

Tyrosine phosphorylation of the human guanylyl cyclase C receptor

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Tyrosine phosphorylation events are key components of several cellular signal transduction pathways. This study describes a novel method for identification of substrates for tyrosine kinases. Co-expression of the tyrosine kinase EphB1 with the intracellular domain of guanylyl cyclase C (GCC) in *Escherichia coli* cells resulted in tyrosine phosphorylation of GCC, indicating that GCC is a potential substrate for tyrosine kinases. Indeed, GCC expressed in mammalian cells is tyrosine phosphorylated, suggesting that tyrosine phosphorylation may play a role in regulation of GCC signalling. This is the first demonstration of tyrosine phosphorylation of any member of the family of membrane-associated guanylyl cyclases.

1. Introduction

A change in tyrosine phosphorylation of proteins is an important mechanism of cellular signal transduction, and can regulate both protein function, as well as interaction with other proteins (Fantl *et al* 1993). Consequently, novel pathways in signalling cascades can be suggested by the identification and characterization of tyrosine phosphorylation sites in proteins. Classically, tyrosine phosphorylation sites have been identified by peptide mapping of phosphoproteins usually radiolabelled *in vivo*. Screening of peptide libraries has led to the identification of consensus substrate sequences for specific protein kinases, making it possible to predict phosphorylation sites based on sequence analysis (Songyang *et al* 1995). In this study we describe another approach for the identification of novel tyrosine phosphorylation sites in a protein. This involves co-expression in *Escherichia coli* of a tyrosine kinase along with domains of a receptor guanylyl cyclase, earlier not known to undergo tyrosine phosphorylation.

Guanylyl cyclase C (GCC), the receptor for the family of heat-stable enterotoxin peptides and the guanylin family of endogenous peptides, is known to play a role in the regulation of fluid and ion balance in the intestinal epithelium (Drewett and Garbers 1994). Ligand mediated activation of GCC leads to an increase in intracellular cGMP levels, which in turn leads to the activation of cyclic nucleotide-dependent protein kinases (Chao *et al* 1994; Vaandrager *et al* 1997). The activated kinases phosphorylate and activate the cystic fibrosis transmembrane conductance regulator (CFTR), thereby leading to an efflux of Cl⁻ ions and fluid from the cells (Chao *et al* 1994). Analysis of GCC expression in the rat and opossum intestine, by *in situ* hybridization (Li and Goy 1993; Krause *et al* 1994), and immunohistochemical studies (Nandi *et al* 1997), has shown that GCC is expressed in certain cell types in the intestine that do not express CFTR (Trezise and Buchwald 1991). This suggests that GCC may have other functions in the cell besides regulation of chloride secretion *via* the CFTR. One such possibility is

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Abbreviations used: GCC: Guanylyl cyclase C; CFTR: cystic fibrosis transmembrane regulator; GST: glutathione S transferase; PKLD: protein kinase-like domain; CTD: C-terminal domain; GCD: guanylyl cyclase domain; PVDF: polyvinylidene difluoride; GCA: guanylyl cyclase A.

that GCC may undergo changes in phosphorylation, and this in turn mediates novel functions of GCC.

The regulation of GCC activity by protein kinase C mediated phosphorylation of a specific serine residue has been described earlier (Roy and Visweswariah 1998). In order to examine whether GCC is a substrate for tyrosine phosphorylation, we have utilized a novel method to examine whether the intracellular domain of GCC is tyrosine phosphorylated, by co-expression of various domains of GCC with the tyrosine kinase, EphB1/Elk (Lhotak *et al* 1991; Eph Nomenclature Committee 1997). GCC was found to be tyrosine phosphorylated under these conditions, indicating that it is a potential substrate for tyrosine kinases. We also demonstrate that GCC expressed in mammalian cells is indeed tyrosine phosphorylated, suggesting that tyrosine phosphorylation of GCC may play a role in GCC mediated cellular signalling.

