Adaptor signalling proteins Grb2 and Grb7 are recruited by human G6f, a novel member of the immunoglobulin superfamily encoded in the MHC

Edwin C. J. M. DE VET, Begoña AGUADO and R. Duncan CAMPBELL[1]

MRC Rosalind Franklin Centre for Genomics Research (formerly the Medical Research Council U.K. Human Genome Mapping Project Resource Centre), Hinxton, Cambridge CB10 1SB, U.K.

The human G6f protein, which is encoded by a gene in the MHC, is a putative cell-surface receptor belonging to the immunoglobulin superfamily. The intracellular tail of G6f is 40 amino acids in length and contains one tyrosine residue (Y281), which is phosphorylated after treatment of cells with pervanadate. This tyrosine residue is found in a consensus-binding motif (YXN) for the Src homology 2 domains of Grb2 and Grb7 (where Grb stands for growth-factor-receptor-bound protein). Glutathione S-transferase pull-down assays showed that the interaction of G6f with both Grb2 and Grb7 is mediated through the Src homology 2 domains of these two proteins and is dependent on the phosphorylation of G6f. Immunoprecipitation experiments showed the interaction of full-length phosphorylated G6f with both full-length Grb2 and Grb7. Antibody cross-linking of G6f expressed in K562 cells resulted in a transient phosphorylation of p42/44 MAP kinase (also known as extracellular-signal-regulated protein kinase-1/2; MAP stands for mitogen-activated protein) which could be prevented by MAP kinase kinase (MEK) inhibitors. These results suggest a coupling of G6f with downstream signal transduction pathways involving Grb2 and Grb7, including the Ras–MAP kinase pathway.

Key words: cell-surface molecule, immunoglobulin superfamily, MHC, signal transduction.

INTRODUCTION

Phosphorylation of cell-surface receptors plays an important role in signal transduction. SH2 (Src homology 2) domains can bind the resulting tyrosine-phosphorylated proteins [1,2]. These domains are found in numerous adaptor signalling molecules including Grb2 (growth-factor-receptor-bound protein 2), a molecule with an SH3–SH2–SH3 domain structure. It links membrane proteins to the Ras–MAP kinase pathway (where MAP stands for mitogen-activated protein) by interacting with the son of sevenless homologue, the guanine nucleotide exchange factor for Ras [1,3]. The SH2 domain of Grb2 recognizes phosphotyrosine residues normally found in the consensus sequence YXN [4,5].

Grb7 is a protein first identified as an interaction partner for the epidermal growth factor receptor [6]. It belongs to a family of proteins also comprising Grb10 and Grb14. All members of this family contain a putative Ras-associating-like domain [7], a PH (pleckstrin homology) domain and a C-terminal SH2 domain [8]. The region between the PH domain and the SH2 domain is called BPS (or phosphotyrosine interacting region) and has been shown to be important for interaction of Grb7 family members with the insulin receptor [9–11]. Many interaction partners have been identified for Grb7, including numerous receptor tyrosine kinases as well as other non-receptor signalling molecules like SHP2 (SH2 domain-containing tyrosine phosphatase-2) and focal adhesion kinase (see [8] and references therein). Most of these interactions are through the SH2 domain of Grb7 with a phosphotyrosine motif in the binding partner. Comparison of various Grb7–SH2-binding motifs shows that most, but not all, phosphotyrosine residues are in a YXN motif, similar to the recognition sequence for the Grb2–SH2 domain [8]. There are indeed examples of tyrosine residues in such a motif binding both Grb2 and Grb7 (i.e. Y580 of human SHP2 [12] and Y936 of c-Kit/stem cell factor receptor [13]), whereas other tyrosine residues are reported to bind to either Grb2 or Grb7 [12,14]. Despite this knowledge, little is known about the more downstream effectors of Grb7-mediated signalling, although a role for Grb7 in cell migration has been suggested [8].

The human G6f protein is a type I transmembrane protein belonging to the Ig (immunoglobulin) superfamily. This superfamily contains a large group of cell-surface proteins, which are involved in the immune system and cellular recognition [15,16]. The *G6f* gene is located in the class III region of the MHC and lies in a cluster of genes encoding cell-surface molecules [17,18]. Susceptibility to a wide range of diseases, mostly autoimmune in nature, has been linked to the human MHC, some of which are due to loci in the class III region [19–22]. The G6f protein contains two extracellular domains, the most N-terminal of which is a putative V-type Ig domain. The intracellular tail of G6f is 40 amino acids in length and contains one tyrosine residue in a consensus-binding motif (YXN) for the SH2 domain of Grb2 and possibly of Grb7. In the present study, we show that the G6f protein is capable of interacting with both Grb2 and Grb7. These interactions are dependent on phosphorylation of G6f on Y281 and are mediated by the SH2 domains of Grb2 and Grb7. In addition, by antibody cross-linking of G6f expressed in K562 cells, we observed enhanced phosphorylation of p42/44 MAP kinase. These results suggest that G6f can link to downstream signal transduction pathways involving Grb2 and Grb7, including the Ras–MAP kinase pathway.

