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The murine haemopexin receptor

Evidence that the haemopexin-binding site resides on a 20 kDa subunit and that receptor recycling is regulated by protein kinase C

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Haemopexin receptors from mouse hepatoma (Hepa) cells were affinity-labelled by cross-linking to haem-^{125}I-haemopexin complexes using two homo-[disuccinimidyl suberate (DSS) and 3,3′-dithiobis(succinimidyl propionate) (DTSSP)] and one hetero-[sulphosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (sulpho-SMPB)] bifunctional cross-linking agents. Analysis of the cross-linked products by SDS/PAGE in the absence of reducing agents revealed that ^{125}I-haemopexin was cross-linked specifically to a protein of apparent molecular mass 85–90 kDa. Upon reduction, haemopexin remained cross-linked to a protein of 20 kDa, suggesting that the murine haemopexin receptor has a subunit structure. Two subunits were identified: α (p65) and β (p20). Furthermore, because haemopexin was cross-linked by all three agents to p20, the shortest cross-linker arm being 1.1 nm (11 Å), we propose that the haem–haemopexin-binding site resides on this subunit. In addition, a cysteine residue of p20 is located near the haemopexin-binding site, since haemopexin, which has no free thiol groups, is cross-linked to this subunit by the hetero-bifunctional agent sulpho-SMPB. Exposure of Hepa cells to the tumour-promoting phorbol ester 4α-phorbol 12-myristate 13-acetate (PMA) causes a rapid redistribution of haemopexin receptors from the cell surface to the cell interior. Within 2–4 min of incubation with 100 nM-PMA, there was an approx. 50% decrease in cell-surface haemopexin receptors, as judged by ligand binding at 0 °C and affinity labelling of the receptor. This time- and dose-dependent down-regulation was fully reversible within 60–90 min after removal of PMA, and the affinity of the remaining receptors was unaltered by PMA. The specificity of PMA was demonstrated by comparison with the non-tumour-promoter 4α-phorbol, which did not affect any of the parameters examined. The amine H-7, a specific inhibitor of protein kinase C, antagonised the receptor redistribution effect of PMA, suggesting that the down-regulation of haemopexin receptors on the cell surface was a consequence of protein kinase C activation. The PMA-induced decrease in surface haemopexin receptors was due to a 2-fold increase in the rate of internalization (from 0.73 min^{-1} to 1.32 min^{-1}), whereas the rate of exocytosis (0.6 min^{-1}) was unchanged. PMA treatment, like binding of the natural ligand, haem–haemopexin, results in a lower steady-state level of surface haemopexin receptors independent of receptor synthesis, and the receptors were not degraded but were recycled back to the cell surface.

INTRODUCTION

The plasma transport proteins haemopexin [1] and transferrin [2] function as extracellular antioxidants as part of their role in the body's defence mechanisms. By binding extracellular haem and iron respectively these proteins inhibit haem- [1] or iron-mediated [2,3] lipid peroxidation, which destroys cellular components. Such oxidation-mediated damage to tissues is exploited by monocyte/macrophages for bacterial killing [4], but metal-catalysed radical-mediated cell damage is also implicated in the pathology of several human diseases and during organ reperfusion after surgery, tissue trauma or ischaemia (for a recent review see [5]).

Haemopexin transports haem to cells, including liver, by a process recently shown to involve receptor-mediated endocytosis of haemopexin [6,7], with recycling of intact protein [7] (for a recent review see [8]). The endocytosis of transferrin has been elegantly defined in many cell types during the past 10 years [9], and several parallels exist between the haemopexin and transferrin systems [7,8]. Using a combination of morphological and biochemical techniques, co-localization of haemopexin and transferrin in the intracellular organelles of the classical pathway of endocytosis has been demonstrated [6,7]. Thus haemopexin and transferrin represent a distinct class of endocytotic transport systems in which the ligand-binding plasma protein is normally not degraded, but recycles and undergoes further rounds of transport. However, structural information on the haemopexin receptor itself is lacking, and this membrane protein is not yet well characterized. We have reported that the receptor from rabbit liver plasma is closely associated with an integral membrane haem-binding protein (MHBP) [10], which plays a pivotal role in intracellular haem transport (A. Smith, unpublished work; [8]). MHBP appears to be oligomeric in structure, and the smallest haem-binding subunit has a molecular mass of 17.5 kDa

Abbreviations used: DSS, disuccinimidyl suberate; DTSSP, 3,3′-dithiobis(succinimidyl propionate); DTT, dithiothreitol; sulpho-SMPB, sulphosuccinimidyl 4-(*p*-maleimidophenyl)butyrate; PKC, protein kinase C; PMA, 4α-phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; EGF, epidermal growth factor; MHBP, membrane haem-binding protein.

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[10]. The pig hepatic haemopexin receptor, isolated by affinity chromatography on haemopexin–haemin–Sepharose [11], chromatographed with an apparent molecular mass of 80 kDa on gel filtration and was composed of a 71 kDa protein and an associated 16 kDa haem-binding protein [11]. The human placental haemopexin receptor was isolated by affinity chromatography using apo-haemopexin–Sepharose [12] and reported to be an 80 kDa single-chain polypeptide [12,13].

Regulation of receptor internalization and recycling is a fundamental question in receptor-mediated endocytosis. However, the signal(s) required for internalization and recycling of receptors or receptor–ligand complexes remain controversial. In a number of transport systems, phosphorylation of the receptor molecule accompanies its internalization. Some receptors, including those for insulin [14] and epidermal growth factor (EGF) [15], contain intrinsic kinase activity which is activated upon ligand binding. Other receptors, in particular the transferrin receptor, are phosphorylated after protein kinase C (PKC) activation by exposure of cells to tumour-promoting phorbol esters [16–18] or to synthetic analogues of diacylglycerol [19]. Incubation of human promyelocytic HL-60 [16,17] or erythroleukaemic K562 [18] cells with 4α-phorbol 12-myristate 13-acetate (PMA), a tumour-promoting phorbol ester which activates PKC [20], results in hyperphosphorylation of the transferrin receptor. Moreover, dephosphorylation precedes exocytosis in HL-60 cells [21]. However, mutant transferrin receptors, lacking the PKC phosphorylation site Ser-24, do undergo endocytosis [22]. Nevertheless, the overall expression of cell surface transferrin receptors is rapidly decreased by PMA in several cell types. However, although this always results from increases in the rate of internalization, the rate of exocytosis may or may not be affected, depending upon cell type. Several groups have suggested that the effect of PMA on the transferrin receptor mimics a naturally occurring mechanism controlling transferrin receptor endocytosis and receptor recycling [16,17,22], since similar changes in receptor redistribution between the cell surface and intracellular pools occur when the natural ligand binds to the receptor.