2. Materials and methods

2.1 Generation of anti-phosphotyrosine antibodies

A polyclonal antiserum to phosphotyrosine was generated as described earlier (Kamps 1991b) and phosphotyrosine specific antibodies purified from the IgG fraction of the antiserum by affinity chromatography using phosphotyrosine linked to Affi-Gel 15 (Bio-Rad). This antibody was extensively characterized to show its specificity for phosphotyrosine (data not shown).

2.2 Construction of plasmids expressing various regions of the intracellular domain of GCC

Subclones of the intracellular domain of GCC, represented diagrammatically in figure 3a, were constructed from pBSK-GCC (Singh *et al* 1991) as follows:

- (i) *GCC-ID1*: The 1626 bp fragment released on digestion of pBSK-GCC by *Bam*HI and *Sma*I was cloned into pGEX2T (Amersham Pharmacia Biotech, UK) to generate a plasmid expressing the recombinant protein designated GCC-ID1. This protein includes the C-terminal 507 amino acids of GCC as a fusion with glutathione S transferase (GST).
- (ii) *GCC-protein kinase-like domain*: A DNA fragment generated by digestion of pRSET-ID6 (Nandi *et al* 1997) with *Pvu*II was cloned into the *Sma*I site of pGEX-5X2. This construct expresses the N-terminal 330 amino acids of the intracellular domain of GCC, encompassing the protein kinase-like domain (PKLD) of GCC, as a GST fusion.
- (iii) *GCC-C-terminal domain*: A 429 bp *Eco*RI fragment from pBSK-GCC, was ligated into the *Eco*RI site of

pGEX-5X2 to generate pGEX-CTD, which expresses the C-terminal 112 amino acids of GCC as fusion with GST.

(iv) *GCC-guanylyl cyclase domain*: The cDNA coding for the guanylyl cyclase domain (GCD) of GCC (from Pro 732 to the C-terminus) was obtained by PCR amplification from pBSK-GCC, and cloned into pTrc99A at *Kpn*I–*Pst*I sites. The primers used for amplification were (1) sense strand: 5' CCA GAA AAG GTA CCA GAT TTC AAA 3', and (2) antisense strand: 5' CAT CTG CAG TTA AAA ATA GGT GCT 3'. A *Kpn*I–*Hind*III fragment from this clone was ligated into pRSET C (Invitrogen, USA). The resulting plasmid was digested with *Bam*HI and *Hind*III, and ligated into *Bam*HI–*Hind*III digested pGEX-CTD to generate a plasmid expressing the catalytic domain (342 amino acids) of GCC as a fusion with GST.

2.3 Expression of fusion proteins in TKB1

The BL21(DE3) pTK strain of *E. coli* (abbreviated TKB1), that harbours a plasmid encoding the tyrosine kinase Elk (EphB1), was obtained from Stratagene (USA). Transformation and induction of TKB1 cells was carried out as per the manufacturers' protocol, with slight modifications. TKB1 cells transformed with the plasmid of interest were grown to an A_{600} of 0.5, in Luria Bertani broth containing antibiotics at 37°C. Cells were collected by centrifugation and resuspended in induction medium, containing 1 mM IPTG, to induce the gene of interest, and 10 µg/ml **b** Indole acrylic acid, to induce expression of the *ephB1* gene. Cells were grown for an additional 3 h at 37°C, collected by centrifugation and sonicated (Virtis 475 sonicator) in 50 mM Tris-Cl pH 8.0, 5 mM 2-mercaptoethanol, 0.1 M NaCl, 2 mM benzamidine, 5 mM EDTA, 100 µM sodium orthovanadate, 50 mM sodium fluoride and 1% Triton X-100. Fractionation of the cell lysates by centrifugation at 10,000 g for 20 min, and analysis by SDS-PAGE revealed that the proteins were predominantly localized to the inclusion body fraction.