Abbreviations used: Grb, growth-factor-receptor-bound protein; GST, glutathione S-transferase; Ig, immunoglobulin; mAb, monoclonal antibody; MAP, mitogen-activated protein; MEK, MAP kinase kinase; NP40, Nonidet P40; ORF, open reading frame; PH, pleckstrin homology; RT, reverse transcriptase; SH2, Src homology 2; BPS, between PH and SH2; SHP2, SH2 domain-containing tyrosine phosphatase-2; for brevity the one-letter system for amino acids has been used, Y281, e.g. means Tyr^{281}.

[1] To whom correspondence should be addressed (e-mail rcampbel@rfcgr.mrc.ac.uk).

The nucleotide sequence reported in this paper has been submitted to the GenBank®/EMBL Database under the accession number AJ496460.

EXPERIMENTAL

RT (reverse transcriptase)–PCR

RNA isolation and cDNA synthesis from human cell lines was performed as described previously [23,24]. Control PCRs with β-actin primers were performed on each cDNA reaction [23]. Primer sequences for PCR amplification of the complete ORF (open reading frame) of G6f were based on the genomic sequence (accession nos. AF129756, AL132713 and AP000504) and by analogy with the sequences of two mouse expressed sequence tags (AA267573 and AA184991). To increase sensitivity, a nested approach was used. In the first round, the forward primer 5′-CAAGAGAACTTGGCAGGCTC-3′ (nt 43167–43186) was used in combination with the reverse primer 5′-ATGGCCAGCGAGACAGCAGG-3′ (nt 39428–39447; all numbering of nucleotides refers to accession no. AF129756). Reactants of the PCR were incubated at 95 °C for 2 min followed by 25 cycles of 95 °C for 45 s, 60 °C for 30 s and 72 °C for 2 min, with a final incubation at 72 °C for 10 min. For the second round of the nested PCR, 0.2 μl from these reactions were used as template with the forward primer 5′-CCCCATGGCAGTCTTATTCC-3′ (nt 43146–43165) and the reverse primer 5′-CACTTCCCAGCAGATCTCAC-3′ (nt 39451–39470). PCR was performed under identical conditions as for the first round. PCR products were analysed on an ethidium bromide-stained agarose gel. Fragments were cloned into the pGEM-T vector (Promega, Chilworth, Southampton, U.K.) and sequenced on an ABI 377 automatic DNA sequencer using BigDye terminators. The codon encoding amino acid 167 (AAG, lysine) was changed into a codon encoding arginine (AGG) by replacing the *Bbs*I–*Xcm*I fragment in the cloned cDNA with the corresponding fragment obtained by PCR on genomic DNA from a healthy individual [25].

Expression of G6f in Cos-7 and K562 cells, glycosidase treatment and immunofluorescence

In all expression studies, the G6f cDNA was used with codon 167 coding for an arginine instead of a lysine. For expression of epitope-tagged G6f in mammalian cells, the ORF of G6f was cloned into the pcDNA3 vector (Invitrogen, Groningen, The Netherlands) fused to a T7 epitope tag (MASMTGGQQMGRDP). For construction of G6f with a C-terminal epitope tag, PCR copies of the complete ORF were made with a reverse primer that removed the stop codon and allowed direct fusion to the T7 epitope tag, as described previously [23]. To express G6f with an N-terminal tag, PCR copies of the ORF lacking the first 11 amino acids of the protein were made and directly cloned into an expression construct that contained the leader peptide of human CD33 followed by the T7 epitope tag in pcDNA3 (described in [23]). A Y281 to F (Y281F) mutant version of this construct was made using the Quickchange™ mutagenesis method (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions.

Proteins were transiently expressed in Cos-7 cells using the DEAE-dextran method as described elsewhere [26]. Cells were harvested 3 days after transfection. K562 cells were transfected with N-terminally tagged G6f and G6f(Y281F) and maintained in G418 containing medium as described previously [23]. Expression of recombinant proteins was analysed by SDS/PAGE [12 % (w/v) gel], followed by Western-blot immunostaining with the anti-T7-tag mAb (monoclonal antibody) (Novagen, Nottingham, U.K.). Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibody followed by detection with enhanced chemiluminescence (ECL®; Amersham Biosciences, Little Chalfont, Bucks., U.K.). Glycosidase treatment of G6f expressed in Cos-7 cells and immunofluorescence localization studies under permeabilizing and non-permeabilizing conditions were conducted as described previously [23].