Here, the results of cross-linking haem–[^{125}I]-haemopexin to its receptor in mouse hepatoma cells and treatment with PMA are presented. These data further characterize the subunit structure of this receptor and provide evidence for the localization of the haemopexin-binding site. In addition, the effects of ligand binding and of PMA treatment on the rates of endocytosis and exocytosis of haemopexin are described. A preliminary report of these results has been presented [23].

MATERIALS AND METHODS

Materials

Apo-haemopexin was purified from rabbit serum (Pelfreeze, Rogers, AK, U.S.A.) using published procedures [24]. Iodination of apo-haemopexin was carried out using Iodobeads supplied by Pierce Chemical Co. (Rockford, IL, U.S.A.), according to the manufacturers' instructions. Haem–haemopexin complexes were made from mesohaem (iron–mesoporphyrin) purchased from Porphyrin Products (Logan, UT, U.S.A.), since this haem analogue is more stable than protohaem, and mesohaem–haemopexin complexes have been shown to be chemically and biologically equivalent to protohaem–haemopexin [25–27]. Pronase (5000 units/mg of protein) was purchased from Calbiochem (La Jolla, CA, U.S.A.). The cross-linking reagents 3,3′-dithiobis(sulphosuccinimidyl propionate) (DTSSP), disuccinimidyl suberate (DSS) and sulphosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (sulpho-SMPB) were obtained from Pierce, and phenylmethanesulphonyl fluoride (PMSF), leupeptin and pepstatin A were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). The PKC inhibitor H-7 was purchased from Seikagaku Koyoso (Koyoto, Japan). PMA and 4α-phorbol were purchased from Sigma (St. Louis, MO, U.S.A.). Fresh stock solutions (1 mM) of H-7 and phorbol ester were made in water or dimethyl sulphoxide respectively and kept in the dark.

Cells and cell culture

Minimal deviation mouse hepatoma cells from the solid tumour line BW 7756 (Hepa) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.35% (w/v) glucose and 2% calf serum in humidified air/CO_2 (19:1) as previously described [28]. Cells were fed every 2–3 days and were always in the logarithmic phase of growth for experimental studies.

Haem–^{125}I-haemopexin binding mouse to Hepa cells

Total specific binding of haem–haemopexin was measured using published procedures [28]. If the cells [$(1.0–1.2) \times 10^6$ cells/well] were to be subsequently incubated with reagents such as PMA, they were first washed free of culture medium by rinsing with Hepes-buffered DMEM (pH 7.2) at 37 °C. The various incubation times are indicated in the Figure legends. After washing at either 4 or 37 °C, 1 ml of ice-cold binding buffer (25 mM-Hepes, pH 7.2, with 0.2% ovalbumin in Hanks balanced salt solution) containing 70 nM-haem–^{125}I-haemopexin [specific radioactivity $(7–8) \times 10^{-4}$ d.p.m./pmol of haemopexin] was added and the incubation was continued for 3 h at 0 °C. After aspiration of binding buffer, the cells were washed three times with ice-cold phosphate-buffered saline (PBS, 10 mM-sodium phosphate buffer, pH 7.4, containing 145 mM-NaCl) and the bound haemopexin was measured as previously described [28]. The specific binding was determined in the presence and absence of a 100-fold excess of unlabelled mesohaem–haemopexin complex to give 0.73 ± 0.22 pmol of haemopexin/10^6 cells or 0.91 ± 0.11 pmol/mg of cellular protein, constituted 60–80% of total binding. The experiments to determine the effect of phorbol esters on receptor regulation involved incubation of the cells with the tumour-promoting phorbol ester PMA, generally at 100 nM for 15 min at 37 °C. An equal concentration of the non-tumour-promoter 4α-phorbol was used as a negative control. Additional details of specific experimental protocols are given in the Figure legends.

Measurement of haemopexin receptor distribution in mouse Hepa cells

The number of cell surface and internalized haemopexin receptors was investigated by measuring the distribution of receptor-bound haem–^{125}I-haemopexin on the cell surface and intracellular compartments using protocols described for the transferrin receptor in A431 epidermoid cells [29]. Hepa cells were incubated in binding buffer with haem–^{125}I-haemopexin (70 nM) at 0 °C for 3 h and subsequently washed three times with ice-cold serum-free DMEM at 0 °C. Subsequent incubations were carried out in binding buffer (previously warmed to 37 °C) in the presence or absence of PMA at 37 °C. At the indicated times one set of cells was washed and lysed in 0.1 M-NaOH for the determination of total binding, while the other set was incubated with Pronase (0.25%) in serum-free DMEM for 15 min at 0 °C. The proteolytic activity of Pronase was quenched by the addition to each well of 100 μl of fetal calf serum. Cells were scraped and transferred to Microfuge tubes, washed three times in ice-cold PBS and lysed in 0.1 M-NaOH, and cell-

associated radioactivity was determined. This Pronase treatment releases 90–95% of the externally bound haem–haemopexin.

Measurement of apo-haemopexin release from the cells

The rate of release of apo-^{125}I-haemopexin from Hepa cells was measured by the pulse–chase technique described for the transferrin receptor [29]. Hepa cells were washed with Hepes-buffered DMEM at 37 °C and then incubated in 1 ml of binding buffer containing 150 nM-haem–^{125}I-haemopexin for 2 h at 37 °C. The cells were washed briefly followed by incubation at 37 °C in fresh binding buffer containing 1 μM unlabelled haem–haemopexin complex in the presence or absence of 100 nM-PMA. At defined times the medium was aspirated, and cells were washed with 3 × 2 ml of ice-cold PBS and harvested for determination of cell-associated radioactivity as described above.

Affinity labelling of the haemopexin receptor by cross-linking haem–^{125}I-haemopexin with the homo-bifunctional agents DTSSP or DSS and with the hetero-bifunctional agent sulpho-SMPB

The Hepa cells (1.2×10^6 cells/well) were precooled on ice and then washed twice with ice-cold DMEM. Haem–^{125}I-haemopexin (70 nM, 1.3×10^5 d.p.m./pmol of haemopexin) in 1 ml of binding buffer was added to the cells and incubation was continued at 0 °C for 2 h. Cells were then washed free of unbound ligand by rinsing with ice-cold PBS before the cross-linking agent (DTSSP or DSS), diluted to the required concentration (0.05–3 mM) in 2 ml of PBS, was added to the cells and incubated for a further 30 min at 4 °C. The cross-linking reaction was terminated by the addition of 20 μl of 2 M-Tris/HCl buffer, pH 8.0. The cells were then washed with cold PBS and dissolved by adding 200 μl of lysis buffer (2 M-urea, 1.5% Triton X-100 and 1 mM-PMSF in 10 mM-Tris/HCl, pH 7.4, containing 0.1 mM-leupeptin and 1 μg of pepstatin A/ml). The cell lysate was centrifuged at 10000 *g* for 5 min at 4 °C and the supernatants were analysed by electrophoresis on SDS/PAGE (7.5% gels). After electrophoresis gels were dried and exposed to X-Omat film (Kodak) at −70 °C using an image intensifying screen (Dupont Cornex Lightning Plus).

