Functional interaction between the ser/thr kinase PKL12 and N-acetylglucosamine kinase, a prominent enzyme implicated in the salvage pathway for GlcNAc recycling

Jose Manuel Ligos†, Teresa Laín de Lera‡#, Stephan Hinderlich‡, Bárbara Guinea†, Luis Sánchez‡, Ramón Roca‡, Alfonso Valencia‡ and Antonio Bernad†

†Departamento de Inmunología y Oncología; ‡Protein Design Group. Centro Nacional de Biotecnología, C.S.I.C., Campus de la Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain. §Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Germany.

# Current address, Centro de Investigaciones Biológicas, E-28049 Madrid, Spain.

Running title: PKL12-GlcNAcK interaction

Footnote: Ligos J.M., Laín de Lera, T., Guinea, B., Martín-Caballero, J., Flores, J., and Bernad, A. (2001) Submitted.
Summary

PKL12 (STK16) is a ubiquitously expressed ser/thr kinase, not structurally related to the well-known subfamilies, with a putative role in cell adhesion control. Yeast two-hybrid protein interaction screening was used to search for proteins that associate with PKL12, to delineate signalling pathways and/or regulatory circuits in which this kinase participates. One positive clone contained an open reading frame highly similar to N-acetylglucosamine kinase (GlcNAcK) of several species. The PKL12/GlcNAcK interaction was further confirmed both in vitro and in vivo. Protein expression analysis of GlcNAcK using a specific rabbit antiserum displayed a ubiquitous pattern in cell lines and animal tissues. Subcellular localisation studies showed that GlcNAcK is a cytoplasmic protein with a dual subcellular localization, distributed between the perinuclear and peripheral cell reservoirs. After overexpression, GlcNAcK localizes in vesicular structures associated mainly with the cell membrane, and co-localizes with the PKL12 protein. GlcNAcK is not otherwise a substrate for PKL12 activity and PKL12 does not appear to influence GlcNAcK activity either in vitro or in vivo. In vitro kinase assays have nonetheless revealed that functional GlcNAcK, although not able to modulate autophosphorylation of PKL12, greatly influences PKL12 kinase activity on a defined substrate protein. These results are interpreted to indicate a potential in vivo role for GlcNAcK in PKL12 translocation, and a tentative regulatory role for PKL12-mediated phosphorylation on substrate proteins.
Introduction

PKL12 (protein kinase expressed in day 12 fetal liver), also known as Krct (kinase related to *cerevisiae* and *thaliana*), EDPK (embryo-derived protein kinase) and MPSK1 (myristoylated and palmitoylated serine-threonine kinase-1), has recently been isolated from several sources and partially characterized (1-4). All correspond to the same mammalian gene, human or murine, for which the denomination STK16 has been proposed (International Committee for Human Nomenclature).

PKL12 protein appears to be the first mammalian member of a new ser/thr kinase subfamily not closely related to those previously reported (1). This subfamily includes the putative homologues from *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, forming a group of four sequences close in size to a theoretical minimal catalytic domain. It has therefore been proposed that PKL12 may be the catalytic subunit of a more complex holoenzyme composed of catalytic and regulatory subunits (1). *E. coli*-expressed PKL12, his-tagged or as a GST-fusion, and a FLAG-tagged PKL12 protein have been shown to have functional kinase activity, able to phosphorylate exogenous substrates (1-3) and to promote autophosphorylation (1, 2) with the sequence-predicted ser/thr specificity (1, 3).

PKL12 mRNA appears to be broadly distributed, both in murine fetal stages (E 6.5-E 18.5) (2, 3) and in adult tissues, at low levels in skeletal muscle, heart and spleen, and with high expression in liver, testis and kidney (1-3). Despite its ubiquitous distribution, *in situ* analysis showed that PKL12 mRNA is preferentially expressed within specific cellular types in several adult tissues, with predominant expression in epithelial compared to mesenchymal compartments (2). Further analyses have confirmed the broad distribution of the PKL12 protein in murine tissues and cell lines, although a lack of correlation between mRNA and protein levels was reported (Ligos et al., 2001), suggesting post-transcriptional regulation (2).

hPKL12 is acylated by myristic acid at glycine residue 2 and by palmitic acid at cysteines 6 and/or 8 (4). It has been proposed that hPKL12 membrane localization via a myristoylation-dependent mechanism is required for the subsequent palmitoylation modification, as demonstrated
in other models (4, 5). A membrane-associated protein kinase, PKL12, must therefore play a role in intracellular signalling, with a general, highly conserved cellular function (Ligos et al., 2001). Subcellular localization analysis has shown that PKL12 is a Golgi-resident enzyme. Transient overexpression of PKL12 in NIH-3T3 cells promotes its accumulation in structures related to filopodia and lamellipodia, inducing redistribution of focal contacts and disorganization of the actin cytoskeleton, but no marked alterations in Golgi (Ligos et al., 2001). A regulatory role is thus hypothesized for PKL12 in the control of extracellular matrix-cell adhesion, mediating the dynamic equilibrium of organization/disorganization of focal adhesion structures and actin cytoskeleton (Ligos et al., 2001). Concurring with this proposal, high level forced expression of PKL12 protein in adherent cell lines appears to be incompatible with their survival, whereas PKL12 can be overexpressed in non-adhesion-dependent cell lines without disturbing growth and survival parameters (Ligos et al., 2001).

