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Expression of a glycosylphosphatidylinositol-anchored ligand, growth hormone, blocks receptor signalling

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Synopsis

We have investigated the interaction between GH (growth hormone) and GHR (GH receptor). We previously demonstrated that a truncated GHR that possesses a transmembrane domain but no cytoplasmic domain blocks receptor signalling. Based on this observation we investigated the impact of tethering the receptor's extracellular domain to the cell surface using a native lipid GPI (glycosylphosphatidylinositol) anchor. We also investigated the effect of tethering GH, the ligand itself, to the cell surface and demonstrated that tethering either the ecGHR (extracellular domain of GHR) or the ligand itself to the cell membrane via a GPI anchor greatly attenuates signalling. To elucidate the mechanism for this antagonist activity, we used confocal microscopy to examine the fluorescently modified ligand and receptor. GH–GPI was expressed on the cell surface and formed inactive receptor complexes that failed to internalize and blocked receptor activation. In conclusion, contrary to expectation, tethering an agonist to the cell surface can generate an inactive hormone receptor complex that fails to internalize.

Key words: cytokine antagonist, growth hormone, growth hormone receptor, glycosylphosphatidylinositol anchor, signal transduction, receptor trafficking

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INTRODUCTION

We have been investigating the interaction of GH (growth hormone) with its receptor and investigating approaches to manipulate receptor signalling. We observed that a patient heterozygous for a mutation in the GHR (GH receptor) had a short stature and GH insensitivity suggesting that the mutation created a dominant negative receptor [1]. The mutation encoded a truncated receptor that had a normal extracellular and transmembrane domain but lacked the essential cytoplasmic signalling domain. We demonstrated that the dominant negative action occurred as the truncated receptor was highly expressed on the cell surface, complexed with the full-length receptor, but the complex failed to signal or internalize [2]. Based on these observations, we proposed that anchoring a truncated receptor to the cell surface would generate an antagonist and by using a synthetic lipid anchor we demonstrated this was the case [3]. Following on from this work we have been examining the impact of anchoring proteins using naturally occurring GPI (glycosylphosphatidylinositol) lipid anchors.

GPI anchors are common components of the eukaryotic cell membrane and examples include ALP (alkaline phosphatase) and DAF (decay-accelerating factor) [4]. GPI-anchored proteins are tethered to the cell membrane through a glycolipid moiety and have no transmembrane or cytoplasmic domains. Recombinant

Abbreviations used: CMV, cytomegalovirus; GFP, green fluorescent protein; GH, growth hormone; GHR, growth hormone receptor; ecGHR, extracellular domain of GHR; GPI, glycosylphosphatidylinositol; HEK, human embryonic kidney; LHRE, lactogenic hormone response element; LL, long linker; PE, phycoerythrin; RFP, red fluorescent protein; RL, *Renilla* luciferase; wtGH, wild-type GH.

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proteins can be engineered so as to contain a GPI-signal sequence, which is post-translationally modified in human cells resulting in attachment onto the plasma membrane via the GPI-anchor. GPI anchoring can be generated by fusing the cDNA of interest with the GPI signal sequence for a naturally occurring GPI-anchored protein such as Thy-1 [5].

GH is a cytokine that engages two identical type 1 cytokine GHR, and the transmembrane domains of two or more GHR molecules must associate to initiate signalling [6]. GH, produced by the pituitary, regulates body composition, with deficiency resulting in short stature and excess in gigantism and acromegaly [7]. In this paper, we have examined the impact on signalling of expressing a fusion of the ecGHR (extracellular domain of GHR) with a GPI anchor and also asked the question; what would happen if we anchored the ligand itself to the cell surface? As expected, anchored receptor blocked signalling but to our surprise we found that the anchored ligand also blocked receptor signalling and internalization.