To determine whether PMA decreased the amount of the haemopexin receptor detected by cross-linking with DTSSP, the cells were first incubated with Hepes-buffered DMEM containing various concentrations of either PMA alone or PMA plus H-7 for 15 min at 37 °C. In the latter case, cells were incubated with the PKC inhibitor H-7 for 10 min at 37 °C before the addition of PMA. This treatment was followed by washing the cells with ice-cold DMEM, incubation with radiolabelled haemopexin and finally addition of cross-linker as described above. For data presentation all experiments were repeated two or three times under the same conditions, generally with triplicate samples for each data point. The results shown are the means ± S.E.M. unless otherwise stated.

RESULTS

Chemical cross-linking analysis of the haemopexin receptor

Haem–^{125}I-haemopexin complex binding to the haemopexin receptor was monitored by cross-linking analysis using two homo-bifunctional and one hetero-bifunctional agent. The first two, DSS and DTSSP, form covalent links between $-NH_2$ groups and have linker lengths of 11.4 and 12.0 Å (1.14 and 1.20 nm) respectively. DSS is a non-thiol-cleavable agent, and DTSSP is a water-soluble membrane-impermeable thiol-cleavable agent. The third cross-linker used, sulpho-SMBP, is a hydrophilic membrane-impermeable agent which reacts with both a primary amine and a thiol and has an extended linker arm of 14.5 Å (1.45 nm) that limits steric hindrance. Importantly, haemopexin contains no free —SH groups [24]; thus the formation of haemopexin homo-dimers is impossible with sulpho-SMPB. In the first series of experiments, mouse Hepa cells were incubated with haem–^{125}I-haemopexin in the presence of various concentrations (0.05–2.5 mM) of the cross-linkers DSS and DTSSP. Analysis of the cell extract by electrophoresis and autoradiography revealed the presence of an iodinated band migrating under non-reducing conditions with an apparent molecular mass of 145–150 kDa from cells incubated with either DSS (Fig. 1*a*, lane 1) or DTSSP (Fig. 1*c*, lane 1; Fig. 1*d*, lanes 2 and 3). No radioactive material was found in this region of the gel when the cross-linker was omitted (for DSS see Fig. 1*a*, lane 2, and for experiments with DTSSP see Fig. 1*d*, lane 1), or when dithiothreithol (DTT) was added to the DTSSP cross-linked cell extract (Fig. 1*c*, lane 2). The specificity of the affinity labelling was further demonstrated by the loss of radioactive cross-linked product when the cells were incubated in the presence of 7 μM non-radiolabelled haem–haemopexin as a competitive inhibitor of the radiolabelled ligand (Fig. 1*d*, lane 4). Interestingly, reduction of the cell extracts with DTT yielded a radioactive DSS-cross-linked product of 80 kDa, which migrated as a sharp well-defined band (Fig. 1*a*, lane 3 and Fig. 1*b*). Formation of this radioactive complex was specifically inhibited by excess unlabelled haem–haemopexin (compare Fig. 1*a*, lane 4 and Fig. 1*b*, lane 3). Under these electrophoretic conditions haemopexin reduced with DTT migrates with a molecular mass of 60 kDa. Therefore these results indicate that the haemopexin receptor in mouse Hepa cells consists of a 85–90 kDa protein composed of 65–70 and 20 kDa subunits.

Evidence that the haemopexin-binding site resides on the 20 kDa subunit

The results from a second series of experiments using several concentrations of the heterologous cross-linker sulpho-SMPB (0.1–2.5 mM) are shown in Fig. 2. As with DSS and DTSSP, under non-reducing conditions the cross-linked product had a molecular mass of 140–145 kDa (Fig. 2, lanes 2 and 3) which was also specifically affinity-labelled (Fig. 2, lane 4). Upon reduction with DTT the radioactive cross-linked material now migrated as a sharp band of 85 kDa (Fig. 2, lanes 5 and 7) which was also specifically labelled (Fig. 2, lanes 6 and 9). Thus these experiments confirmed the presence of two disulphide-linked subunits, and furthermore demonstrated that haemopexin can be cross-linked to the small subunit of the receptor via a thiol group on the latter, since sulpho-SMPB contains both a primary amine-reactive and thiol-reactive group. In addition, this cross-linker yielded a higher molecular mass band (~ 240 kDa), suggesting that the receptor may exist as a dimer (Fig. 2, lane 2). However, this was not regularly observed.

Effect of the phorbol ester PMA on the number of cell-surface haemopexin receptors

Incubation of Hepa cells with up to 200 nM-PMA for 30 min at 37 °C resulted in a time- and dose-dependent loss of cell-surface haemopexin receptors, i.e. a down-regulation of unoccupied receptors, as measured by the decreased specific binding of haem–^{125}I-haemopexin (Figs. 3*a* and 3*b*). This effect of PMA was rapid, with haemopexin binding decreasing by 45–50% within 2–4 min of exposure to 100 nM-PMA (Fig. 3*a*). In addition, receptor down-regulation was maintained for at least 30 min at 37 °C. In contrast with the effect of PMA, treatment of Hepa cells with 4α-phorbol did not alter the number of haemopexin

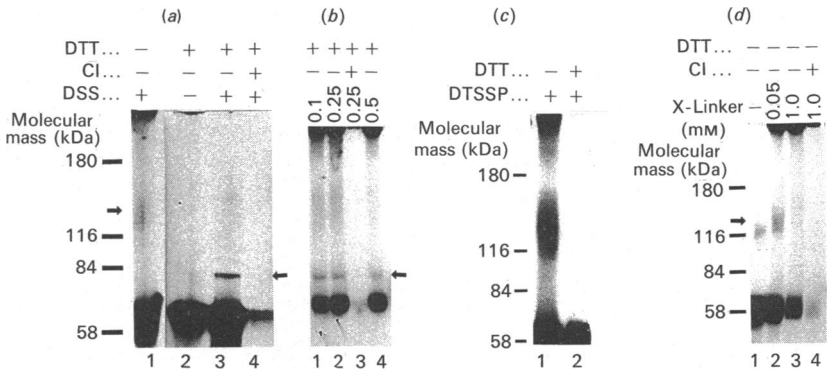

Fig. 1. Affinity cross-linking of the haemopexin receptor with haem–^{125}I-haemopexin using DSS and DTSSP