2.4 [³²P]-labelling of TKB1 cells

TKB1 cells expressing GCC-ID1 were grown in Luria Bertani broth to an A_{600} of 0.5, collected by centrifugation and resuspended in 50 mM HEPES, pH 7.5, 19 mM NH₄Cl, 8 mM NaCl, 20 µM CaCl₂, 1 mM MgSO₄, 0.2% glucose, 0.1% casamino acids, 0.00005% thiamine, 100 µg/ml of ampicillin, and 12.5 µg/ml tetracycline. [³²P]-orthophosphate (500 µCi per 10 ml culture, specific activity 8500 Ci/mmol, NEN Life Science Products) was added to the medium, cells grown at 37°C for 1 h and induced for an additional 3 h by the addition of IPTG and **b** Indole-acrylic acid. Cells were harvested and lysed as described above, and [³²P]-labelled protein purified from

the inclusion body fraction by electroelution from polyacrylamide gels (Nandi *et al* 1997).

2.5 Immunoprecipitation, phosphoamino acid analysis and phosphopeptide mapping of radiolabelled GCC-ID1

Approximately 20 µg of [³²P]-labelled purified GCC-ID1, was diluted in RIPA buffer (50 mM Tris-Cl, pH 8.0, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 150 mM NaCl), containing 1 mg/ml BSA, 5 mM EDTA, 50 mM sodium fluoride, 1 mM benzamide, 100 µM sodium orthovanadate and 1 µg/ml leupeptin. The protein was incubated with normal mouse IgG (7 µg, Sigma), or GCC monoclonal antibody, GCC:C8 (7 µg; Bakre *et al* 2000), normal rabbit IgG (12 µg) or affinity purified anti-phosphotyrosine polyclonal IgG (12 µg) at 4°C for 12 h, and the immune complexes precipitated with Protein A Sepharose beads (Life Technologies, USA). The Protein A beads were washed sequentially with RIPA buffer and phosphate buffered saline, boiled in SDS sample buffer, and subjected to SDS-PAGE in a 10% polyacrylamide gel. The gel was fixed and stained prior to exposing to Kodak XK-5 film at -70°C.

For phosphoamino acid analysis, GCC-ID1 inclusion body fraction containing the radiolabelled protein, was resolved by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore). The radiolabelled protein on the membrane was hydrolysed (Kamps 1991b), the resulting phosphoamino acids separated by one-dimensional thin-layer electrophoresis at pH 3.5 (Boyle *et al* 1991) and visualized by autoradiography.

The inclusion body fraction containing radiolabelled GCC-ID1 was subjected to reduction and carboxyamidation in the presence of 1% SDS, 5 mM DTT and 25 mM iodoacetamide, according to standard procedures (Carne 1994). The reduced and carboxyamidated proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The band of interest was subjected to tryptic digestion and phosphopeptide mapping (Boyle *et al* 1991).

2.6 Detection of tyrosine phosphorylation of GCC in HEK293-GCC cells

We have earlier reported the generation of the cell line HEK293-GCC that expresses high levels of human GCC (Bakre *et al* 2000). HEK293-GCC cells were lysed by sonication in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DDT, 5 mM EDTA, 1 mM sodium orthovanadate, 50 nM okadaic acid, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 80 µM *b*-glycerol phosphate, 5 µg/ml soyabean trypsin inhibitor, 5 µg/ml aprotinin and 5 µg/ml leupeptin. The membrane fraction