EXPERIMENTAL

Plasmids

The reporter construct pUC18–LHRE (lactogenic hormone response element)–Luc, containing STAT5 (signal transducer and activator of transcription 5) binding element, LHRE fused to the minimal tk (thymidine kinase) promoter and the firefly luciferase cDNA (GHR) has been described previously [2]. The phRL-CMV expression vector, encoding RL (*Renilla* luciferase) under control of the CMV (cytomegalovirus) promoter, was from Promega. The expression vector pCR3.1gpi, encoding the mammalian Thy-1 GPI signal sequence under control of the CMV promoter was a gift from C. Beghadi (University of Lausanne, Lausanne, Switzerland). The cDNAs encoding human GH and the ecGHR were cloned without stop codons upstream of the Thy-1 GPI signal sequence in pCR3.1gpi to generate the ecGHR–GPI and GH-GPI constructs. The human GH cDNA was cloned in the same vector to generate the wtGH (wild-type GH) construct, encoding non-anchored wtGH. GH–LL (long linker)–GPI was generated by insertion in GH–GPI of a sequence encoding a flexible LL consisting of five repeats of a Gly^4Ser (tetraglycine-serine) motif between the coding sequences of GH and the Thy-1 signal sequence. The GHR–GFP (green fluorescent protein) construct, encoding the full-length GHR fused at its C-terminal end to the GFP, was generated by inserting the open GHR coding sequence into the pTagGFP vector (Evrogen). The GH–RFP (red fluorescent protein) construct, encoding GH fused at its C-terminal end to the RFP, was made by inserting the open GH coding sequence into pTagRFP. Adding the Thy-1-GPI signal to GH–RFP then generated the GH–RFP–GPI construct. All the constructs were subjected to DNA sequencing carried out within our Core Genetics Facility (Faculty of Medicine, Dentistry and Health, University of Sheffield).

Flow cytometry

HEK (human embryonic kidney)-293 cells were transiently transected using calcium phosphate (Invitrogen). Detection was by monoclonal antibodies to GHR (2C8) and GH (7F8) both kind gifts from Professor C.J. Strasburger (Campus Mitte Charite-Universitatsmedizim, Berlin, Germany). Briefly, about 3×10^5 cells were washed with PBS/1 % BSA (wash buffer) and incubated with 5 μg of 2C8 or 7F8 antibodies for 30 min on ice. The cells were washed again and incubated with 1 μg of biotinylated goat anti-Mouse IgG (Calbiochem). The cells were then washed and incubated with 14 μl of streptavidin conjugated to R-PE (phycoerythrin, Serotec). A final three washes were performed to remove the residual unbound stain. PE was excited at 488 nm and the emitted light was detected through a 585 ± 21 nm band pass filter. Data were acquired on a FACSort flow cytometer (Becton Dickinson) using the CellQuest data acquisition and analysis software housed in our Core Flow Cytometry Facility (Faculty of Medicine, Dentistry and Health, University of Sheffield).

Triton X-114 phase partitioning

Transfected HEK-293 cells (1×10^6) producing ecGHR–GPI, GH–GPI or GH–LL–GPI were washed in PBS and lysed in 250 μl of lysis buffer (20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 2 % Triton X-114 and 0.0005 % Bromophenol Blue). The cells were then left on ice with frequent stirring for 20 min. The resulting lysate was centrifuged at 13 000 ***g*** for 5 min at 0°C to remove cellular debris. The supernatant was incubated at 30°C for 5 min and then centrifuged at 4000 ***g*** for 3 min at room temperature to separate the clear aqueous phase from the detergent phase containing Bromophenol Blue.

Western blotting

Samples were separated by SDS/PAGE (12 % gels) and blotted on to the PVDF membrane. The primary antibodies were mouse anti-GHR mAb263 (Biogenesis used at a 1:2500 dilution ratio) and anti-human GH (rabbit) polyclonal antibody [NIH (National Institutes of Health), used at 1:10000 dilution]. The secondary antibodies were anti-mouse or anti-rabbit HRP (horseradish peroxidase)-linked IgG (Amersham Pharmacia Biotech) used at 1:10000 dilution. They were detected using an enhanced chemiluminescence substrate (Roche Diagnostics) and light sensitive X-ray films.

GH signalling bioassay

The luciferase reporter GH bioassay was performed as described previously except that the phRL-CMV vector was used as a transfection control instead of the pCH110 vector [2]. Assays were carried out in HEK-293 cells that permanently express GHR, called HEK-293–GHR or HEK-293 Hi unless stated otherwise. The cells were plated in 12-well plates and transiently transfected with either reporter constructs alone (50 ng/well of pUC18–LHRE–Luc and 1 ng/well of phRL-CMV) or with reporter constructs plus 100 ng/well of cDNA expression plasmid, using calcium phosphate. The cells were stimulated with GH