After washing to remove culture medium, Hepa cells were incubated with haem–^{125}I-haemopexin (70 nM, 1.2×10^5 d.p.m./pmol of haemopexin) followed by cross-linking with DSS (*a* and *b*) or with DTSSP (*c* and *d*) using the experimental protocols described in the Materials and methods section. After detergent extraction of cells, the cross-linked product was analysed by electrophoresis on a 7.5% acrylamide gel under reducing and non-reducing conditions and the radioactive haemopexin was detected by autoradiography. Molecular mass markers (reduced with DTT) were electrophoresed simultaneously on the same gel and their positions are indicated. The positions of radioactive haemopexin–receptor complexes are indicated by arrows. (*a*) Results of the cross-linking experiments using the non-thiol-cleavable agent DSS at 0.1 mM. Electrophoresis under non-reducing conditions (lane 1) and under reducing conditions (lane 3) is shown. In lane 2 the cross-linker was omitted. In lane 4 unlabelled haem–haemopexin was added as competitive inhibitor (CI) to the cell incubation medium before the cross-linker. (*b*) Results of varying the concentration of DSS from 0.1–0.5 mM; in lane 3, unlabelled complex was added before the cross-linker. Some cell lysis occurred at 0.5 mM-DSS, resulting in decreased recovery of radioactive cross-linked material. (*c*) and (*d*) Results of a series of experiments using the thiol-cleavable cross-linker DTSSP. Electrophoresis under non-reducing conditions (*c*, lane 1 and *d*, lane 2) and reducing conditions (*c*, lane 2) is shown. Occasionally, a very small amount of haemopexin was observed even in the absence of cross-linking agents under non-reducing conditions, migrating with a molecular mass of 120 kDa, just below the cross-linked material (*d*, lane 1). Unlabelled haem–haemopexin was added to the cell incubation medium before addition of cross-linker (*d*, lane 4). Under these electrophoretic conditions, haemopexin migrates with molecular mass of 60 kDa under non-reducing conditions and with a slightly higher molecular mass upon reduction, since it contains three disulphide bonds.

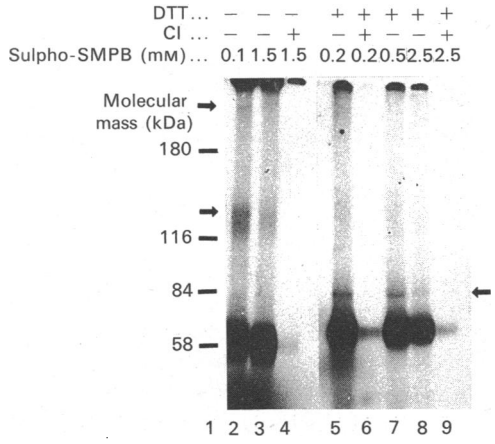

Fig. 2. Evidence for subunit structure of the haemopexin receptor using the heterologous cross-linking agent sulpho-SMPB

After washing to remove culture medium, Hepa cells were incubated with haem–^{125}I-haemopexin (70 nM, 1.2×10^5 d.p.m./pmol of haemopexin) followed by cross-linking with several concentrations of sulpho-SMPB (0.1–2.5 mM) using the experimental conditions and protocols described in the legend to Fig. 1 and in the Materials and methods section. Electrophoresis under non-reducing conditions (lanes 2–4) and under reducing conditions (lanes 5–9) are shown. In lanes 4, 6 and 9, unlabelled haem–haemopexin was added as competitive inhibitor (CI) of the radiolabelled ligand to the cell incubation medium before the sulpho-SMPB. Somewhat decreased recovery of radioactive cross-linked material was observed at the higher concentrations of sulpho-SMPB. The positions of radioactive haemopexin–receptor complexes are indicated by arrows.

receptors on the cell surface (Fig. 3*a*). There was no detectable change in the affinity of the haemopexin receptor for its ligand after these treatments (results not shown).

Reversibility of the PMA-induced down-regulation of the haemopexin receptor and evidence for recycling of haemopexin receptors

Hepa cells were first incubated in DMEM containing cycloheximide (at 10 μmg/ml) for 2 h at 37 °C, followed by a 10 min incubation at 37 °C in DMEM containing either PMA or 4α-phorbol. The cells were then washed free of these reagents and recovery of cell-surface receptors was monitored upon further incubation at 37 °C and by the specific binding of haem–^{125}I-haemopexin at 0 °C. Control levels of haemopexin receptors were measured in cells incubated for the same period of time in DMEM alone or exposed to 4α-phorbol. Within 30 min of removal of PMA from the incubation medium, nearly 80% of the haem–^{125}I-haemopexin surface binding had returned (Fig. 3*c*), and complete recovery to control levels took place within 60–90 min. This replenishment was not due to newly synthesized receptors, since the cells were exposed throughout to concentrations of cycloheximide known to inhibit protein synthesis in these cells [27]. If the down-regulation of haemopexin receptors is due to an irreversible internalization, then the number of haem–^{125}I-haemopexin-binding sites would continue to decline upon continued incubation with PMA. Clearly this is not the case, as the number of surface haemopexin receptors declined to 50% of those originally present regardless of the length of incubation with PMA (Figs. 3*a* and 3*b*). Thus PMA treatment appears to cause a new steady-state level of surface haemopexin receptors, and after PMA-induced down-regulation the haemopexin receptors were not degraded but were recycled back to the cell surface.

Inhibition of PKC antagonizes the PMA-induced down-regulation of haemopexin receptors

Hepa cells were incubated with increasing amounts of the amine H-7 [30], a specific inhibitor of PKC, before treatment