Based on a two-hybrid analysis, we have identified and demonstrated functional interaction in vitro and in vivo between PKL12, a Golgi-resident ser/thr kinase, and a recently cloned enzyme (6) of amino sugar metabolism, N-acetylglucosamine kinase (GlcNAcK). Although GlcNAcK is not a substrate of PKL12, nor does PKL12 influence GlcNAcK activity either in vitro or in vivo, we have found that both enzymes co-localize in vivo upon overexpression, being a functional GlcNAcK capable to influence PKL12 kinase activity on exogenous substrates. These results indicate a potential in vivo role for GlcNAcK in PKL12 translocation and a tentative regulatory role for PKL12-mediated phosphorylation on substrate proteins.
Experimental Procedures

Cell lines

NIH-3T3 and SV40-transformed NIH-3T3 cells were obtained from the American Type Culture Collection (Manassas, VA); BA/F3, A.20, FL5.12, WEHI3b, ST2 and EL4 cells were kindly provided by Dr. C. Martínez-A (CNB, Madrid, Spain); Cos-1 cells were a kind gift of Dr. J. Ortín (CNB, Madrid, Spain). NIH-3T3, Cos-1 and ST2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 2 mM L-glutamine (Merck, Darmstadt, Germany), streptomycin (0.1 mg/ml, Sigma, St. Louis, MO) and penicillin (100 U/ml, Sigma). A.20, BA/F3, FL5.12, WEHI3b and EL4 cells were maintained in RPMI 1640 (Gibco-BRL) supplemented as above. BA/F3 stable clones (K3 and K8) overexpressing PKL12 protein have been described (Ligos et al., 2001).

Yeast two-hybrid analysis

The yeast two-hybrid protein interaction screen was carried out essentially as described (7) using the HF7c yeast strain (Clontech, Palo Alto, CA). Murine PKL12 was fused to the GAL4 DNA-binding domain in the pGBT8 vector (kindly provided by Dr. M. Serrano), generating the pGBT8-PKL12 vector. The coding sequence for the murine PKL12 orf was amplified by PCR from the pcDNA3-PKL12 plasmid, using specific primers (sense primer: 5´-GATGTCGAATTCTTAATGGGCCACGC-3´; antisense primer: 5´-CGACTGAGATCTTCAGATTGGTTGTG-3´) for simultaneous elimination of the PKL12 orf initiation codon and creation of an EcoRI site for in-frame fusion with the GAL4 DNA binding domain in pGBT8. PCR conditions were as follows: 94°C, 30 sec; 60°C, 1 min; 68°C, 10 min for 12 cycles, using the Taq/Pow thermostable enzyme mixture (Expand Long Template System; Roche, Indianapolis, IN) and a 2400 Perkin Elmer Thermocycler (Foster City, CA). The amplified products were digested with EcoRI and BglII, and ligated to the pre-digested vector and transformed in E. coli XL1-blue competent cells.
An NIH-3T3 cDNA library generated in the pGAD424 vector (provided by Dr. M. Serrano) was used in the screening. Yeast stably transformed with the pGBT8-PKL12 vector, and thus able to grow in tryptophan-free medium, were obtained. Stable expression of GAL4bd-mPKL12 protein was confirmed by western blot (not shown). Yeast were then transformed with the NIH-3T3 cDNA library in the pGAD424 vector and selected for growth in minimal medium without tryptophan, leucine or histidine. As positive controls, pGBT8-p16 and pGAD424-ckd4 plasmids were cotransfected in yeast; as negative control the pGBT8 vector plasmid was cotransfected with the pGAD424-ckd4 plasmid. Yeast clones obtained after selection in tryptophan-, leucine- and histidine-free medium were also tested for β-galactosidase expression. After isolation of pGAD424-derivative plasmids from clones positive for both selective criteria, they were re-confirmed by direct cotransfection with the pGBT8-PKL12 vector in HF7c yeast, and inserts were directly sequenced using the vector primers.

**mSIP16(GlcNAcK) full-length cDNA cloning**

Specific oligonucleotides (sense primer: 5´-AGGCGACACAGGGGCGAGAGA-3´; antisense primer: 5´-GAAAGCGGTGCCTCAACTCCTC-3´) were synthesized (Isogen, Maarssen, The Netherlands) based on the 5´ and 3´ sequences of the mSIP16 obtained from the ESTs data bank and our data. Total mRNA was prepared from NIH 3T3 cells and used to obtain cDNA. PCR was carried out on cDNA derived from NIH 3T3 cells with the primers indicated above (94°C, 1 min; 60°C, 1 min; 72°C, 1,5 min; 30 cycles), using Taq/Pow as above. The fragment obtained, of the predicted size, was cloned in the pGEM-T (Promega) plasmid. Several clones were obtained and fully sequenced. The final clone selected was termed pGEM- GlcNAcK.

**Expression plasmids**

The pcDNA3-PKL12 plasmid was obtained by subcloning mPKL12 into the pcDNA3 eukaryotic expression plasmid (InvitroGen, Carlsbad, CA) as described (Ligos et al., 2001). pcDNA3-HA GlcNAcK plasmid was obtained by subcloning the GlcNAcK orf isolated from the pGEM- GlcNAcK plasmid. Specific oligonucleotides were synthesized (sense primer, 5´ TACTGAGATCTTTGGCCGCGCTTTATGGTG; antisense primer, 5´ GAAAGCGGTGCCTC
AACTCCTC 3´) to create an EcoRI site for in-frame SIP16 orf fusion with the hemagglutinin epitope (HA) in the pcDNA3.1/Neo-HA plasmid. PCR conditions were 94ºC, 30 sec; 60ºC, 1 min; 68ºC, 10 min for 12 cycles. The amplified products were digested with EcoRI, ligated to the EcoRI-digested vector and transformed in E. coli XL1-blue competent cells. Several clones were obtained, and their sequences completely confirmed. All plasmids were purified using the Plasmid Maxi Kit (Quiagen, Valencia, CA) following manufacturer’s instructions.