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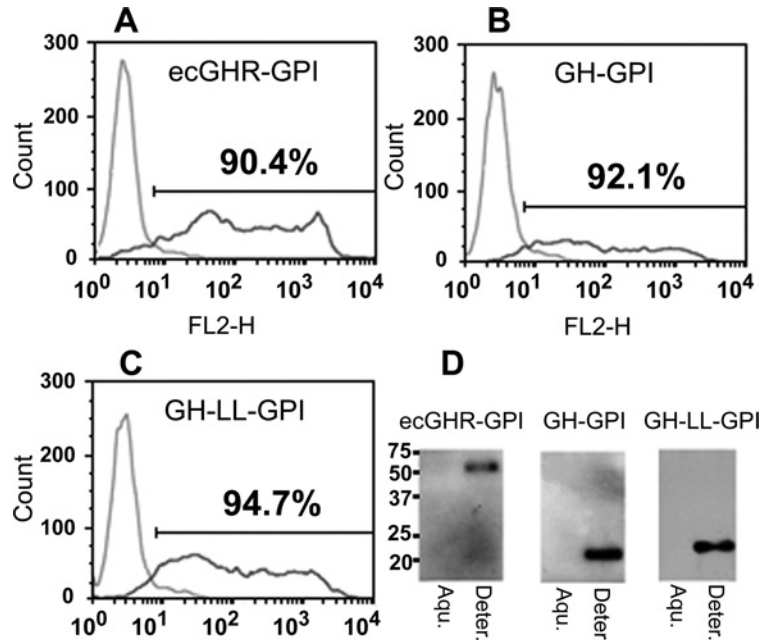

Figure 1 GPI-anchored proteins associate with plasma membrane
(**A**–**C**) Detection of GPI-anchored proteins at the surface of transfected cells. HEK-293 cells were transfected with ecGHR–GPI (**A**), GH–GPI (**B**) or GH–LL–GPI (**C**), labelled with antibodies to GHR or GH and subjected to flow cytometry. Histograms from transfected cells (dark grey curves) are shown overlaid onto control histograms from untransfected cells (light grey). (**D**) Phase partitioning and molecular masses of GPI-anchored proteins. Cells transfected with ecGHR–GPI, GH–GPI or GH–LL–GPI were subjected to phase partitioning and the resulting detergent (Deter.) and aqueous (Aqu.) phases were probed by Western blotting with antibodies to GHR or GH, as appropriate. The positions and molecular masses (kDa) of standard proteins are shown next to the first immunoblot.

18 h after transfection, then lysed after a further 6 h. The firefly and RL activities were measured using the Promega dual luciferase reporter assay kit. The firefly luciferase values were normalized to those of the phRL-CMV-encoded RL to correct for variations in transfection efficiency and cell number. The results were expressed as fold-induction relative to the unstimulated control of each plate. For experiments requiring autocrine GH stimulation, HEK-293–GHR cells were transfected twice. The first transfection used 100 ng/well (12-well plate) of wtGH construct. The other cDNA expression constructs and the reporter constructs were co-transfected in the second transfection 18 h later.

Laser scanning confocal microscopy

HEK-293 cells were grown on poly-L-lysine-coated glass-bottomed microwell culture dishes (MatTek Corporation) for 16 h before being transfected using Lipofectamine2000 (Invitrogen). Confocal microscopy was carried out 2 days after transfection using a Zeiss 510 NLO laser scanning confocal microscope fitted with ×20 NA 0.8 and ×40 NA 1.2W (water) objective lenses. Fluorescence images of GFP were obtained using 488 nm laser excitation, NFT490 dichroic and LP505 emission filter. Images of RFP were obtained using 543 nm laser excitation, NFT545 dichroic and LP560 emission filters. Images were acquired at 1024×1024 (pixel dwell time 0.8 μs) pixels and 1.3 μm optical slices. Background fluorescence was subtracted from the images using ImageJ 1.37c software (NIH) [20]. Co-localization of fluorophores was identified by Intensity Correlation Analysis, determining the positive product of the difference of the means with the ImageJ plug-in developed at the Wright Cell Imaging Facility, Toronto, Canada.

RESULTS

GPI-anchored GH proteins demonstrate cell membrane expression

We wished to examine the impact of anchoring ecGHR and GH to the cell surface and we also raised the question whether the length of linker between GH and its anchor would alter biological activity. The following constructs were tested: wtGH with no anchor (wt GH), GH linked to GPI either through a short linker (GH–GPI) or LL (GH–LL–GPI), and ecGHR linked to GPI (ecGHR–GPI). Flow cytometry of HEK-293 cells transfected with the GPI-anchored proteins: GH–GPI, GH–LL–GPI and ecGHR–GPI showed that transfection efficiencies were >90 % and that for all the GPI-anchored proteins there was a high but variable cell surface expression (Figures 1A–1C). The biophysical properties of the recombinant proteins were then