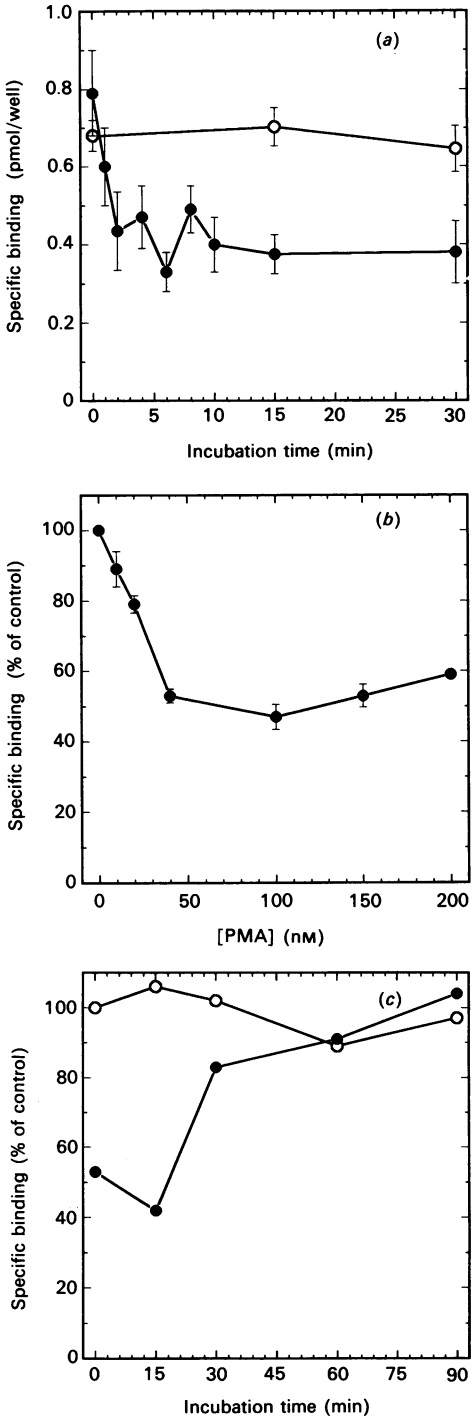

Fig. 3. Effect of PMA on surface haemopexin receptors of mouse Hepa cells

(*a*) Time course of PMA-induced down-regulation of haemopexin receptors. Mouse Hepa cells (1.2×10^6 cells/well) were washed and incubated in Hepes-buffered DMEM containing either 100 nM-PMA (●) or 100 nM-4α-phorbol (○) for up to 30 min at 37 °C. After the indicated time periods the cells were washed with ice-cold DMEM and specific surface binding of haem–^{125}I-haemopexin was measured at 0 °C for 3 h as described in the Materials and methods section. (*b*) Concentration-dependence of PMA-induced down-regulation of haem–^{125}I-haemopexin binding. Hepa cells were incubated with up to 150 nM-PMA at 37 °C for 10 min, and specifically bound haem–^{125}-haemopexin was measured as described in the Materials and methods section. (*c*) Reversibility of the phorbol-ester-mediated decrease in haem–^{125}I-haemopexin binding. Hepa cells were first incubated in the presence of either 100 nM-PMA (●) or 100 nM-4α-phorbol (○) at 37 °C for 10 min. The cells were then washed twice with DMEM followed by incubation in binding buffer at 37 °C. At the indicated times, the specific binding of haem–^{125}I-haemopexin (70 nM) was measured as described.

Table 1. Effect of the PKC inhibitor H-7 on the PMA-induced down-regulation of haemopexin receptors

Hepa cells were incubated in the presence or absence of 100 nM-PMA at 37 °C for 10 min before rinsing with ice-cold DMEM and determination of the specific binding of haem–haemopexin as described in the Materials and methods section. In experiments with H-7, the inhibitor was added to the cells 15 min before PMA and incubation with both agents was continued for 10 min, then the number of surface haemopexin receptors was determined using the standard binding assay. The results are presented as specific binding (pmol/mg of protein), means ± S.E.M. The numbers in parentheses indicate the numbers of experiments, not replicates.

Agent	Specific binding (pmol/mg of protein)
None	0.75 ± 0.05 (n = 4)
4α-Phorbol	0.70 ± 0.07 (n = 8)
PMA	0.37 ± 0.06 (n = 7)
PMA + 50 μM-H-7	0.55 ± 0.07 (n = 8)
PMA + 100 μM-H-7	0.67 ± 0.20 (n = 4)
PMA + 150 μM-H-7	0.70 ± 0.15 (n = 6)

with 100 nM-PMA or 4α-phorbol. The cells were then washed and the number of cell-surface haemopexin receptors was quantified. The PMA-induced down-regulation of haemopexin receptors was significantly inhibited in a dose-dependent manner by the simultaneous presence of H-7 (Table 1), and complete protection was afforded by 100–150 μM-H-7, consistent with the relative affinities of these agents for PKC [31,32]. Therefore the down-regulation of haemopexin receptors by PMA treatment of cells results from an activation of PKC, but whether the haemopexin receptor itself is phosphorylated is not yet known.

Haem–haemopexin down-regulates the expression of surface haemopexin receptors

To compare the down-regulation of surface haemopexin receptors by PMA and by the natural ligand, haem–haemopexin, mouse Hepa cells were incubated with up to 5 μM haem–haemopexin for 30 min at 37 °C, and the number of cell-surface receptors was assessed using the standard specific binding assay. Incubation of cells with 0.5–5.0 μM-haem–haemopexin produced a decrease of 50–55 % in the surface haemopexin receptors (Fig. 4). In these experiments the ligand concentrations used were of sufficient magnitude to ensure full occupancy of the surface receptors by ligand (K_d for the haem–haemopexin–receptor interaction is 17 nM [28]) and to avoid receptor occupancy becoming rate-limiting for receptor internalization.

PMA treatment increases endocytosis but not exocytosis of the haemopexin receptor

The rates of endocytosis and of exocytosis of the haemopexin receptor were determined by measuring the internalization of haem–^{125}I-haemopexin and release of apo-^{125}I-haemopexin from control and PMA-treated cells, as described for the transferrin receptor (see below). Since the rapid decline in surface haemopexin receptors caused by PMA is not due to an irreversible internalization, the decrease in cell-surface receptors may reflect changes in the recycling kinetics of the receptor, i.e. in the rate of internalization or of exocytosis. The endocytotic rate constant of

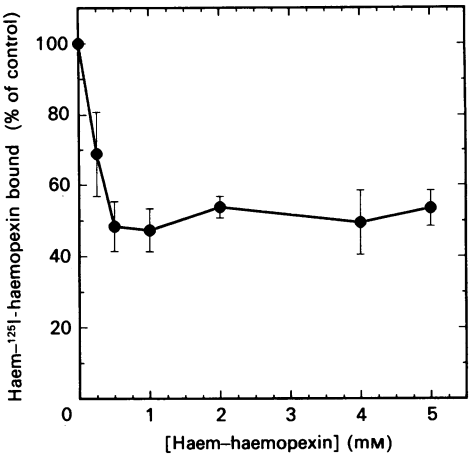

Fig. 4. Effect of binding of the natural ligand, haem–haemopexin, on the number of surface haemopexin receptors

Mouse Hepa cells (1.2×10^6 cells/well) were incubated with 0–5.0 μM-haem–haemopexin in binding buffer for 30 min at 37 °C. After this incubation the cells were washed thoroughly with ice-cold DMEM to dissociate any remaining surface-bound haemopexin, and receptor expression was determined by measuring the specific binding of haem-^{125}I-haemopexin at 4 °C.