was obtained from the cell lysate by centrifugation at 10,000 *g* for 1 h, and solubilized in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 1% Triton-X100, 0.5% Nonidet P 40, 150 mM sodium chloride, 1 mM EDTA, 200 µM sodium orthovanadate, 50 nM okadaic acid, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 80 µM *b*-glycerol phosphate, 5 µg/ml soyabean trypsin inhibitor, 5 µg/ml aprotinin and 5 µg/ml leupeptin). The solubilized sample was pre-cleared by incubation with 5 µg/ml normal mouse IgG for 10 h at 4°C followed by 10 µl Protein A Sepharose beads for 2 h at 4°C. Immunoprecipitation was carried out by incubation with 5 µg/ml GCC:4D7 monoclonal antibody raised to the PKLD of GCC (unpublished results), for 10 h at 4°C, followed by Protein A Sepharose beads for 2 h at 4°C. The beads were washed extensively with immunoprecipitation buffer and boiled in SDS sample buffer. The immunoprecipitated proteins were resolved by SDS-PAGE in a 7.5% polyacrylamide gel and transferred to a PVDF membrane. Western blot analysis was carried out as described earlier (Bakre *et al* 2000) using the pY20 monoclonal antibody (Transduction Labs, USA), followed by detection using ECL Plus reagent (Amersham Pharmacia Biotech, UK). Blots were subsequently stripped and re-probed with the GCC:C8 monoclonal antibody.

3. Results

3.1 Demonstration of tyrosine phosphorylation on GCC

To examine whether GCC is a potential substrate for tyrosine kinases, we over-expressed the intracellular domain of GCC in TKB1. We were unable to obtain transformants of the plasmid expressing the entire intracellular domain of GCC in TKB1 cells, and therefore expressed GCC-ID1, a GST fusion of a part of the intracellular domain of GCC (aa 567-1073), in TKB1 cells. Expression of GCC-ID1 in TKB1 cells in the presence of [³²P]_i yielded [³²P]-labelled GCC-ID1, which was purified from the inclusion body fraction (figure 1a, lane 1). The identity of the labelled protein was confirmed by immunoprecipitation with a monoclonal antibody specific to the PKLD of GCC (figure 1a, lane 3). Moreover, immunoprecipitation of the radiolabelled protein was observed with phosphotyrosine antibodies (figure 1a, lane 5). Phosphoamino acid analysis of [³²P]-labelled GCC-ID1 revealed the presence of only radiolabelled phosphotyrosine, with no detectable amounts of phosphoserine and phosphothreonine (figure 1b).

3.2 Analysis of tyrosine phosphorylation sites in GCC-ID1

Tryptic digestion and two-dimensional phosphopeptide mapping of [³²P]-labelled GCC-ID1 indicated at least 12