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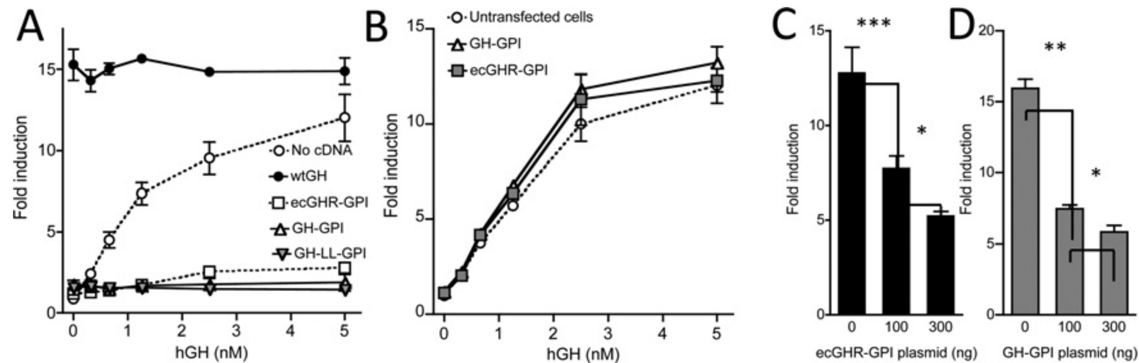

Figure 2 Inhibition of signalling by GPI-anchored proteins
(**A**) HEK-293–GHR cells were co-transfected with the pUC18–LHRE–Luc and phRL–CMV reporter constructs, with or without the wtGH, ecGHR–GPI, GH–GPI or GH–LL–GPI cDNA construct, as indicated, and stimulated 18 h later with various doses of exogenous GH. Normalized reporter levels are expressed relative to values in cells transfected with reporter constructs only and not exposed to GH. Means ± S.E.M. of $n = 4$ (ecGHR–GPI, GH–LL–GPI) or $n = 6$ are shown. (**B**) HEK-293–GHR cells were transfected with the reporter constructs but no cDNA and were then challenged with conditioned media from untransfected cells or cDNA-transfected cells expressing ecGHR-GPI or GH–GPI, as indicated. Stimulation with exogenous GH, reporter assays and data analysis were carried out as in (**A**). Means ± S.E.M. of $n = 3$ are shown. (**C**) To test the inhibition of the autocrine GH response, HEK-293–GHR cells were pre-transfected with 100 ng of wtGH construct 18 h before being transfected a second time with the reporter constructs and various amounts of plasmid encoding ecGHR-GPI, as indicated. Data analysis was carried out as in (**A**) and (**B**) and one-way ANOVA with Bonferroni's *post-hoc* comparison was performed. Means ± S.E.M. of $n = 6$ are shown. One, two or three asterisks correspond to $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively. (**D**) As in (**C**), except that the GH–GPI expression construct was used instead of ecGHR–GPI.

assessed by phase partitioning (separation of protein into either a detergent or aqueous phase) to demonstrate the presence or absence of a GPI anchor (Figure 1D). The recombinant proteins ecGHR–GPI, GH–GPI, GH–LL–GPI migrated at their predicted molecular masses and all partitioned into the detergent-enriched phase with no detectable protein in the aqueous phase, as is expected for GPI-anchored proteins. These results confirmed that all the three GPI-anchored proteins were expressed at the cell surface and retained their GPI lipid moiety.

GPI-membrane-anchored GH and ecGHR block GH signalling

A dual luciferase promoter–reporter bioassay for functional ligand–receptor-mediated signalling was employed to examine the action of GPI-anchored proteins. HEK-293 cells stably expressing human GHR (HEK-293–GHR) and transiently transfected with the bioassay reporter constructs showed a dose-dependent response to exogenously administered GH (Figure 2A). When the wtGH expression plasmid was co-transfected with the reporter constructs, high reporter expression was seen independent of exogenous GH, indicating that the expression of wtGH led to autocrine/paracrine stimulation. In contrast, HEK-293–GHR cells transfected with the anchored ecGHR, ecGHR–GPI, showed no increase in basal reporter expression and no response to the exogenously administered GH (Figure 2A). This demonstrated that, as expected, ecGHR–GPI acted as an antagonist. To our surprise, cells transfected with GH–GPI did not behave like the wt-GH-transfected cells but instead showed no increase in basal reporter expression and, like the ecGHR–GPI-transfected cells, no response to the exogenously added GH (Figure 2A). We questioned whether an LL between GH and GPI could overcome any steric hindrance and restore signalling; however, the GH–LL–GPI construct inhibited the bioassay as effectively as GH–GPI (Figure 2A). Thus, both the GH–GPI and GH–LL–GPI constructs behaved as antagonists of GH signalling.

We considered the possibility that the inhibitory effects of the GPI-linked proteins might be caused by the partial release of these proteins into the culture medium. To examine this, we challenged untransfected cells with GH in the presence of conditioned media from cells transfected with either ecGHR–GPI or GH–GPI. The conditioned media did not inhibit the response to GH (Figure 2B), indicating that the ecGHR–GPI- and GH–GPI-expressing cells were not secreting any detectable soluble inhibitor of GH signalling.