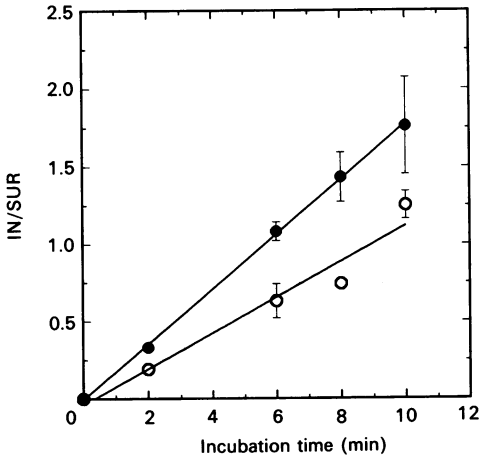

Fig. 5. Effect of PMA on the rate of haemopexin receptor endocytosis

Cells, incubated in the presence (●) or absence (○) of 100 nM-PMA for 15 min at 37 °C, were then incubated at 37 °C with a saturating amount of haem-^{125}I-haemopexin (150 nM) before the distribution of haem-^{125}I-haemopexin on the cell surface and intracellular compartments was assessed by Pronase sensitivity to distinguish surface-bound from internal ligand as described in the Materials and methods section. The internalization of haem-^{125}I-haemopexin is shown using an IN/SUR (intracellular/surface) analysis [33], and the endocytotic rate constants were calculated from the initial slopes.

the haemopexin receptor in control and PMA-treated cells was determined under steady-state conditions as described by Wiley & Cunningham [33] for the epidermal growth factor (EGF) receptor. Measurements of the rate of haem-^{125}I-haemopexin internalization were made in the presence of saturating amounts of haem-^{125}I-haemopexin to ensure complete occupancy of surface receptors and were limited to the first 2–6 min at 37 °C, since other experiments have established that there is minimal release of endocytosed haemopexin (i.e. exocytosis) during this time period [7]. Intracellular accumulation of ligand is

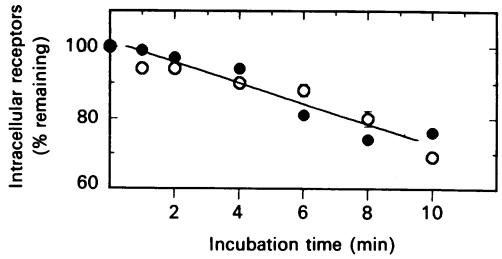

Fig. 6. Effect of PMA on the rate of haemopexin receptor exocytosis

After incubating cells in binding buffer containing a saturating concentration (150 nM) of haem-^{125}I-haemopexin for 2 h at 37 °C to drive the endocytosis of haemopexin, the cells were washed free of unbound ligand and incubated in the presence (●) or absence (○) of 100 nM-PMA in binding buffer containing 1 μM unlabelled haem–haemopexin for up to 15 min at 37 °C. At the indicated times the amount of intracellular haem-^{125}I-haemopexin, representing the population of receptors remaining within the cell, was determined. The rate of haemopexin receptor exocytosis was estimated by measuring the decline in endocytosed haemopexin.

demonstrated by the linear increase with time for the first 10 min of the IN/SUR (internal/surface) ratio described by Wiley & Cunningham ([33]; see Fig. 5). After 10 min, the rate of increase in the IN/SUR ratio decreases somewhat, presumably due to the release of accumulated haemopexin as apo-^{125}I-haemopexin. The first-order endocytotic rate constant was calculated to be 0.73 ± 0.31 and 1.32 ± 0.30 min^{-1} (means ± S.D.) in control and PMA-treated cells respectively.

The down-regulation of the haemopexin receptors observed in PMA-treated cells could also be affected by a decrease in the rate of receptor exocytosis, thus slowing down the overall recycling time of the receptor, as shown for the transferrin receptor in human promyelocytic HL-60 cells [16]. The rate of exocytosis of the haemopexin receptor was investigated by incubating cells with haem-^{125}I-haemopexin and measuring the release of apo-^{125}I-haemopexin from cells in the presence and absence of PMA as described by Davis *et al.* [29] for transferrin receptor. The amount of cell-associated ^{125}I-haemopexin radioactivity was the same in both PMA-treated and control cells, suggesting that PMA did not affect the rate of release of apo-^{125}I-haemopexin (Fig. 6). Assuming that exocytosis of haemopexin occurs from a single intracellular pool of haemopexin receptors, the first-order rate constant for exocytosis of the haemopexin receptor is 0.60 min^{-1}. During active recycling, the half-life of the haemopexin receptors on the cell surface would be 2–3 min (calculated as ln 2/rate of endocytosis [34]).

Redistribution of haemopexin receptors by haem–haemopexin

Since PMA caused a redistribution of haemopexin receptors from the cell surface to an internal pool, we next addressed whether haem–haemopexin similarly affected receptor distribution and whether PMA altered ligand-induced receptor distribution. Control and PMA-treated (for 15 min at 37 °C) Hepa cells were rapidly cooled and incubated at 4 °C with radiolabelled haemopexin to label surface receptors, followed by rapid warming to 37 °C without removing excess ligand. Surface binding was determined as a function of time, in parallel sets of cells, by Pronase digestion. Haem–haemopexin caused a ~ 25% decrease in surface receptors within 10–12 min in control cells (Fig. 7*b*), with a concomitant increase in internal ligand with time (Fig. 7*c*). This is also consistent with a rapid redistribution of receptors between surface and intracellular locations.

In spite of a 2-fold difference in surface receptors after the initial incubation with PMA (Fig. 7*b*), by 15 min of subsequent

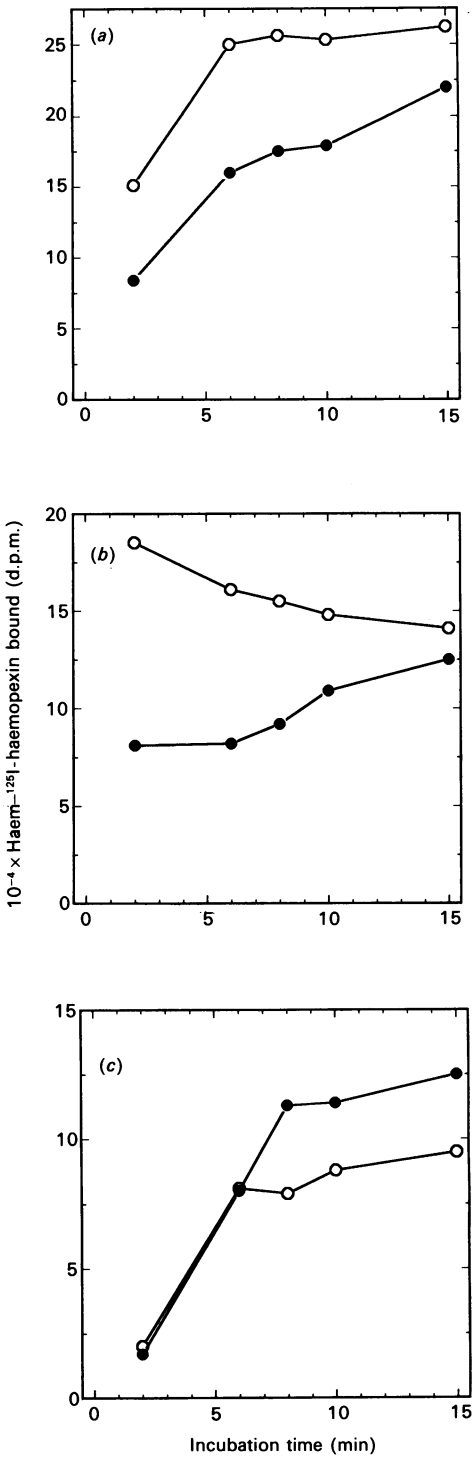

Fig. 7. Effect of PMA and of haem–haemopexin on the redistribution of the haemopexin receptor