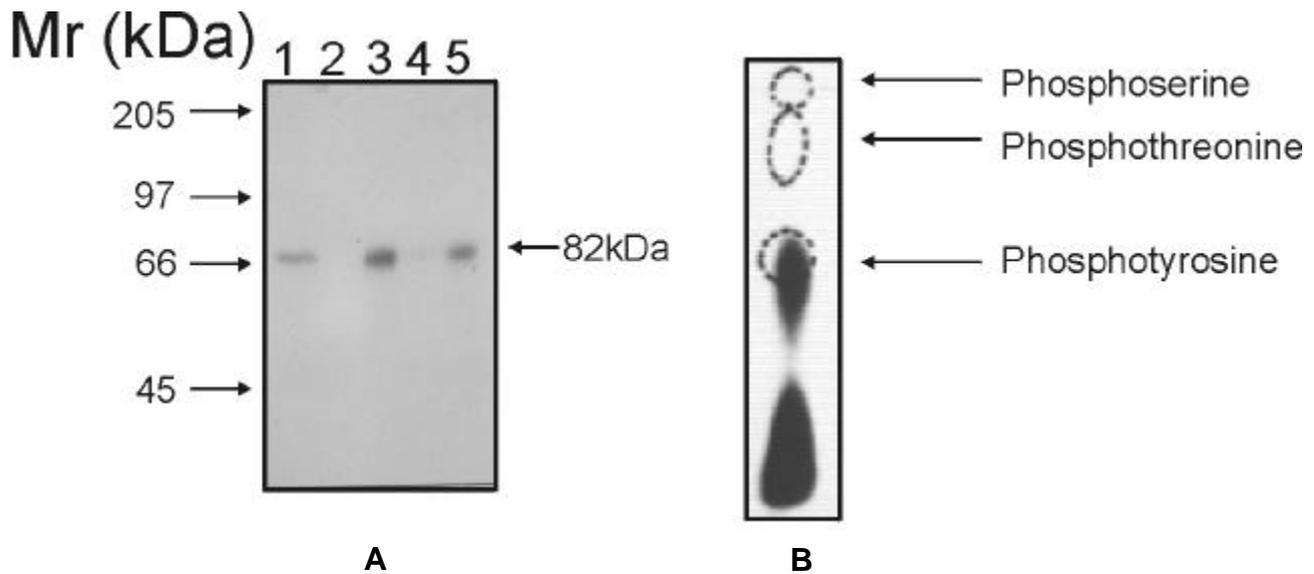


Figure 1. Immunoprecipitation of [^{32}P]-labelled GCC-ID1 and phosphoamino acid analysis. (A) [^{32}P]-labelled GCC-ID1 expressed in TKB1 cells was purified from the inclusion body fraction by electroelution (lane 1). This purified protein was incubated with either normal mouse IgG (lane 2), GCC:C8 monoclonal antibody (lane 3), normal rabbit IgG (lane 4) or polyclonal anti-phosphotyrosine antibody (lane 5) and the immunoprecipitates analysed by SDS-PAGE followed by autoradiography. (B) [^{32}P]-labelled GCC-ID1 was subjected to hydrolysis as detailed in the text and products separated by electrophoresis on cellulose TLC plates, followed by autoradiography. Migration of phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) standards are indicated by dotted circles.

major spots (figure 2). This could correspond to partially digested phosphopeptides, but could also indicate the presence of multiple sites of tyrosine phosphorylation in the intracellular domain of GCC. To investigate this possibility, we constructed various sub-clones of the intracellular domain, (figure 3a), expressed these proteins in TKB1 and monitored tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibodies (figure 3b). As demonstrated earlier by phosphoamino acid analysis, GCC-ID1 was tyrosine phosphorylated when expressed in TKB1 cells, but not when expressed in the BL21(DE3) strain. GST alone, expressed in TKB1 cells was not tyrosine phosphorylated, indicating that the phosphorylation detected in GCC-ID1 was present only in the GCC fragment of the protein. The lack of phosphorylation of GST by EphB1 has also been reported earlier by the use of a similar expression system (Larose *et al* 1993).

The PKLD of GCC, (GCC-PKLD), expressed in TKB1, was phosphorylated on tyrosine (figure 3b), as was the sub-clone GCC-GCD, which spans the guanylyl cyclase domain. Since there are only two tyrosine residues in the 53 amino acid overlap between GCC-PKLD and GCC-GCD (Pro732 to Gln 784), it is possible that sites phosphorylated in PKLD and GCD may be distinct. The GCC sub-clone spanning the C-terminal domain, GCC-CTD, was also phosphorylated in TKB1. It can be concluded,

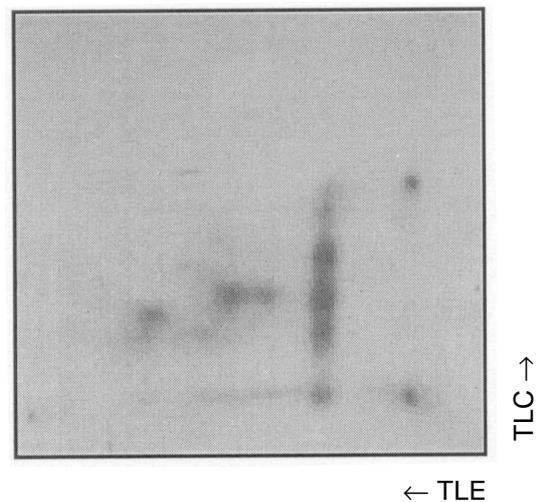


Figure 2. Phosphopeptide map analysis of GCC-ID1 expressed in TKB1. [^{32}P]-labelled GCC-ID1 was digested with trypsin as detailed in the text and the resulting phosphopeptides spotted onto cellulose TLC plates and separated in two dimensions. Spots were visualized following autoradiography.