Tyrosine phosphorylation of G6f

K562 cells expressing either N-terminally tagged G6f or G6f(Y281F) were treated with or without pervanadate for 10 min, as described previously [23]. Cells were lysed in NP40 (Nonidet P40) lysis buffer [10 mM Tris/HCl (pH 7.5), 1 % NP40, 150 mM NaCl, 0.02 % sodium azide, 1 mg/ml BSA and protease inhibitor cocktail (Sigma P8340)]. For pervanadate-treated cells, this buffer was supplemented with 1 mM sodium vanadate and 50 mM sodium fluoride. Lysates were cleared by centrifugation for 15 min at 4 °C and the proteins were immunoprecipitated with the T7 mAb essentially as described previously [23]. Immunoprecipitates were analysed by Western blotting using either the anti-T7-tag mAb horseradish peroxidase conjugate (Novagen) or the anti-phosphotyrosine mAb (4G10) coupled with horseradish peroxidase (Upstate Biotechnology, Waltham, MA, U.S.A.).

GST (glutathione S-transferase) pull-down assays

To express GST fusions with various domains of Grb2, Grb7 and Grb10, cDNA fragments encoding these domains were amplified by RT–PCR using various human cDNA preparations as template and cloned into the pGEX-2T vector in frame with GST. The following constructs were created: the SH2 domain of Grb2 (amino acids 54–153), the BPS domain (amino acids 343–428) and the SH2 domain (amino acids 421–532) of Grb7 and the SH2 domain of Grb10 (amino acids 426–536). Dominant-negative mutant constructs of the SH2 domains of Grb2 (R86K) and Grb7 (R458K) were made using the Quickchange™ mutagenesis method (Stratagene). The constructs were checked by sequencing and all GST fusions were expressed in *Escherichia coli* using standard procedures. Bacterial cells were lysed by sonication in NP40 lysis buffer containing 0.2 mg/ml lysozyme, and the GST-fusion proteins were purified from these lysates with glutathione–agarose beads (Sigma). K562 cells expressing N-terminally tagged G6f and G6f(Y281F) were treated with or without pervanadate and lysed in NP40 lysis buffer. Lysates were cleared by centrifugation, and aliquots of these lysates were incubated with purified GST-fusion proteins bound to glutathione–agarose beads at 4 °C for 2 h. Beads were washed with lysis buffer and eluted with SDS/PAGE sample buffer at 95 °C for 2 min. Eluates were examined by Western-blot immunostaining for the presence of T7 epitope-tagged G6f using the T7 mAb.

Interaction of full-length G6f with Grb2 and Grb7

A nucleotide fragment encoding the c-Myc (a protein which is the product of the c-*myc* gene) epitope tag (MEEQKLISEEDLGIRGR) was cloned into the pcDNA3 vector with the initial methionine in a Kozak consensus sequence for initiation of translation [27]. Copies of the cDNAs, encoding full-length Grb2 and Grb7, were obtained by PCR using a human K562 cDNA sample as template and cloned directly into the vector described above as fusions with the c-Myc tag at the N-terminus. The N-terminally T7-tagged G6f and G6f(Y281F) were transiently expressed in Cos-7 cells with the DEAE-dextran method as described above and cells were treated with or without pervanadate before lysis in ice-cold NP40 lysis buffer. Lysates were cleared by centrifugation and mixed with lysates of Cos-7 cells transfected with c-Myc-tagged Grb2 and Grb7. The mixtures were incubated overnight at 4 °C and immunoprecipitated with a

c-Myc mAb (Clontech, Palo Alto, CA, U.S.A.). Immunoprecipitates were analysed on Western-blot immunostaining with the anti-T7 mAb peroxidase conjugate for the presence of G6f and anti-c-Myc polyclonal antibodies (Upstate Biotechnology) for detection of Grb2 and Grb7.

For co-immunoprecipitation of endogenous Grb2 with G6f, K562 cells stably transfected with T7-tagged G6f and G6f(Y281F) were treated with or without pervanadate and lysed as described above. The co-immunoprecipitation was performed with an anti-Grb2 polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and immunoprecipitates were analysed by Western-blot immunostaining with the anti-T7 mAb and anti-Grb2 mAb (Santa Cruz Biotechnology).

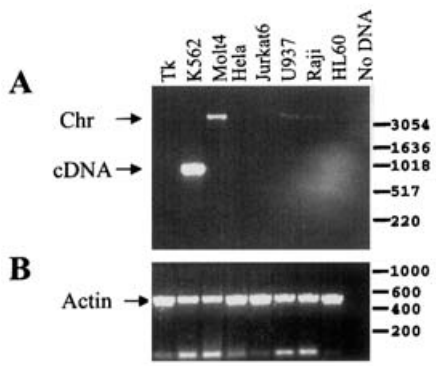
Antibody cross-linking and p42/44 MAP kinase phosphorylation