Hepa cells were incubated in the presence (●) or absence (○) of 100 nM-PMA for 15 min at 37 °C, rinsed with ice-cold DMEM and then incubated with 70 nM-haem-^{125}I-haemopexin in the presence or absence of non-radiolabelled haem–haemopexin for 2 h at 0 °C. The cells were rapidly warmed to 37 °C in binding buffer containing 7 μM non-radioactive haem–haemopexin, and at the indicated times in parallel sets of cells, the total cell-associated (*a*) and Pronase-sensitive (representing surface-bound ligand, *b*) haem-^{125}I-haemopexin was determined. In (*c*), the Pronase-resistant counts, calculated from the difference between the total and Pronase-sensitive radioactivity and representing internalized ligand, are shown. The results presented are the means of 4–6 observations from a representative experiment, and similar results were obtained in two other experiments.

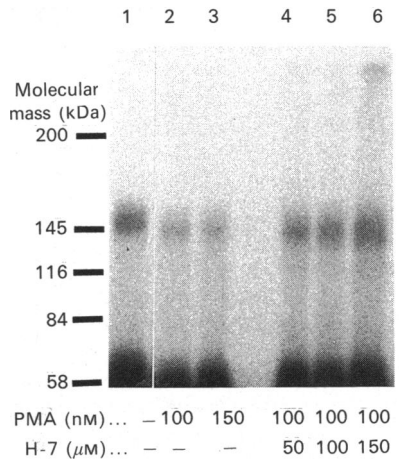

Fig. 8. Affinity cross-linking of surface haemopexin receptors after PMA treatment

Mouse Hepa cells (1.2×10^6 cells/well) were incubated with 0, 100 or 150 nM-PMA (lanes 1–3 respectively) or with 100 nM-PMA and H-7 (50–150 μM, lanes 4–6) for 10 min at 37 °C. The cells were cooled by washing with ice-cold DMEM and incubated with 70 nM-haem-^{125}I-haemopexin for 2 h at 0 °C to label the surface haemopexin receptors. After washing the cells free of unbound ligand, 1 mM-DSS was added to cross-link haemopexin to its receptor as described in the Materials and methods section. The cross-linked haem-^{125}I-haemopexin–receptor complexes were detected by autoradiography after electrophoresis of the total cell extract on a 10% (w/v) acrylamide gel.

incubation at 37 °C the total cell-associated haemopexin reached 85% of control values in PMA-treated cells (Fig. 7*a*). This suggests that most of the haemopexin receptors redistributed from the surface intracellularly by PMA continue to play a role in endocytosis of haemopexin and to recycle. In addition, there appears to be no difference in the number of receptors taking part in endocytosis, as judged by similarities in internal haemopexin levels between the control and PMA-treated cells during the first 8 min (Fig. 7*c*), and the internal pools of receptors seem to be equivalent (Fig. 7*c*). Thus no further redistribution of receptors takes place upon addition of haem–haemopexin to PMA-treated cells. This suggests that PMA treatment of Hepa cells results in the mobilization to intracellular sites of a population of surface haemopexin receptors which would normally be involved in transporting the natural ligand, haem–haemopexin. An increase in surface haemopexin receptors is apparent after 8 min of incubation in the absence of PMA. This may reflect changes in the phosphorylation state of the receptor, presumably induced by withdrawal of PMA even though ligand is present.

Cross-linking of surface haemopexin receptors of PMA-treated cells

To confirm and further evaluate the effect of PMA on surface expression of haemopexin receptors, haemopexin receptor–ligand complexes were identified on the cell surface by cross-linking haem-^{125}I-haemopexin by DTSSP as described above. Incubation of mouse Hepa cells with 100 or 150 nM-PMA for 10 min before addition of radioactive haem–haemopexin and DTSSP resulted in a significant decrease in the intensity of radiolabelled cross-linked product (Fig. 8, lanes 2 and 3) compared with the untreated control cell extract (Fig. 8, lane 1), consistent with the loss of surface haemopexin receptors detected by binding assays. Furthermore, addition of H-7 to the cells prevented the PMA-induced loss of cross-linked product in a dose-dependent manner (Fig. 8, lanes 4–6).

DISCUSSION

An initial analysis of the murine haemopexin receptor structure was facilitated by affinity cross-linking of haemopexin to the haemopexin receptor. In sum, these observations indicate that the murine haemopexin receptor is an 85–90 kDa protein, consists of two disulphide-linked peptides of 65 and 20 kDa, designated α and β subunits respectively, by convention with the usual nomenclature for receptors, and may exist as a disulphide-linked dimer. Haemopexin was cross-linked to the β-subunit, suggesting that at least part of the haemopexin-binding site is located on this subunit. Moreover, since haemopexin was cross-linked to this subunit by sulpho-SMPB (containing both a primary amine-reactive and a thiol-reactive group), and because haemopexin has no free thiol groups, a cysteine residue must be in the vicinity [within 10 Å (1 nm)] of the haemopexin-binding site on the β subunit (Fig. 9). This description of the receptor requires a stoichiometry of binding of 1 mol of haemopexin per receptor. A ~ 145 kDa radioactive species could result from a 20 kDa receptor molecule binding two molecules of haemopexin (because of the similarity in migration between haemopexin and the postulated 65 kDa subunit). However, the two-subunit model is supported by preliminary evidence from ligand-blotting experiments (results not shown), using detergent extracts of intact mouse Hepa cells and rabbit liver plasma membranes as sources of hepatic haemopexin receptor. Iodinated haemopexin is found to bind to a ~ 90 kDa protein as well as to a ~ 20 kDa protein.