therefore, that at least two sites for tyrosine phosphorylation could be present in the intracellular domain of GCC. As can be seen, utilization of the TKB1 strain allowed us to suggest that a protein not hither to known to be

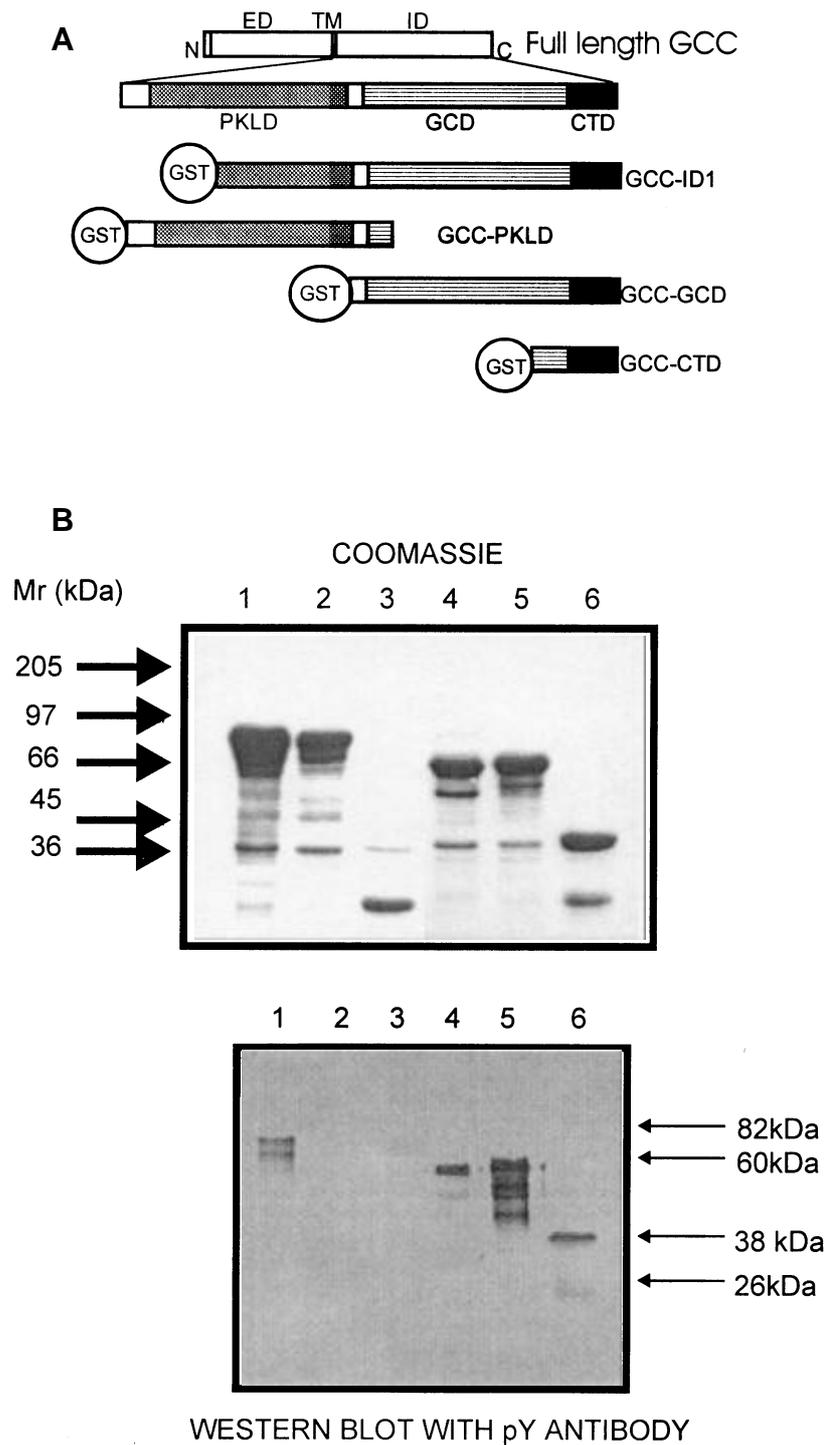


Figure 3. (A) Schematic representation of various subclones of the intracellular domain of GCC. GCC: guanylyl cyclase C; ED: extracellular domain; TM: transmembrane domain; ID: intracellular domain; N: N-terminus; C: C-terminus; PKLD: protein kinase-like domain; GCD: guanylyl cyclase domain; CTD: C-terminal domain; GST: glutathione S-transferase. Open boxes in the intracellular domain represent possible linker regions between the defined domains. (B) Expression of different regions of the intracellular domain of GCC in TKB1. *Top panel:* Coomassie Blue stained gel picture of inclusion body fractions (10 µg each) of GCC-ID1 expressed in TKB1 (lane 1), GCC-ID1 expressed in BL21(DE3) (lane 2), GST expressed from the pGEX-5X plasmid in TKB1 (lane 3), GCC-PKLD in TKB1 (lane 4), GCC-GCD in TKB1 (lane 5) and GCC-CTD in TKB1 (lane 6), subjected to SDS-PAGE. Molecular weight standards are indicated on the left. *Lower panel:* The inclusion body fractions (0.5 µg each) as in the top panel were blotted onto nitrocellulose membranes and probed with anti-phosphotyrosine antibody (0.6 µg/ml). Molecular weights of the expressed proteins are indicated by arrows.

phosphorylated on tyrosine may undergo tyrosine phosphorylation in mammalian cells.

3.3 GCC expressed in HEK-293 cells is tyrosine phosphorylated