**E. coli expression and purification of histidine- and GST- tagged proteins**

Histidine-tagged PKL12 was expressed in E. coli strain M15 and purified to homogeneity using Ni-NTA agarose resin (Qiagen), as described (1); GST-SIP16 protein expression and purification was performed in a similar manner. The coding sequence for the orf corresponding to murine SIP16 was PCR amplified from the pGAD424-SIP16 plasmid using specific primers (sense primer: 5´-TACCTGAGATCTACAGACCAGTGTGG-3´; antisense primer: 5´-ATACG AATTCACTAGAAGGTATAGGAATA-3´) for simultaneous elimination of the SIP16 ORF initiation codon and creation of a BamHI site for in-frame fusion with the glutathione S-transferase (GST) protein in the pGEX-2T plasmid (Amersham Pharmacia Biotech). PCR conditions were 94ºC, 30 sec; 60ºC, 1 min; 68ºC, 10 min for 12 cycles. Amplified products were BamHI-digested, ligated to the digested vector and transformed in E. coli XL1-blue competent cells. Several clones were obtained and their sequences completely confirmed. GST-tagged SIP16 protein was expressed in E. coli (XL1B strain) and purified using the GST Gene Fusion System (Amersham Pharmacia Biotech) following manufacturer’s guidelines. E. coli extracts were prepared as described (1); those containing the expressed GST-SIP16 protein were loaded in a prepacked glutathione Sepharose 4B column (Amersham Pharmacia Biotech). After extensive washing, retained protein was eluted with buffer E (50 mM Tris-HCl pH 8.0, 10 mM glutathione). Purification was monitored by SDS-PAGE (8) of aliquots of the fractions obtained, followed by Coomassie blue staining or western blot analysis with an anti-GST antibody (Santa Cruz, Santa Cruz, CA). A parallel negative control purification was performed using E. coli cells transformed with the vector pGEX-2T plasmid.
Several independent induction/purification experiments rendered an almost pure fraction of GST-SIP16 protein, with two protein bands (see Results, Fig. 3). Both bands were recognized by anti-GST antibody and the specific anti-SIP16 antiserum; we thus concluded that in E. coli or during the purification process, the SIP16 protein is proteolyzed at a specific carboxy-terminal point, rendering both products. These bands are not present in negative control purified fractions.

Control GST and GST-SP (a fusion of GST with a protein that is a substrate for the kinase activity of the PKL12 protein; (Ligos et al., 2001) proteins were expressed and purified essentially as described for GST-SIP16. Protein concentration of purified fractions was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with a bovine serum albumin standard.

**Rabbit antiserum generation and testing**

Purified GST-tagged SIP16 protein was prepared as above. Outbred New Zealand rabbits were injected intradermally in multiple sites using 250 µg of purified protein emulsified with an equal volume of Freund’s complete adjuvant. Two 125 µg intramuscular boosts of the same material in incomplete adjuvant were given 4 and 7 weeks later. Sera were collected 7 and 10 days after the last injection and tested in ELISA; the IgG serum fraction was purified as described (1).

**Eukaryotic cell transfections**

Cells (2 x 10⁶) were transfected by electroporation (200V, 960 µF in 0.2 ml) in a Gene Pulser (Bio-Rad) with 20 µg of pcDNA3-PKL12 or pcDNA3-HA-GlcNAcK plasmids, then cultured in standard conditions or on coverslips. After 18 h, cells were processed for western blot analysis or fluorescence microscopy.

**Preparation of cell lysates**

Cells (2 x 10⁶) were lysed in 100 µl of RIPA buffer (137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml aprotinin) for 30 min at 4°C and cellular debris was removed by centrifugation (18000 x g, 20 min). Protein content was determined using the Bio-Rad protein assay (Bio-Rad).
Western blotting

Protein (20 µg) was separated in SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1 h in Tris-buffered saline (25 mM Tris; TBS) plus 0.1% Tween, with 5% non-fat dry milk, followed by incubation with primary antibody for 2 h and secondary antibody for 40 min. Western blots were developed using the ECL system (Amersham, Aylesbury, U.K.). The polyclonal anti-PKL12 and anti-GlcNAcK antisera were used at 1:2000 and 1:3000 dilution, respectively.

Immunoprecipitation assays

Cells were transfected as above, cultured for an additional 24 h and lysed in 100 µl of IP buffer (150 mM NaCl, 100 mM Tris-HCl pH 7.4, 10% glycerol, 1% NP-40, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml aprotinin) for 30 min at 4°C and cellular debris removed by centrifugation. Protein content was determined as above. Protein (200 µg) in 0.5 ml of IP buffer was incubated with 5 µl of polyclonal anti-PKL12 antibody, or 5 µl of polyclonal anti-GlcNAcK antibody (16 h, 4°C), followed by incubation with protein A-Sepharose beads (Sigma) for 1 h. After extensive washing with IP buffer, immune complexes were analyzed by SDS-PAGE and western blotting.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde (10 min, room temperature), then washed three times in PBST (PBS containing 0.1% Tween). Preparations were incubated with PBS plus 20% FCS (1 h, room temperature) followed by primary antibody (1 h). After washing with PBST and staining with secondary antibody (40 min), cells were washed and mounted in ProLong Antifade mounting medium (Molecular Probes, Eugene, OR). Polyclonal anti-PKL12 and anti-GlcNAcK antisera were used at 1:150 and 1:100 dilutions, respectively. Murine anti-HA mAb (Roche), was used at 1:1000 dilution. Secondary antibodies Alexa 488-conjugated goat anti-rabbit IgG, Cy3-goat anti-rabbit IgG and Cy2-goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used following manufacturer’s instructions.
In vitro protein kinase assay