We next examined the effect of the GPI-anchored proteins on autocrine GH signalling. The cells were subjected to a first transfection with wtGH and 18 h later to a second transfection with reporter constructs and various amounts of cDNA construct encoding either ecGHR–GPI (Figure 2C) or GH–GPI (Figure 2D). Control transfections in which the GPI-fusion constructs were omitted showed that autocrine action of wtGH led to a 12-fold increase in reporter expression relative to cells not expressing the cytokine. This response was inhibited when either ecGHR–GPI or GH–GPI was included in the second transfection and in each case, the degree of inhibition was greater as the amount of transfected GPI construct was increased (Figures 2C and 2D).

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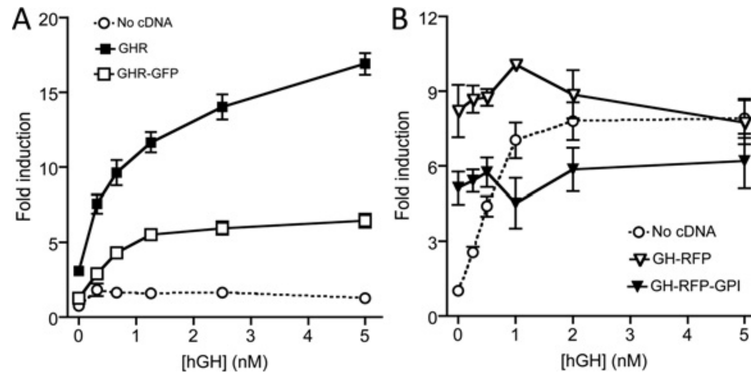

Figure 3 Biological activities of the fluorescent proteins
(**A**) HEK-293 cells were subjected to the promoter–reporter bioassay in the absence of co-transfected cDNA or in the presence of cDNA constructs encoding wt GHR or GHR–GFP, as indicated. (**B**) The activities of fluorescent labelled GH-RFP and GH–RFP–GPI proteins were tested with the bioassay in the HEK-293–GHR-expressing cell line as had been done for the non-fluorescent wtGH and GH–GPI in Figure 2(A). All the data were analysed as in Figure 2 and are means ± S.E.M. of $n = 4$ (**A**) or $n = 6$ (**B**).

Signalling properties of fluorescent fusion proteins

As a preparation to investigating the mechanisms of inhibition of GH action by the GPI fusion proteins, we generated a red-fluorescent GPI-anchored GH (GH–RFP–GPI), a red-fluorescent non-anchored GH (GH–RFP) and a green fluorescent receptor (GHR–GFP) and assessed the biological activities of the fluorescent proteins. The activity of fluorescent labelled GHR–GFP was assessed with the dual-luciferase promoter–reporter bioassay in the HEK-293 cells, which endogenously only express low levels of GHR. In the absence of a co-transfected GHR cDNA, the cells only show weak activation of reporter expression in response to exogenously administered GH (maximum fold activation 1.65 ± 0.26), but the response to the GH is dramatically increased when they are transfected with a wt GHR cDNA construct (maximum fold activation 16.9 ± 1.5, Figure 3A). A similar but quantitatively smaller increase was observed when the fluorescent receptor construct, GHR–GFP, was substituted for the wtGHR (maximum fold induction 6.5 ± 0.9, Figure 3A).

The activities of fluorescent labelled GH–RFP and GH–RFP–GPI proteins were tested in the HEK-293–GHR cell line (Figure 3B). Cells expressing GH–RFP showed constitutively high levels of reporter expression (8.2 ± 1.5-fold activation of reporter expression relative to cells expressing no cDNA) and this was not significantly increased when the cells were exposed to increasing amounts of exogenously administered GH (Figure 3B). Expression of GH–RFP–GPI activated reporter expression, albeit to a lesser extent than that observed in cells expressing GH–RFP (5.7 ± 0.7-fold activation). This intermediate level of reporter activity suggested that the expression of GH–RFP–GPI partially activated the GH signalling machinery; however, no significant increase in reporter activation was observed when the cells expressing GH–RFP–GPI were exposed to increasing doses of exogenous GH (Figure 3B). This suggested that while GH–RFP–GPI differed from GH–GPI and GH–LL–GPI by being a partial agonist, it shared with the other GPI constructs the ability to block the action of exogenously administered GH.