The results presented so far suggest that GCC is a substrate for phosphorylation by the EphB1 tyrosine kinase, and may perhaps be a substrate for other tyrosine kinases when expressed in mammalian cells. We immunoprecipitated GCC from membranes prepared from HEK293-GCC cells, using a monoclonal antibody to GCC (GCC:4D7). Western blot analysis using an anti-phosphotyrosine antibody, confirmed that GCC was indeed tyrosine phosphorylated in mammalian cells (figure 4). Bands reacting with phosphotyrosine antibody co-migrated with the doublet of immunoreactive proteins (130 kDa and 145 kDa) detected by a GCC monoclonal antibody. The monoclonal antibody GCC:4D7 did not immunoprecipitate any proteins from COS7 cells (known not to express GCC) indicating that the bands detected with the phosphotyrosine antibody were specific to GCC, and not to any non-specific co-immunoprecipitating protein (data not shown).

4. Discussion

To our knowledge, the present study is the first report of the use of the TKB1 *E. coli* strain to demonstrate tyrosine phosphorylation of a mammalian protein that was to date not known to be phosphorylated on tyrosine residues, and suggests the utility of the TKB1 system to identify novel substrates for tyrosine kinases. In principle, expression of a protein of interest along with any tyrosine kinase in *E. coli* cells could be employed to identify substrates for other tyrosine kinases. Such an expression system allows the generation of large amounts of tyrosine phosphorylated protein, which can in turn be used to identify interacting proteins, perhaps containing SH2 domains. A similar expression strategy using EphB1 expression in bacteria has been used by Larose *et al* (1993) to identify residues in the platelet derived growth factor receptor that bind to the SH2 domain of phospholipase C- α 1.