In contrast with the evidence for haemopexin receptor subunits presented here, Taketani and co-workers reported the haemopexin receptor from human placenta [12] and HL-60 cells [13] to be an 80 kDa single polypeptide chain, and found no change in the molecular mass upon reduction of the putative receptor cross-linked to haemopexin by DSS in HL-60 cells [13]. However, the pig haemopexin receptor has been reported to be composed of two subunits, a 75 kDa subunit and a 14–16 kDa haem-binding subunit [11]. This raises the question of whether the β subunit of the murine receptor is the MHBP, which plays a pivotal role in haem transport through the plasma membrane (A. Smith, unpublished work; [8]). However, in rabbit liver plasma membranes and mouse Hepa cells, no evidence that MHBP is disulphide-linked to a 65 kDa protein has been found (A. Smith, unpublished work). Furthermore, MHBP is present in rabbit liver membranes in an approx. 10-fold excess over the receptor. Based on these considerations, the 20 kDa receptor subunit of Hepa cells does not appear to be the 17.5 kDa MHBP, although definitive proof is lacking. Interestingly, antibodies to MHBP block both haemopexin binding to the receptor and haem transport (A. Smith, unpublished work), suggesting that MHBP is closely associated with the haemopexin receptor.

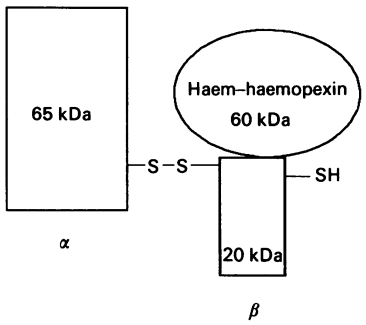

Fig. 9. Model of the murine haemopexin receptor

Based on the present results, a schematic diagram of the hepatic haemopexin receptor has been developed. The murine receptor appears to be an 85–90 kDa protein, composed of at least two subunits. The larger subunit of 65–70 kDa is designated the α subunit, by convention with other receptors. The binding site for haemopexin appears to be located on the smaller (20 kDa) subunit, designated β, which is disulphide-linked to the α subunit. A cysteine residue lies close to the haemopexin-binding site, since haemopexin, which lacks a free —SH group, is linked by sulpho-SMBP which contains both reactive primary amine and thiol groups.

In sum, the data presented here (and on receptor recycling below) add to the evidence for the existence of a specific receptor for haemopexin and provide evidence that uptake of intact haemopexin is not mediated by the asialoglycoprotein receptor. Recent reports by Muller-Eberhard, Reed, Sinclair and their collaborators [35–37] have questioned the existence of a specific haemopexin receptor. Moreover, the implication [35] that haemopexin isolated by lectin chromatography is desialylated and consequently that published reports on haemopexin-mediated haem uptake by a haemopexin receptor merely reflect uptake via the asialoglycoprotein receptor are also clearly precluded by the results of these cross-linking studies, as well as by other observations. The rat asialoglycoprotein receptor occurs predominantly as a 41.5 kDa protein [38], quite distinct from the structure presented here for the murine haemopexin receptor, as well as those reported for the pig [11] and human [12] haemopexin receptors. Binding studies *in vitro* showed that native haem–haemopexin interacts only with the haemopexin receptor and that haem–asialohaemopexin interacts with both the haemopexin-specific and asialoglycoprotein-specific receptors on liver plasma membranes [39]. Native haemopexin recycles intact both *in vivo* [25,40] and *in vitro* [6,7,26,41], whereas asialohaemopexin is cleared from the circulation in minutes [24,39,42] and catabolized via interaction with the asialoglycoprotein receptor [39,42,43]. Importantly, haem–iron from haem–asialohaemopexin is not accumulated in the liver to the same extent as is haem–iron from native haemopexin complexes [29].

The effects of a tumour-promoting phorbol ester (PMA) on the cellular distribution of haemopexin receptors demonstrate that phorbol esters rapidly down-regulate the number of surface haemopexin receptors in a dose-dependent reversible manner. In addition, this decrease is not due to catabolism of the receptors, since recovery from down-regulation is not inhibited by cycloheximide. The time course of the response of haemopexin receptor surface expression by PMA is rapid and resembles that observed for the transferrin receptor after exposure of HL-60 [16,21] and K562 [18] cells to phorbol esters, which bind to and activate PKC and cause its redistribution from the cytosol to the membrane. PKC involvement is supported by the abrogation of the effects of phorbol ester by simultaneous incubation with H-7, a specific inhibitor of PKC, and the specificity of the effects of PMA is indicated by the lack of effect of 4α-phorbol. Moreover, activation of PKC appears to produce the same redistribution of the haemopexin receptor as does binding of the natural ligand haem–haemopexin.

The down-regulation of the haemopexin receptor shown here is due to a doubling of the rate of endocytosis, without a change in the rate of exocytosis, and is similar to the transferrin receptor in HL-60, Hep G2 [44,45] and K562 cells but different from that of receptors such as the asialoglycoprotein receptor in Hep G2 cells, in which phorbol ester treatment decreases receptor affinity but not the number of surface receptors [45]. The role of phosphorylation by PKC in receptor recycling remains unclear and appears to be complicated. For example, when mutant human transferrin receptors lacking the PKC phosphorylation site, Ser-24, are expressed in transfected 3T3 cells, they undergo endocytosis with recycling [22], but very little down-regulation of the constitutive receptor occurs, indicating that cell-specific factors are also important for receptor endocytosis [22,46]. In

addition, a decrease in surface transferrin receptor number always results from an increase in endocytosis, but can be accompanied by either a decrease (e.g. in HL-60 cells [16]) or an increase (e.g. in Chinese hamster ovary fibroblast cells [47]) in exocytosis. Thus whether the rate of exocytosis of the transferrin receptor is affected appears to depend on cell type. Whether haemopexin receptor redistribution after ligand binding and phorbol ester treatment reflects a general perturbation of cellular membrane traffic or involves only a subclass of membrane proteins of which both the haemopexin and transferrin receptors are members remains to be determined.

The mechanism by which receptor movements are regulated and intracellular receptor pools are maintained remains a fundamental question in receptor-mediated endocytosis. In some systems, for example EGF, both receptor and ligand are degraded [48], whereas more commonly only the ligand is degraded and the receptor recycles back to the cell surface. This is the case for the asialoglycoprotein [49], low-density lipoprotein [50], α_2-macroglobulin [51] and insulin [52] receptors. In the cases of transferrin and haemopexin, the receptors recycle to the cell surface in a manner similar to that of the insulin receptor [14]; but, unlike insulin, both receptor and apo-transport protein recycle for another round of transport. Both the insulin and EGF receptors have intrinsic tyrosine kinase activity, and recently studies on mutant EGF receptors have provided evidence that its tyrosine kinase domain plays a role in lysosomal targeting of the receptor, and mutant receptors lacking this activity recycle to the plasma membrane together with the transferrin receptor [53]. However, the precise function of phosphorylation in endocytosis and exocytosis of the transferrin receptor remains unknown. Importantly, since haemopexin and transferrin co-localize intracellularly in hepatoma cells [6,7], the data here provide additional evidence that these two systems have similar mechanisms of receptor-mediated endocytosis.

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