GPI-anchored GH forms cell surface complexes with wt receptor

To examine the interaction between GH–GPI and GHR, we monitored the localization of the fluorescent fusion proteins by laser scanning fluorescence microscopy. We first characterized the distribution of each fluorescent protein when transfected by itself (Figure 4). Cells transfected with GHR–GFP showed fluorescence both on the cell membrane and within the cytoplasm (Figure 4A), in a pattern comparable with previous data showing trafficking of GFP-tagged GHR from the ER (endoplasmic reticulum) via the Golgi apparatus to the cell membrane [8]. In cells transfected with the non-anchored GH–RFP fusion protein, red fluorescence was detected predominantly in discrete structures within the cytoplasm. We considered that this distribution could be either GH–RFP packaged for secretion or GH–RFP that had been secreted and then bound receptor and internalized (Figure 4B). To discriminate between these two alternatives, we applied conditioned media from GH–RFP transfected cells to untransfected HEK-293–GHR cells. The untransfected cells then showed the presence of GH–RFP in discrete intracellular structures, which confirmed that the fluorescent agonist had been secreted by the transfected cells and internalized by the imaged cells (Figure 4C). Similar localization of the red fluorescence in Figures 4(B) and 4(C) suggests that in the GH–RFP-expressing cells (Figure 4B) at least a fraction of the intracellular fluorescence probably corresponds to GH–RFP internalized after secretion. In contrast to GH-RFP, the GH–RFP–GPI fusion protein was localized predominantly on the cell membrane with only a small proportion present within the cytoplasm, suggesting little or no internalization (Figure 4D).

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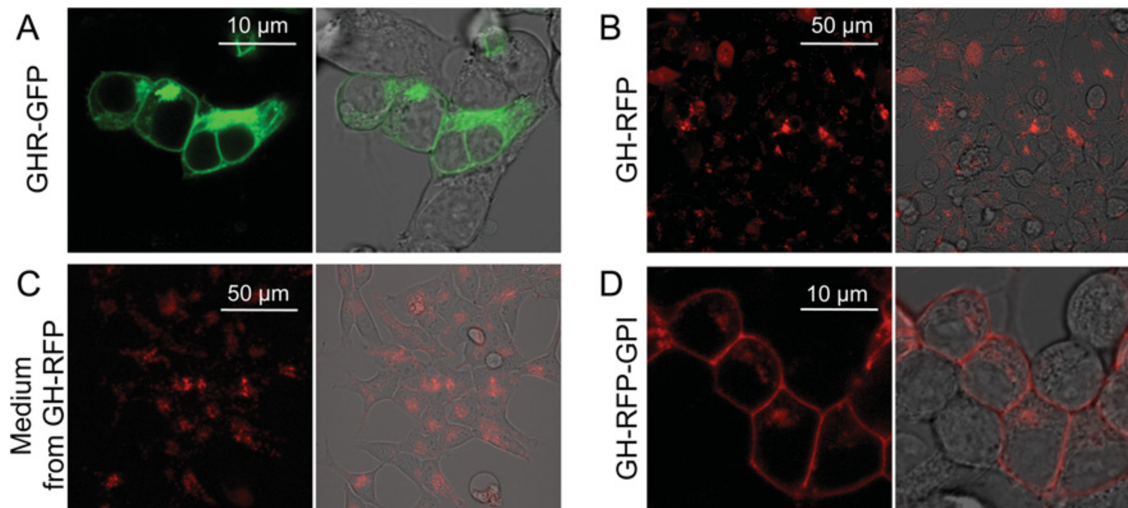

Figure 4 Intracellular localization of fluorescent fusion proteins
(**A**, **B**) HEK-293 cells imaged after transfection with either the green fluorescent GHR fusion construct, GHR–GFP (**A**) or the red fluorescent cytokine construct, GH–RFP (**B**). (**C**) HEK-293 cells that have not been transfected were imaged after a 3 h incubation in conditioned media from the GH–RFP-expressing cells. The intracellular red fluorescence indicates that the cells had internalized GH–RFP present in the conditioned media from the GH–RFP-expressing cells. (**D**) HEK-293 cells imaged after transfection with the GPI-anchored fluorescent GH construct, GH–RFP–GPI. Each confocal fluorescence image is shown both alone (left) and overlaid onto a phase contrast image of the same field (right). In contrast, anchored GH–RFP–GPI is predominantly found on the cell surface (**C**).