In vitro kinase assays using purified proteins were performed as follows: 35 µl of reaction buffer containing the indicated amount of recombinant histidine-tagged PKL12, 50 mM Tris-HCl pH 7.4, 10 mM MnCl₂ and 10 mM MgCl₂, 10 mM ATP and 10 µCi/µl of [γ³²P] ATP, 3000 Ci/mmol (Amersham), were preincubated (30°C, 1 min), then mixed with 10 ml of the same buffer containing the indicated amounts of the purified putative substrate and/or modulator proteins. Reactions were incubated (30°C, 30 min), terminated by addition of Laemmli sample buffer, and proteins separated in 10% SDS-PAGE gels. After Coomassie blue staining, the gel was dried and autoradiographed. As a negative control, equivalent fractions purified from E. coli vector-transformed clones were assayed.

GlcNAcK assay

BAF/3 PKL12-stable clones cells (2 x 10⁶) or transiently transfected NIH-3T3 or Cos-1 cells (2 x 10⁶) were prepared as described (Ligos et al., 2001). PBS-washed cells were lysed by hypotonic shock in 200 µl of 10 mM sodium phosphate pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF. The lysate was centrifuged (30,000 x g, 20 min), the supernatant assayed for GlcNAcK activity and analyzed for GlcNAcK expression in western blot.

In vitro GlcNAcK activity was determined as described (7). In brief, GlcNAcK assays were performed in a final volume of 225 µl containing 60 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 10 mM GlcNAc, and variable amounts of protein extract. Incubations were carried out (37°C, 2 h), and the reaction terminated by adding 350 µl ethanol. Radiolabeled substrates were separated by descending paper chromatography and measured by liquid scintillation analysis.

Amino acid sequence comparison

Initial comparative sequence searches were performed using the BLAST algorithm (9) on a non-redundant database (EMBL NRDB). Additional remote homologue searching was done by HMM (hmm search, default parameters) over protein nrdb database (10). Sequences were aligned with CLUSTALW software (11). Alignment visualization was performed with Belvu (E.
Sonnhammer, <http://www.cgr.ki.se/cgr/groups/sonnhammer/Belvu.html). Complementary data and alignments are available on request at: http://www.cnb.uam.es/~cnbprot/priv/STK/SIP16.
RESULTS

Interaction between PKL12 and GlcNAcK in yeast two-hybrid

We used the yeast two-hybrid protein interaction screen to search for proteins able to associate with the murine PKL12 (STK16) ser/thr kinase and to delineate signalling pathways and/or regulatory circuits in which this kinase participates. PKL12 is expressed ubiquitously and NIH-3T3 cells are a study model (1-3, Ligos et al., 2001). HF7c yeast was stably transformed with the GAL4bd-PKL12 plasmid (pGBT8-PKL12); a NIH-3T3 library fused to the GAL4 activator domain in the pGAD424 plasmid was then transformed and screened in minimal medium. Several clones were selected that grew under the selection conditions and also expressed lacZ, the second transactivatable marker gene. pGAD424 plasmids harbored by the positive yeast clones were isolated; positive interaction was confirmed by individual cotransformation of HF7c yeast with pGBT8-PKL12 plasmid or the negative controls pGBT8-p16 (12) and pGBT8. Positive clones were isolated and fully sequenced. Several positive clones (representing 23% of sequences obtained) were found containing partial sequences corresponding to the same gene. The clone containing the longest ORF corresponded to a 334-amino-acid protein (38 kDa) in phase with GAL4db. This clone, pGAD424-SIP16Δ, was selected for some of the further experiments.

This protein was denominated SIP16 (STK-16 interacting protein), as sequence homology analysis showed that it has a yet-undescribed function. Homologous EST sequences from several murine and human tissues, and a C. elegans (WO6B4.2) protein (unknown function) showed significant similarity to the SIP16Δ encoded orf. Based on the data bank-annotated EST sequences, we identified the putative 5´ UTR sequence and the transcription initiation codon of the mSIP16 orf. We designed specific oligonucleotides to clone the full-length cDNA, which was obtained from NIH-3T3 cells. mSIP16 protein consists of 343 amino acids (approximate M, 40 kDa) and hSIP16 was electronically assembled from the data bank EST sequences. Similarity searches using human and murine sequences (92% similarity) revealed the presence of ortholog sequences in several other eukaryotic organisms (Fig. 1), including Drosophila melanogaster and Streptomyces coelicolor (43% and 27% similarity, respectively), and partial EST sequences in pig.
bull, rat and xenopus (not shown). All sequences are related by the presence of a conserved ATP binding site, with the recognized subdomains (I-V) (13). Remarkably, all sequences are similar in size, around 330 amino acids, except that of C. elegans, which contains an additional N-terminal domain (1-192 amino acids) not shared by the other putative orthologs. Comparison with prokaryotic sequences by HMM search (10) showed significant relationship with members of the ROK family containing sugar kinases (13). Figure 1 shows the amino acid sequence comparison between eukaryotic enzymes and the most representative members of the prokaryotic family, including the YHCl, YAJF, YCFX and ALSK enzymes from E. coli, and SCRK from B. subtilis.

These data strongly suggested that SIP16 is an eukaryotic sugar kinase. When preparing the first version of the manuscript, a final search in the data banks revealed that new sequences were included (NP_062415; NP_060037; CAB61849) with high similarity (98%) to mSIP16. These sequences corresponded to the murine and human GlcNAcK proteins (6); a PKL12 (STK16) interaction with GlcNAcK was therefore suggested.