Our study is the first to demonstrate tyrosine phosphorylation of a member of the family of receptor guanylyl cyclases. Earlier studies on the ANF receptor (guanylyl cyclase A, GCA), another member of this family of receptors, have demonstrated that GCA expressed in mammalian cells is phosphorylated on serine and threonine residues but not on tyrosine (Koller *et al* 1993; Potter and Garbers 1992, 1994). Although there is significant sequence similarity between the intracellular domains of the natriuretic peptide receptors and GCC

(deSavage *et al* 1991; Singh *et al* 1991), this difference in tyrosine phosphorylation could indicate that tyrosine phosphorylation is a unique feature of GCC and does not occur in other members of this family.

Tyrosine phosphorylation of the intracellular domain of GCC by EphB1 within bacterial cells suggests that GCC may be a substrate for the Eph family of tyrosine kinases in mammalian cells as well. Although EphB1 is not expressed in the intestine (Tang *et al* 1995), which is the major site of GCC expression, other members of this family, including EphA2 (Eck; Lindberg and Hunter 1990), and the mouse homologues EphB4 (MDK2) and EphB3 (MDK5; Ciossek *et al* 1995), are present in the intestine. Also, EphB1 is detected in the testis and kidney (Tang *et al* 1995), where the expression of GCC has been demonstrated (Forte *et al* 1989; Laney *et al* 1992), and perhaps also in HEK293 cells, where we have demonstrated GCC tyrosine phosphorylation. A family of gut-associated tyrosine kinases (Sunitha and Avigan 1996) are highly expressed in cell types in the intestine, which also express GCC (Nandi *et al* 1997). Furthermore, high levels of pp60c-src have been reported in colonic carcinoma cells, including T84 cells that express GCC (Cartwright *et al* 1989). It would be interesting to explore the possibility that GCC may be a substrate for these tyrosine kinases in the intestine and perhaps in other tissues.

We have been able to demonstrate that GCC is tyrosine phosphorylated in mammalian cells. The role of tyrosine phosphorylation in GCC function now remains to be examined. It is possible that changes in tyrosine phosphorylation may modulate GCC activity. Tyrosine phosphorylation

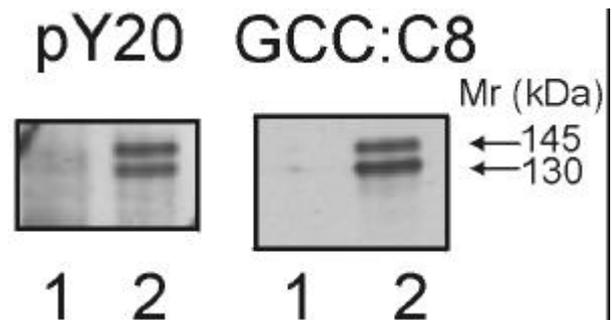


Figure 4. Tyrosine phosphorylation of GCC in HEK293-GCC cells. The solubilized membrane fraction from HEK293-GCC cells was incubated with normal mouse IgG (lane 1) or GCC:4D7 monoclonal antibody (lane 2) and immune complexes collected on protein A beads. Immunoprecipitated proteins were subjected to Western blot analysis with phosphotyrosine antibodies (*left panel*; pY20), followed by reprobing of the blot with a GCC-specific monoclonal antibody (*right panel*; GCC:C8). Molecular weights represent the sizes of the two differentially glycosylated forms of GCC in HEK293-GCC cells.

of GCC may also mediate interaction of GCC with other pathways in the cell. On phosphorylation, certain tyrosine residues may serve as sites for interaction of GCC with other intracellular proteins containing SH2 or PTB domains (Shoelson 1997), thereby leading to additional signal transduction events involving GCC. Answers to these questions will be available only when individual sites for tyrosine phosphorylation in GCC are characterized, and mutagenesis studies carried out to understand the role of GCC phosphorylation at such sites.

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