To analyse the interactions between the GH constructs and the GH receptor, we then co-transfected the GH–RFP expression plasmid with the GH-based constructs (Figure 5). In cells co-transfected with GHR–GFP and GH–RFP, the recombinant receptor showed both membrane and cytoplasmic localizations (Figure 5A). However, in contrast to what had been observed in the single transfection experiments (Figure 4A), the intracellular pool of GHR–GFP molecules was not evenly distributed throughout the cytoplasm but appeared instead to accumulate in discrete intracellular locations (Figure 5A) similar to that seen for internalized GH–RFP from media (Figure 4C). Overlaying the red and green fluorescent images showed that a fraction of the GHR–GFP pool co-localizes with GH–RFP (Figure 5C), and this was confirmed by the co-localization analysis (Figure 5D). Similar results were observed when culture media containing non-anchored GH–RFP were added to GHR–GFP-transfected cells (Figures 5G–5K). These results are entirely consistent with the distributions of GH–RFP observed previously in cells that were not expressing GHR–GFP (Figures 4B and 4C). They indicate that co-localization of GH–RFP and the receptor reflects internalization of the receptor–hormone complex.

We then observed the distributions of co-transfected GHR–GFP and GH–RFP–GPI (Figures 5I–5L). The results show that the two pools of fluorescent proteins are entirely co-localized, with the majority of each construct being located on the cell membrane and only a minor fraction of each pool adopting an intracellular distribution (Figure 5I–5L). Thus, GH–RFP-GPI forms a complex with GHR–GFP on the cell surface, which does not internalize.

DISCUSSION

We have examined the interaction between GH and its receptor and investigated the impact of anchoring either the extracellular domain of the receptor or the GH ligand itself to the cell surface via attachment of an endogenous GPI anchor. Both the receptor's extracellular domain and ligand blocked receptor signalling when tethered to the cell membrane through a GPI anchor.

GH binding to its receptor results in a conformational change that includes rotation in the transmembrane domain and triggers signalling and internalization [9]. We questioned whether increasing the length of linker to the cell membrane could restore agonistic activity to our GPI-anchored hormone, as too short a linker could restrict the movement of the molecule. However, the GH–LL–GPI construct inhibited GH signalling as efficiently as GH–GPI despite the presence of a 70 Å (1 Å = 0.1 nm) long glycine/serine-rich linker that was intended to relieve any conformational or spatial restraints between the GH moiety and the GPI domain.

Experiments with the fluorescent receptor, GHR–GFP and the non-anchored fluorescent ligand, GH–RFP, showed that fluorescent tagging of GH and its receptor did not interfere with trafficking of the receptor to the cell surface, formation of receptor–cytokine complexes and internalization of the complexes. GH–RFP–GPI anchored to the cell surface still formed a ligand–receptor complex but was not internalized. The partial activation of signalling seen by GH–RFP–GPI could be a steric action of RFP or the introduction of RFP might result in a small proportion of the molecule being proteolysed and releasing free GH.

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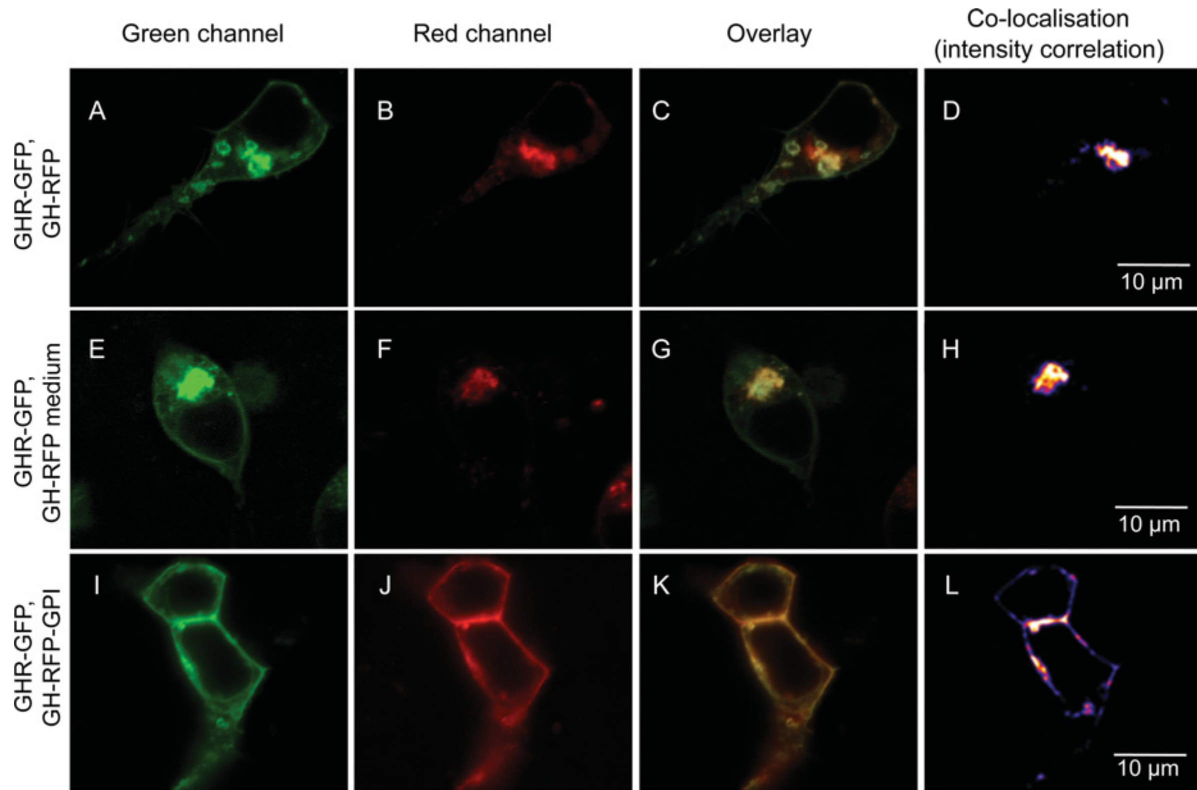