K562 cells expressing either N-terminally tagged G6f or G6f(Y281F) were spun down, washed and resuspended in PBS [$(1–1.5) \times 10^7$ cells/ml]. Cells were incubated with or without a combination of the anti-T7 mAb (1 μg/ml) and goat anti-mouse IgG (2.3 μg/ml) (Sigma) for the indicated period of time at 37 °C. As control, K562 cells transfected with an empty pcDNA3 vector underwent the same treatment. To examine the effect of the MEK (MAP kinase kinase) inhibitors PD098059 (20 μM) and U0126 (20 μM) (Sigma), antibody cross-linking was performed in the presence of these two compounds. Cells were directly lysed in SDS/PAGE sample buffer and analysed by Western-blot immunostaining with polyclonal antibodies against p42/44 MAP kinase and against phospho-p42/44 MAP kinase (T202/Y204) (New England Biolabs, Hitchin, Herts., U.K.). The latter antibody recognizes p42/44 MAP kinase only when phosphorylated at T202 and Y204 for p44 and at T185 and Y187 for p42. Total lysates after antibody cross-linking were analysed by Western-blot immunostaining with the anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology).

RESULTS

RT–PCR

The cDNA encoding the G6f ORF was obtained using a nested PCR approach. As can be seen in Figure 1(A), a mature transcript of approx. 900 bp could only be amplified from the human K562 cell line. However, we obtained the control β-actin PCR products in all the cDNA samples tested (Figure 1B). The exon structure of the G6f ORF is the reverse of nt 43 161–43 110 (exon 1), 42 610–42 281 (exon 2), 42 197–41 934 (exon 3), 40 041–39 886 (exon 4), 39 715–39 649 (exon 5) and 39 500–39 476 (exon 6) (for all nucleotide numbers refer to GenBank® accession no. AF129756). Nucleotide sequencing of the PCR fragment revealed a difference in the three available genomic sequences affecting the amino acid sequence. Codon 167 codes in the PCR fragment for lysine (arginine in genomic sequences AF129756, AL132713 and AP000504). To resolve this discrepancy, we PCR-amplified the corresponding genomic regions from three healthy humans [25] (results not shown). In all the three cases, codon 167 codes for an arginine. Based on these results combined with the genomic data in the GenBank®/EMBL database, we concluded that arginine is the wild-type residue at position 167 and that the lysine found at position 167 in the PCR product derived from the K562 cell line might represent a less abundant single-nucleotide polymorphism in the *G6f* gene. This R167K polymorphism has been detected before by denaturing HPLC [25] with a frequency of 12.5 % in the samples studied. In the present study, we used for expression the most common G6f isoform with arginine at position 167.



C
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  1  MAVLFLLLFL CGTPQAADNM QAIYVALGEA VELPCPSPPT LHGDEHLSWF
 51  CSPAAGSFTT LVAQVQVGRP APDPGKPGRE SRLRLLGNYS LWLEGSKEED
101  AGRYWCAVLG QHHNYQNWRV YDVLVLKGSQ LSARAADGSP CNVLLCSVVP
151  SRRMDSVTWQ EGKGPVRGRV QSFWGSEAAL LLVCPGEGLS EPRSRRPRII
201  RCLMTHNKGV SFSLAASIDA SPALCAPSTG WDMPWILMLL LTMGQGVVIL
251  ALSIVLWRQR VRGAPGRDAS IPQFKPEIQV XENIHLARLG PPAHKPR
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Figure 1 RT–PCR analysis and predicted amino acid sequence of human G6f

(**A**) Second round of the nested PCRs on human cDNA preparations using G6f-specific primers. The cell lines from which the cDNA samples were derived are denoted above the gel. Chr corresponds to the band derived from chromosomal contamination. (**B**) β-actin control of the cDNA preparations. Molecular-mass markers (bp) are indicated on the right. (**C**) Predicted amino acid sequence of G6f. The putative signal sequence is shown in bold-italic face. The two cysteine residues predicted to form the disulphide bridge in the V-type Ig domain are in boldface. The predicted transmembrane segment is underlined. Y281 is in boldface and underlined.

The derived amino acid sequence of G6f contains a putative leader peptide (amino acids 1–16), an extracellular part (amino acids 17–234), a transmembrane segment (amino acids 235–257) and a 40-amino-acid-long cytoplasmic tail (amino acids 258–297) (Figure 1C). BLAST searches with the G6f amino acid sequence indicated that residues 15–125 showed homology with V-type Ig domains of other proteins. In addition, manual inspection of the sequence confirms that the major key residues that are characteristic for Ig domains are present [28,29], including the two cysteine residues that form a disulphide link (C35 and C106). This V-type segment of G6f is encoded by one exon (exon 2) flanked at both ends by phase I introns, a feature often found in the genes of Ig superfamily members [30–32]. The rest of the putative extracellular domain (amino acids 126–234) shows no significant homology with a characterized protein. It is, however, large enough to form an independent globular domain. The protein analysis tool PIX (http://www.hgmp.mrc.ac.uk/Registered/Webapp/pix/), which combines four different secondary-structure prediction programs, predicts that this stretch probably comprises β-strands and loop structures, but hardly any helix. More specifically, at least seven β-strands can be predicted in this segment (results not shown). This would fit with the assumption that this part of the polypeptide chain is a globular all-β domain, possibly an Ig-like domain. Another feature is the presence of one tyrosine residue (Y281) in the cytoplasmic tail that could potentially undergo tyrosine phosphorylation.