**Functional interaction of PKL12 and GlcNAcK in vitro and in vivo**

The specificity of the PKL12-GlcNAcK interaction was first studied in a cell-free system. A fusion protein (GST-SIP16Δ) consisting of glutathione S-transferase fused to the specific sequence contained in the pGAD424-SIP16Δ clone isolated from the two-hybrid screening, or the control GST proteins were expressed in bacteria and crude extracts incubated with purified his-PKL12 protein (1). GST-SIP16Δ protein was recovered from the mixtures on glutathione-Sepharose beads; the resulting precipitates were resolved in SDS-PAGE and analyzed by western blot with rabbit polyclonal anti-PKL12 antiserum. The specific PKL12 band was observed only when bacterial extracts containing the GST-SIP16Δ protein were included in the incubation mixture with purified his-PKL12 protein (Fig. 2A).

The specificity of the PKL12/GlcNAcK interaction was also studied in mammalian cells. Full-length mGlcNAcK cDNA was obtained and fused in the amino terminus to the hemagglutinin epitope (HA-GlcNAcK). NIH-3T3 cells were cotransfected with the pcDNA-3-HA-GlcNAcK vector and the pcDNA3-PKL12 plasmid (1); PKL12 was co-immunoprecipitated with anti-HA-
PKL12-GlcNAck interaction

GlcNAcK protein (Fig. 2B). These results confirm the interaction of PKL12 and GlcNAcK revealed by the two-hybrid system.

**GlcNAcK is a ubiquitously expressed cytoplasmic protein in cell lines and murine tissues**

Northern blot analysis showed broad distribution of GlcNAcK mRNA in cell lines and murine tissues (our unpublished results; 6). To confirm this at the protein level, we generated a rabbit antiserum specific for GST-GlcNAcK. The anti-GlcNAcK antiserum detected both purified GST-GlcNAcK expressed in *E. coli* (Fig. 3A) and the endogenous murine protein (Fig. 3B) in several cell lines. Antibody specificity in western blot was confirmed by competition for the signal with an excess of purified GST-GlcNAcK protein (Fig. 3A). The double band revealed by the anti-GlcNAcK antiserum corresponded to the presence of two protein species in the purified GlcNAcK fraction, probably due to carboxy-terminal proteolytic processing of the intact enzyme in *E. coli*. The endogenous GlcNAcK protein levels were variable in the cell lines analyzed, with a surprising near-absence of GlcNAcK protein in the A20 pro-B cell line. This result implies that GlcNAcK activity is not absolutely required for cell viability.

Subcellular localization of endogenous GlcNAcK protein in interphase NIH-3T3 cells was evaluated by indirect immunofluorescence using the rabbit anti-GlcNAcK antiserum. GlcNAcK is a cytoplasmic enzyme located predominantly in the perinuclear area and the cell periphery (Fig. 4A); it was clearly detected in cell membrane structures similar to leading edges and filopodia. Competition experiments with recombinant GST-GlcNAcK protein and staining with a preimmune serum (not shown) confirmed signal specificity. A similar pattern was observed in several other cell lines, including Cos-1 and NRK cells (not shown). Endogenous GlcNAcK is therefore a cytoplasmic protein with a dual subcellular localization. For comparison, Fig. 4A also shows the endogenous localization of PKL12 associated mainly with the Golgi area, co-localizing partially with GlcNAcK. Following overexpression of HA-GlcNAcK (Fig. 4B, green) and PKL12 (Fig. 4B, red) in NIH-3T3 cells, both proteins presented a highly vesicular pattern, different from their endogenous distribution. A strong level of co-localization was observed (Fig. 4B, yellow), especially in peripheral structures in which PKL12 accumulates when overexpressed (Ligos et al.,...
These results provide evidence for the functional relevance of the PKL12/GlcNAcK interaction, probably in translocation.

**GlcNAcK is not a substrate of the PKL12**

We analyzed whether the GlcNAcK protein could be used as a substrate by the intrinsic kinase activity of the PKL12 protein (1). Purified his-PKL12 was incubated with purified GST-SIP16Δ protein in the standard assay conditions (see Experimental Procedures). The autoradiograph obtained after SDS-PAGE resolution of the products of this *in vitro* kinase assay is shown in Fig. 5A; controls are described in the figure legend. No significant phosphorylation was detected in the GlcNAcK band, indicating that at least *in vitro*, GlcNAcK is not a substrate for the PKL12 kinase activity. None of these bands was observed in the negative control fraction (his-control). In addition, no significant alteration was noted in the PKL12 autophosphorylation band (further tested; see Fig. 6). Similar results were obtained when GST- GlcNAcK or purified rat GlcNAcK proteins were used in the assay (not shown).

**GlcNAcK is able to regulate PKL12 kinase activity on exogenous substrates**

Once established that GlcNAcK is neither a substrate for PKL12 kinase nor influences its autophosphorylation capacity, we tested whether GlcNAcK affects PKL12 activity on an exogenous substrate (GST-SP) protein. Full-length GlcNAcK fused to GST (GST-GlcNAcK) was compared with the previously described GST-SIP16Δ construct. Both purified proteins were tested for their intrinsic kinase activity (see Experimental Procedures) in comparison with purified rat GlcNAcK. As predicted, GST-GlcNAcK showed a specific activity (20 U/mg) very similar to that of the rat enzyme. The GST-SIP16Δ protein was completely inactive, due to the lack of highly conserved amino acids of the ATP-binding motif shared by all sugar kinases (13).