Figure 5 GPI-anchoring of GH blocks GHR internalization
This Figure examines the co-localization of GHR and GH with and without GPI-anchored GH–GPI. In cells co-expressing the non-anchored GHR–GFP (**A**, **C**, **D**) and GH–RFP (**B**–**D**) proteins, a fraction of the GHR–GFP pool co-localizes with the GH–RFP inside cells (**A**–**D**). Co-localization is indicated by yellow colouring in the overlay image (**C**) and is confirmed in the intensity correlation image (**D**). The same distributions are observed when cells expressing GHR–GFP only are incubated with exogenously provided GH–RFP (**E**–**H**), indicating that intracellular co-localization reflects the internalization of the receptor–ligand complexes. By contrast, when GHR–GFP is co-expressed with the anchored GH–RFP–GPI (**I**–**L**), neither protein is internalized.

The cytoplasmic domain of the GHR is essential not only for activating the GHR but also for triggering internalization of the hormone receptor complex [10]. However, signalling is not required for receptor internalization as a receptor with a mutated cytoplasmic domain that fails to signal still internalizes [11], and the GH antagonist Pegvisomant binds the receptor dimer, prevents signalling but is internalized [12]. Truncated receptors that lack a cytoplasmic domain act as dominant negative inhibitors of signalling not only because they heterodimerize with the full-length receptor but also because they prevent internalization and therefore accumulate on the cell surface [2]. It has been reported that both GH and its receptor may translate to the nucleus and correlate with proliferative activity [13]; thus a failure of the hormone receptor complex to internalize could again prevent the biological activity of GH. Our results suggest that GH–GPI not only occupies the receptor to prevent signalling but also holds the receptor complex at the cell surface. GHR is present in caveolae and lipid rafts which are lipid-rich microdomains of the plasma membrane [14,15]. Various membrane proteins are concentrated in these lipid microdomains including GPI-anchored proteins such as the Thy-1 cell surface antigen [16]. Thus, GPI anchoring of GH or ecGHR increases the probability that the GPI-anchored proteins will co-segregate with the native GHR on the cell membrane and constrain the GHR in a conformation that cannot be readily internalized.

In some cell types, autocrine actions of GH are more potent than exogenously administered GH [17–19]. Moreover, autocrine GH producing cells become unresponsive to externally administered GH [20,21]. Both GH–GPI and ecGHR–GPI inhibited the action of intracellularly generated GH, and by increasing the ratio of GH–GPI to wtGH, the inhibition was increased although never complete. However, it should be recognized that transfection experiments result in relatively high expression levels of endogenous GH compared with physiological conditions. We attempted to purify ecGHR–GPI and GH–GPI and investigate whether the GPI fusions could be re-inserted into the cell membranes, however despite repeated attempts we were unable to purify the proteins (results not shown). This may relate to either the presence of a lipid moiety or the low level of expression of cell surface proteins.

Our findings show that tethering a cytokine agonist to the cell surface using endogenously generated GPI anchors results in

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an antagonist. Such a strategy could potentially be applied to a wide range of cytokines that act through the cell surface receptors providing a technology that could be used to manipulate cytokine signalling in the laboratory.

AUTHOR CONTRIBUTION

François Guesdon, Yahia Kaabi, Aiden Riley and Ian Wilkinson undertook all the molecular and cell biology testing and analysis. Colin Gray was responsible for confocal microscopy. David James, Peter Artymiuk, Jon Sayers and Richard Ross were responsible for the concepts, data analysis and writing of the paper. Richard Ross supervised the project.

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