence the intracellular distribution and regulatory effects of iron. It is likely, because of links between haem and iron metabolism demonstrated here and elsewhere, that metabolism of haem and haemopexin and haemopexin-mediated haem transport will also change in response to conditions known to affect iron metabolism. For example, elevated temperatures, reflecting the fever found in certain inflammatory responses, induce haem oxygenase (Muller *et al.*, 1987), but decrease haemopexin synthesis in a rat cell line (McCraken, 1984) and in the Hepa cells used here (results not shown). Enhanced haemopexin-mediated haem transport may supply iron during conditions of induction of DNA synthesis, e.g., in liver regeneration, when ribonucleotide reductase activity is increased. Importantly, cell processes normally considered to be regulated by the iron status of the cell, including cell growth, are also likely to be influenced by factors affecting intracellular haem and haem-protein metabolism.

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