Figure 6 shows the autoradiograph obtained after SDS-PAGE resolution of the products of a kinase assay, in which variable amounts of purified his-PKL12 protein were pre-incubated with an excess of GST-GlcNAcK or GST-SIP16Δ proteins. This confirmed that neither of these proteins (GST-GlcNAcK or GST-SIP16Δ) significantly affected PKL12 autophosphorylation capacity. PKL12 activity on the GST-SP substrate protein is not modified by the presence of
GST-SIP16Δ protein in the kinase assay, however GST-GlcNAcK appears to downregulate GST-SP phosphorylation. In conclusion, although GlcNAcK protein interacts with PKL12, this association is not sufficient for the regulatory effect observed. GlcNAcK must be completely functional to be able to influence the phosphorylation capacity of PKL12 on exogenous substrates.

**PKL12 does not influence in vitro and in vivo GlcNAcK activity**

The influence of PKL12 on the activity of GST-GlcNAcK was tested *in vitro*. Purified GST-GlcNAcK (0.05 µg) was preincubated (30 min, 37°C) with up to a 10-fold excess of purified his-PKL12 under conditions of the protein kinase assay. GlcNAcK activity was then determined as described (Experimental Procedures). No differences in GlcNAcK activity were observed in samples preincubated with his-PKL12 and the corresponding control samples (not shown).

To determine whether PKL12 overexpression affected cellular GlcNAcK activity, we used two different cell models with significant differences in endogenous GlcNAcK activity levels, BA/F3 cell clones stably transfected for overexpression of PKL12 (Ligos *et al.*, 2001) and NIH-3T3 or Cos-1 cells with transient PKL12 overexpression. We measured GlcNAcK activity in the BA/F3 (K8) clone, which expresses a 25-fold increase in PKL12 protein compared to endogenous levels (BA/F3, clone C1) (Fig. 7). Both cell populations showed comparable GlcNAcK activity and no difference in GlcNAcK protein expression levels (Fig. 7, lower insert). Concurring with this, when PKL12 protein was overexpressed in NIH-3T3 or Cos-1 cells by transient transfection with the pcDNA3-PKL12 plasmid, no significant changes were measured in cellular GlcNAcK activity or in GlcNAcK protein levels. These results suggest that PKL12/GlcNAcK association is not involved in regulation of cellular GlcNAcK activity.
DISCUSSION

Using the yeast two-hybrid system, we identified a protein (SIP16) that interacts with the ubiquitous ser/thr kinase PKL12, recently isolated by our group and others (1-4), which appears to have an important role in the control of cell adhesion (Ligos et al., 2001). PKL12 is a small ser/thr kinase with no detectable regulatory domain in its primary structure; it was thus proposed that it could be the catalytic subunit of a more complex holoenzyme (1). The detection of the SIP16 interacting protein challenged this hypothesis. SIP16/PKL12 interaction was confirmed both in vitro and in vivo, and co-localization of the proteins was described, partially in the Golgi area of interphase cells and, more prominently, following overexpression in NIH-3T3 cells (Fig. 4). Preliminary sequence homology searches in data banks revealed several human and murine ESTs clearly corresponding to SIP16, which allowed us to assemble the virtual human full-length cDNA; it was also distantly related to orf from C. elegans and D. melanogaster. These sequences did not show notable similarity with other characterized eukaryotic proteins, although they showed a clear relationship with bacterial sugar kinases (Fig. 1). Murine and human sequences for GlcNAcK were recently included in data banks (NP_062415, NP_060037, CAB61849), and subsequently published (6) demonstrating that mSIP16 is nearly identical (98%) to murine GlcNAcK.

GlcNAc is a major component of complex carbohydrates, found in glycoproteins (14), glycolipids (15) and proteoglycans (16). GlcNAcK catalyzes GlcNAc phosphorylation at carbon atom 6. It is the key enzyme in the salvage pathway for recycling GlcNAc derived from lysosomal degradation of oligosaccharide moieties or nutritional sources, into GlcNAc 6-phosphate. GlcNAc 6-phosphate can then enter a catabolic route that links hexosamine metabolism with the glycolytic pathway (17), or may enter an anabolic pathway leading to UDP-GlcNAc formation (18).

De novo UDP-GlcNAc synthesis follows the sequence of intermediates: fructose 6-phosphate $\rightarrow$ glucosamine 6-phosphate $\rightarrow$ N-acetylglucosamine 6-phosphate $\rightarrow$ N-acetylglucosamine 1-phosphate $\rightarrow$ UDP-GlcNAc (18). The extent to which redundancy in UDP-GlcNAc generation is used by cells to respond to distinct external stimuli is unclear, as is the manner in which activity of the respective enzymes is regulated (19). The pathways of glycolysis
PKL12-GlcNAck interaction and UDP-GlcNAc de novo and salvage synthesis appear to be interconnected; it has been proposed that UDP-GlcNAc displays a cellular sensor of energy availability, modulating gene expression in response to nutrient availability (17, 20). The effects of UDP-GlcNAc are mediated via modification of the O-GlcNAcylation status of critical proteins as a counterpart of ser/thr phosphorylation (reviewed in 21). Alterations in the natural equilibrium between the metabolic pathways have been implicated in diabetes pathogenesis (22).

We thus examined a potential regulatory role for the GlcNAcK-PKL12 interaction. We analyzed whether GlcNAcK is a substrate for PKL12 kinase activity, mediating in the regulation of GlcNAcK catalytic activity. In vitro kinase assays using the recombinant GST-SIP16Δ protein as exogenous substrate showed that GlcNAcK contains no substrate site for PKL12 activity (Fig. 5). These results were confirmed using GlcNAcK purified from rat liver (not shown). In addition, we studied the effect of PKL12 overexpression on cellular levels of GlcNAcK protein and activity, both in transient transfection of adherent cells and in stable PKL12 clones generated in non-adhesion-dependent cell lines (Fig. 7). We detected no significant differences in cellular GlcNAcK activity or protein mass in either model, independent of the amount of PKL12 expressed. We conclude that there is no regulation of GlcNAcK and consequently, no direct regulation of the UDP-GlcNAc pool by PKL12.