Expression in Cos-7 and K562 cells, glycosylation, immunofluorescence and tyrosine phosphorylation

Expression of N- and C-terminal epitope-tagged G6f in Cos-7 cells yielded a protein with an apparent molecular mass of approx. 29 kDa when run under reducing conditions on SDS/PAGE (Figure 2A), which is similar to the calculated molecular mass

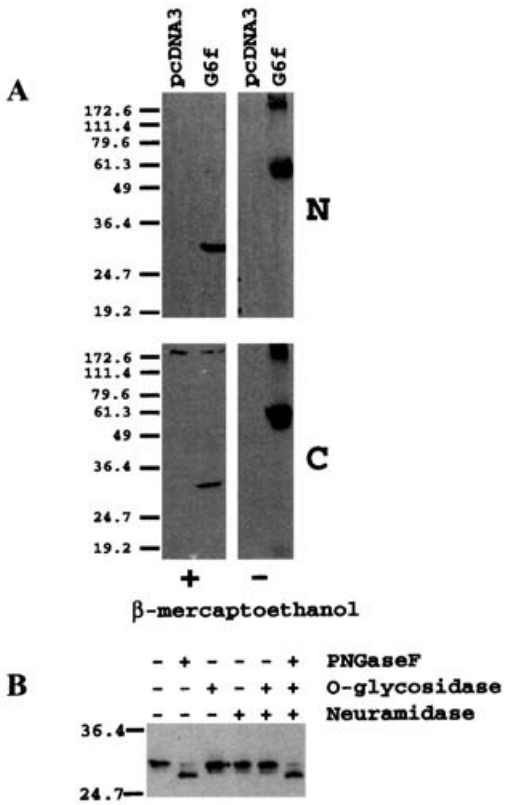

Figure 2 Expression of G6f in Cos-7 cells and glycosidase treatment

(**A**) The N-terminal (N) and C-terminal (C) T7 epitope-tagged G6f constructs were transiently expressed in Cos-7 cells. Expression was analysed by SDS/PAGE under reducing (+) and non-reducing (−) conditions followed by Western-blot immunostaining. The negative control cells were transfected with empty pcDNA3 vector. (**B**) Lysates of Cos-7 cells expressing N-terminally T7 epitope-tagged G6f were treated with various glycosidases alone or in combination as indicated and analysed by Western-blot immunostaining. PNGase F, peptide N-glycosidase F. Molecular-mass markers (kDa) are indicated on the left.

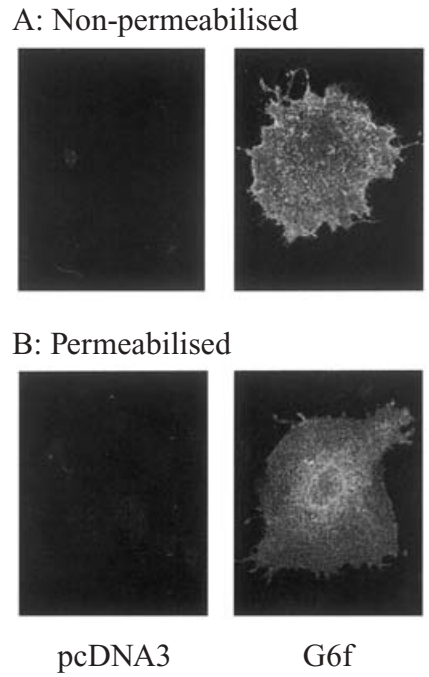

Figure 3 Immunofluorescence of G6f

G6f was expressed in Cos-7 cells as an N-terminal T7 epitope-tagged-fusion protein. The staining was performed under non-permeabilizing (**A**) as well as under permeabilizing conditions (**B**). As negative controls, staining was also performed on cells transfected with an empty pcDNA3 vector.

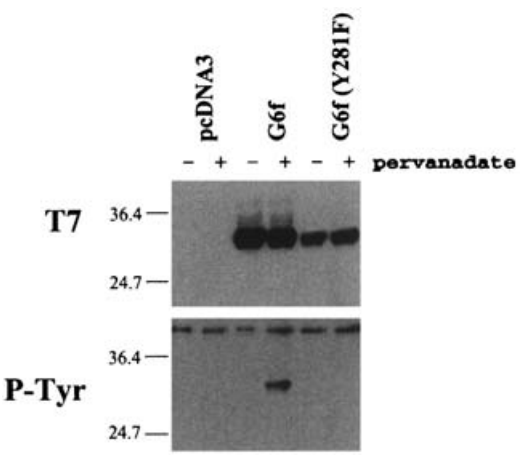

Figure 4 Pervanadate treatment of K562 cells expressing G6f and G6f(Y281F)

Cells expressing T7 epitope-tagged G6f and G6f(Y281F) were not treated (−) or treated (+) with pervanadate and immunoprecipitations were performed with the anti-T7 mAb. Immunoprecipitates were analysed by Western-blot immunostaining with the anti-T7 mAb (upper panel) and the anti-phosphotyrosine mAb (lower panel). As negative control, cells transfected with the empty pcDNA3 vector underwent the same treatment. Molecular-mass markers (kDa) are indicated on the left.

for epitope-tagged G6f (33 kDa). However, under non-reducing conditions, the molecular mass of the protein is about twice as large (approx. 60 kDa), indicating that G6f forms a disulphide-linked dimer. Glycosidase treatment of G6f showed that the protein is N-glycosylated (Figure 2B), in line with the presence of a consensus N-glycosylation site [NX(S/T)] at positions 88–90 in the amino acid sequence. No evidence for the presence of O-glycosylation was obtained.

Immunofluorescence with N-terminally tagged G6f under non-permeabilizing conditions (Figure 3A) showed a clear localization of the protein at the cell surface. A similar overall stain of the cell was observed under permeabilizing conditions (Figure 3B), indicating an efficient transport of G6f to the cell surface.

To investigate whether the only tyrosine in the cytoplasmic tail of G6f(Y281) can be phosphorylated, K562 cells expressing G6f or a variant form of G6f, where Y281 is changed into an F [G6f(Y281F)], were treated with pervanadate. As shown in Figure 4, G6f becomes tyrosine-phosphorylated. However, the Y281F mutant does not, thereby proving that Y281 is indeed the residue to undergo phosphorylation.

GST pull-down assay and co-immunoprecipitation

To investigate whether the SH2 domains of Grb2 and Grb7 recognize G6f in a phosphorylation-dependent manner, GST pull-down assays were performed with phosphorylated and non-phosphorylated G6f derived from stably transfected K562 cells treated with or without pervanadate respectively. The SH2 domains of Grb2 (wild-type and R86K dominant-negative mutant), Grb7 (wild-type and R458K dominant-negative mutant) and Grb10, and the BPS domain of Grb7, were expressed as GST

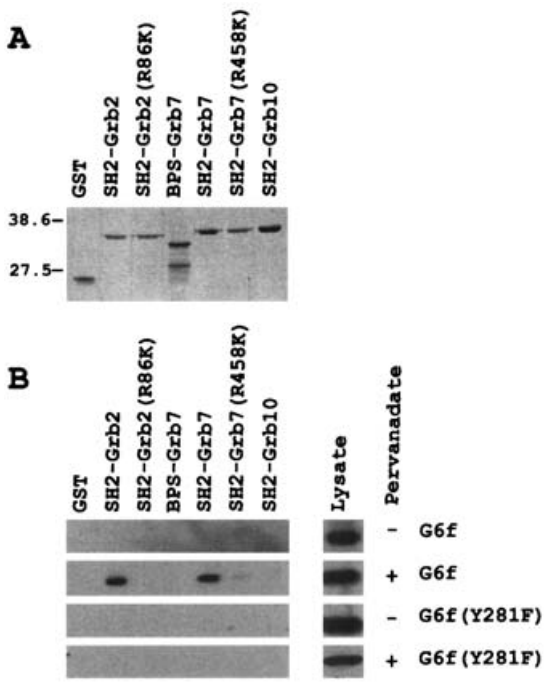

Figure 5 GST pull-down assay of T7-tagged G6f and G6f(Y281F) with domains of Grb2 (SH2), Grb7 (BPS and SH2) and Grb10 (SH2)

(**A**) Coomassie-Blue-stained gel of purified GST-fusion proteins. Molecular-mass markers (kDa) are indicated on the left. (**B**) Western blot with the anti-T7 mAb of GST pull-downs. K562 cells expressing either T7-tagged G6f or G6f(Y281F) were either untreated (−) or treated (+) with pervanadate as indicated and lysed. The GST-fusion proteins used to pull-down G6f from these lysates are indicated at the top of the blot. Amounts of G6f and G6f(Y281F) present in the cell lysates are shown on the right.