GlcNAcK does not appear to regulate the in vitro kinase autophosphorylation activity of PKL12 either positively or negatively (Fig. 5, 6). A negative regulatory effect was nonetheless shown on the PKL12-kinase activity over the exogenous E. coli recombinant GST-SP substrate when a fully active GST-GlcNAcK was included in the assay (Fig. 6). The regulatory effect of GlcNAcK on PKL12 is not only due to binding of the two proteins, since addition to the kinase assay of the truncated GlcNAcK (GST-SIP16Δ), which binds to PKL12 (Fig. 1), does not alter PKL12 activity on the exogenous substrate. It can be speculated that PKL12 harbors O-linked GlcNAc residues, and that GlcNAcK may phosphorylate these O-GlcNAc residues to regulate PKL12 activity. Several attempts to detect such a modification in PKL12-purified eukaryotic cells were unsuccessful, however, indicating that PKL12 is not an O-GlcNAc-modified enzyme. For
regulation of PKL12 activity by full-length GlcNAcK, a structurally intact, rather than an enzymatically active protein may be necessary. Point mutations of GlcNAcK, which only affect the enzymatic activity, will clarify this in the future.

We next studied subcellular localization of endogenous GlcNAcK in NIH-3T3 cells (Fig. 4). The enzyme accumulates mainly in two areas, in the perinuclear region and, predominantly, at the cell leading edge in membrane-associated structures similar to filopodia. This contrasts clearly with the subcellular localization of glucosamine 6-phosphate acetyltransferase (EMeg32), an enzyme involved in de novo synthesis of UDP-GlcNAc (23). Since nucleotide sugar synthesis is believed to take place in the cytoplasm, it was therefore consistent that the enzyme associates with the cytoplasmic face of various intracellular membranes (Golgi and late endosome/lysosome). It was thus tempting to assume that membrane association of EMeg32 to the cytoplasmic leaflet of Golgi and other intracellular membranes may facilitate local cytoplasmic release of GlcNAc-6-P; no further factors would be needed to make this compound available for the last two steps leading to synthesis of UDP-GlcNAc, which is finally transported to ER and Golgi by specific proteins (24). Here we show that another enzyme involved in UDP-GlcNAc synthesis localizes principally in a very different cellular area, near the leading cell edge. We thus suggest that the cellular UDP-GlcNAc pool may be compartmentalized and controlled by the coordinated function of the two principal synthetic routes, which are separated spatially. In this model, both pathways (de novo and recycling) may have unique roles in specific cellular functions, independent of equilibrium conditions.

This hypothesis is supported by conclusions extracted from the study of recently generated EMeg32-deficient mouse embryos and cells (19). Homozygous mutant embryos have a general proliferation defect during development and die at E7.5; this defect was also observed in in vitro-differentiated EMeg32−/− ES cells (19). Analysis of deficient mouse embryonic fibroblasts (MEF) also showed a proliferation defect linked to reduced adhesiveness and altered responses to apoptotic stimuli; all defects were ameliorated by nutritional restoration of UDP-GlcNAc pools (19). Strikingly, reduced UDP-GlcNAc levels translated predominantly into decreased O-GlcNAc
modifications of cytosolic and nuclear proteins, and were thus not limiting for N-GlcNAc modifications of glycoproteins or GPI-linked proteins (19). This phenomenon may be explained by the effect of a global reduction in the UDP-GlcNAc pool, by to a preferential effect on a compartmentalized UDP-GlcNAc pool dedicated mainly to the O-GlcNAc modification of cytosolic and nuclear target proteins by the specific O-GlcNAc transferase enzyme (OGT), essential for embryonic stem cell viability and mouse ontogeny (25).