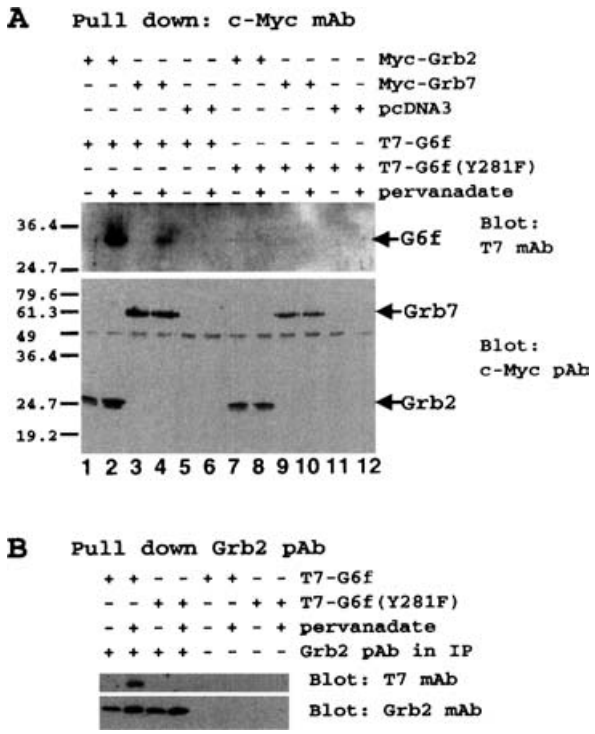

Figure 6 Co-immunoprecipitation of full-length G6f and G6f(Y281F) with full-length Grb2 and Grb7

(**A**) Cos-7 cells were transfected with either Myc-tagged Grb2, Myc-tagged Grb7 or empty vector (pcDNA3) as indicated. Lysates of these cells were mixed with lysates of Cos-7 cells [untreated (−) or treated (+) with pervanadate] expressing either G6f or G6f(Y281F). Immunoprecipitation was performed with the c-Myc mAb and immunoprecipitates were analysed on Western-blot immunostaining with the T7 mAb (upper panel) or c-Myc pAb (lower panel). (**B**) Co-immunoprecipitation of endogenous Grb2 with G6f. K562 cells stably transfected with either T7 epitope-tagged G6f or G6f(Y281F) were either untreated or treated with pervanadate. Immunoprecipitations with or without the Grb2 pAb were performed on the lysates and immunoprecipitates were analysed by Western-blot immunostaining with the anti-T7 mAb and a Grb2 mAb as indicated.

fusions in *E. coli* and purified with glutathione–agarose beads (Figure 5A). As shown in Figure 5(B), the SH2 domains of Grb2 and Grb7 recognize phosphorylated G6f, whereas the dominant-negative mutant forms of those domains do not (Grb2-R86K) or show strongly reduced binding (Grb7-R458K). Furthermore, the SH2 domain of Grb10 does not interact with phosphorylated G6f either. The BPS domain of Grb7 showed no interaction with G6f and although the GST–BPS-fusion protein sample contains two bands (Figure 5A), the upper band corresponds to undegraded protein as judged by its mass. As expected, the G6f(Y281F) protein did not interact with any of the GST fusions studied. We failed to express soluble GST-fusion proteins in *E. coli* that contained the PH domain of Grb7 as well as the GST fusions with full-length Grb2 and Grb7. Although a construct encoding the SH2 domain of Grb14 was also prepared, this protein was not expressed for unknown reasons.

To establish whether G6f is capable of recognizing full-length Grb2 and Grb7, full-length c-Myc-tagged Grb2 and Grb7 were expressed in Cos-7 cells, mixed with either unphosphorylated or phosphorylated G6f and a co-immunoprecipitation was performed with the c-Myc mAb. As is shown in Figure 6(A), phosphorylated G6f (derived from pervanadate-treated cells) interacts with both the full-length Grb2 and Grb7 (lanes 2 and 4), whereas unphosphorylated G6f (derived from cells not treated with pervanadate) does not (lanes 1 and 3). As expected, G6f(Y281F) does not interact with either Grb2 or Grb7 (lanes 7–10).

In K562 cells stably transfected with T7-tagged G6f, endogenous Grb2 co-immunoprecipitated with G6f, but not with the G6f(Y281F) mutant (Figure 6B). Owing to the low levels of endogenous Grb7 in K562 cells detectable by commercially available antibodies (results not shown), a similar interaction could not be shown for this molecule with G6f.

Antibody cross-linking

The interaction of G6f with Grb2 suggests a coupling of G6f with the Ras–MAP kinase pathway. For this reason, the phosphorylation status of the p42/44 MAP kinase (extracellular-signal-regulated protein kinase-1/2) was examined in K562 cells expressing N-terminally tagged G6f or G6f(Y281F) after antibody cross-linking. An increased phosphorylation of p42/44 MAP kinase is observed in G6f expressing cells as a result of antibody cross-linking (Figure 7A). This increase is not observed in cells expressing G6f(Y281F) and in cells transfected with empty vector. A time-course experiment showed that the enhanced phosphorylation of p42/44 MAP kinase after antibody cross-linking of G6f is transient and reached a peak at approx. 5 min (Figure 7B). MEK inhibitors PD098059 and U0126 both inhibit this antibody cross-linking-induced phosphorylation (Figure 7C).

To examine whether more proteins undergo tyrosine phosphorylation after antibody cross-linking of K562 cells stably transfected with G6f, total cell lysates were analysed on Western-blot immunostaining with the anti-phosphotyrosine antibody.

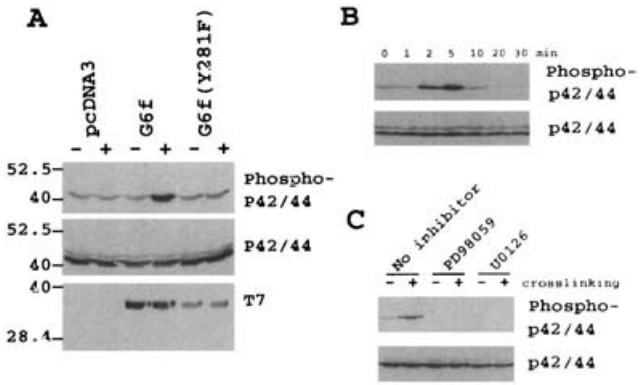

Figure 7 Effect of antibody cross-linking of G6f and G6f(Y281F) on phosphorylation of p42/44 MAP kinase in K562 cells

(**A**) Cells expressing T7 epitope-tagged G6f and G6f(Y281F) were untreated (−) or treated (+) with a combination of the anti-T7 mAb and anti-mouse IgG for 5 min. As negative control, cells transfected with the empty pcDNA3 vector underwent the same treatment. Total cell lysates were analysed for the presence of phosphorylated p42/44 MAP kinase (upper panel), total p42/44 MAP kinase (middle panel) and T7 epitope-tagged G6f (lower panel). Molecular-mass markers (kDa) are indicated on the left. (**B**) G6f expressing K562 cells were subjected to antibody cross-linking for the times indicated. Total lysates were analysed for phospho-p42/44 MAP kinase and p42/44 MAP kinase levels. (**C**) G6f expressing K562 cells were either untreated (−) or subjected to antibody cross-linking (+) for 5 min in the absence or presence of the MEK inhibitors PD098059 and U0126 as indicated. Total lysates were analysed for phospho-p42/44 MAP kinase and p42/44 MAP kinase levels.

Only two proteins showed a clear increase in tyrosine phosphorylation on antibody cross-linking: one with a molecular mass of approx. 42 kDa (most probably the p42 MAP kinase) and an unidentified protein of approx. 120 kDa (results not shown). This increase was not observed in cells expressing G6f(Y281F) or cells transfected with empty vector after the same treatment. This experiment provides evidence that G6f can couple with the Ras–MAP kinase pathway in living cells and that Y281 of G6f is essential for this coupling.

DISCUSSION

We have established that the cytoplasmic tail of G6f can bind the SH2 domain containing signalling adaptor proteins Grb2 and Grb7 and that these interactions are dependent on the phosphorylation of Y281 of G6f. SH2 domains are specialized in binding phosphotyrosine motifs and comprise a central antiparallel β-sheet sandwiched between two α-helices [1,2]. The phosphotyrosine moiety is bound by a positively charged pocket on one side of the β-sheet and a surface on the other side that binds residues C-terminal to the phosphotyrosine residue. It has been clearly established that residues $+1$ to $+3$, C-terminal to the phosphotyrosine, determine the specificity of binding and that the mere presence of a phosphotyrosine residue is in itself not sufficient for binding to an SH2 domain [2]. To illustrate this, we included the SH2 domain of another Grb7 family member, i.e. Grb10, which shows that this particular SH2 domain does not interact with the phosphorylated G6f under the experimental conditions used. Furthermore, the absence of binding of phosphorylated G6f to dominant-negative mutants of the SH2 domains of Grb2 and Grb7, in which the highly conserved arginine residues that bind the phosphate are mutated, shows the specificity of the interaction.

The sequence surrounding the phosphotyrosine of G6f(Y281ENI) is very similar to the consensus sequence of [Y(M/E)NW] for high-affinity binding to the SH2 domain of Grb2 [5]. Furthermore, similar motifs found in human LAT (Y226ENL) and SHP2 (Y580ENV) have been shown to interact with Grb2 [12,33]. Interestingly, Y580 of SHP2 has also been shown to bind the SH2 domain of Grb7 [12], in line with our results. One can tentatively conclude from these results that the presence of a glutamate residue at the $+1$ position (in combination with an asparagine residue at $+2$ and a hydrophobic residue at $+3$) is diagnostic of a motif that will interact with both the SH2 domains of Grb2 and Grb7. However, to our knowledge there are no reports on a possible association between Grb7 and LAT.

The interaction between phosphorylated G6f and Grb2, the transient increase in phosphorylation of p42/44 MAP kinase after antibody cross-linking of G6f in K562 cells, and the inhibition of that phosphorylation by MEK inhibitors strongly suggest a coupling of G6f with the Ras–MAP kinase pathway. This pathway is involved in a variety of tasks such as proliferation, differentiation, survival and apoptosis [34]. The downstream pathways that connect with Grb7 are not well defined although its association with focal adhesion kinase in a cell-adhesion-dependent manner suggests a role for Grb7 in integrin signalling [35]. Therefore further work is required to elucidate the effect of G6f activation on cellular function.

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