We previously demonstrated that PKL12 kinase is located in the cell perinuclear region, colocalizing with several Golgi markers (Ligos et al., 2001; Fig. 4A). In addition, when PKL12 is overexpressed in NIH-3T3 cells, the protein is transiently accumulated in areas that clearly resemble the endogenous location of GlcNAcK (Ligos et al., 2001; Fig. 4B). PKL12 overexpression in adherent cells appears to be incompatible with viability, as it causes disorganization of actin cytoskeleton and focal adhesion contacts, detaching cells from the substrate (Ligos et al., 2001). We interpret these results as indicating that GlcNAcK may have a role as a docking or scaffold molecule that could recruit PKL12 to its site of physiological action following activation. Such a dual role also has been suggested for EMeg32, which co-purifies with the cdc48 homologue p97/VCP, a protein implicated in mitotic membrane fusion events (19). A regulatory or scaffold function for the ATPase activity of p97/VCP has also been proposed (19). The other potential function of the PKL12/GlcNAcK translocation mechanism is GlcNAcK transport to filopodia and lamellipodia. This would establish UDP-GlcNAc biosynthesis independent of the de novo pathway in a specific subcompartment, as discussed above. Taken together, our results strongly favor novel roles for PKL12 and GlcNAcK, completely different from previously described properties of these enzymes, including a regulatory role for GlcNAcK on PKL12-mediated kinase activity over certain specific substrates.
REFERENCES
1. Ligos, J.M., Gerwin, N., Fernandez, P. Gutierrez-Ramos, J.C., and Bernd, A. (1998) Biochem. Biophys. Res. Commun. 249, 380-384
2. Stairs, D.B., Perry Gardner, H., Ha, S.I., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Chodosh, L.A. (1998) Hum. Mol. Genet. 7, 2157-166
3. Kurioka, K., Nakagawa, K., Denda, K., Miyazawa, K., and Kitamura, N. (1998) Biochim. Biophys. Acta 1443, 275-284
4. Berson, A.E., Young, C., Morrison, S.L., Fuji, G.H., Sheung, J., Wu, B., Bolen J.B., and Burkhard, A.L. (1999) Biochem. Biophys. Res. Commun. 259, 533-538
5. Berthiaume, L., and Resh, M.D. (1995) J. Biol. Chem. 270, 22399-22405
6. Hinderlich, S., Berger, M., Schwarzkopf, M., Effertz, K., and Reutter W. (2000) Eur. J. Biochem. 267, 3301-3308
7. McNabb, D.S., and Guarantee, L. (1996) Curr. Opin. Biotechnol. 7, 554-9
8. Laemmli, U. K. (1970) Nature 227, 680-685
9. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) J. Mol. Biol. 215, 403-410
10. Durbin, R., Eddy, S., Krogh, A., and Mitchison, G. (1998) The theory behind profile HMMs: Biological sequence analysis: probabilistic models of proteins and nucleic acids. Cambridge University Press, Cambridge, UK
11. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) Nucl. Acid Res. 24, 4876-4882
12. Serrano, M., Hannon, G.J., and Beach, D. (1993) Nature 336, 704-707
13. Bork, P., Sander, C., and Valencia, A. (1992) Proc. Natl. Acad Sci USA 89, 7290-7294
14. Kornfeld, R., and Kornfeld, S. (1976) Annu Rev Biochem. 45, 217-37.
15. Kopitz, J., Sinz, K., Brossmer, R., and Cantz, M. (1997) Eur. J. Biochem. 248, 527-534.
16. Kjellen L, Lindahl U. (1991) Annu Rev Biochem. 60, 443-475.
17. Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A. M., Fralich, T. J., Schnaar, R. L., and Snyder, S. H. (1998) FASEB J. 12, 91-99
18. Reutter, W., Stäsche, R., Stehling, P., and Baum, O. (1997). In: Glycosciences, Status and Perspectives (Gabius, H.-J. and Gabius, S., eds.), pp. 245-259, Chapman & Hall, Weinheim
19. Boehmelt, G., Wakeham, A., Elia, A., Sasaki, T., Plyte, S., Potter, J., Yang, Y., Tsang, E., Ruland, J., Iscove, N.N., Dennis, J.W., and Mak, T.W. (2000) EMBO J. 19, 5092-5104
20. Wang, J., Liu, R., Hawkins, M., Barzilai, N., and Rossetti, L. (1998) Nature 393, 684-688
21. Comer, F.I., and Hart, G.W. (1999) Biochim. Biophys. Acta 1473, 162-165
22. Du, X-L, Edelstein, D., Rossetti, L., Fantus, I.-G., Goldberg, H., Ziyadeh, F., Wu, J., and Brownlee, M. (2000) Proc. Natl. Acad Sci USA 97, 12222-12226
23. Boehmelt, G., Fialka, I., Brothers, G., McGinley, M.D., Patterson, S.D., Mo. R., Hui, C.C., Chung, S., Hubert, L.A., Mak, T.W., and Iscove N.N. (2000) J. Biol. Chem. 275, 12821-12832
24. Hirschberg, C.B., Robbins, P.W., and Abeijon, C. (1998) Annu. Rev. Biochem. 67, 49-69
25. Shafi, R., Yver, S.P., Ellies, L.G., O’Donnell, N., Marek, K.W., Chui, D., Hart, G.W., and Marth, J.D. (2000) Proc. Natl. Acad. Sci. USA 97, 5735-5739
FIGURE LEGENDS

Figure 1. Amino acid sequence alignment of SIP16 eukaryotic orthologs with some members of the bacterial ROK family. Amino acid sequence of the human SIP16 protein (Sip16_Human) was compared with putative ortholog proteins identified in mouse (Sip16_Mouse), D. melanogaster (Sip16_DroMe), C. elegans (Sip16_CaEl) and S. coelicolor (GLK SRTCO), which show 92%, 43%, 27%, and 23% amino acid similarity, respectively. Multiple sequence alignments were carried out against bacterial data banks. Some of the most representative members of the ROK family from E. coli (YHCI, YAJF, YCFX and ALSK) and B. subtilis (SCRK) are included in the figure; all show limited, although significant, amino acid similarity (9-11%) in comparison with the human SIP16 sequence. Highly conserved residues (present in more than 75% of the aligned sequences) are denoted by white letters in black boxes; less conserved residues are included in grey areas. Numbers indicate the relative position of the adjacent residue with respect to the protein N-terminus. Location is indicated of functional domains (I-V) characteristic of an ATP-binding site (13). Multiple alignment of SIP16-related sequences was done using PILEUP and PRETTY programs from the University of Wisconsin Genetics Computer Group, and adjusted manually to reinforce the specific similarity of the subfamily described (see Experimental Procedures).

Figure 2. Functional interaction of PKL12 and GlcNAcK in vitro and in vivo. A. Whole extracts (20 µg) of bacterial clones overexpressing the GST-GlcNAcK protein, and of control bacteria (GST) were incubated (room temperature, 15 min, with gentle shaking) with his-PKL12 purified protein (100 µg) or the corresponding negative control fraction (his-control). After incubation, GST-GlcNAcK protein was precipitated by glutathione beads and analyzed in western blot using the anti-PKL12 antibody (1:2000). PKL12 protein mobility is indicated at the right (arrow) and the relative mobility of protein markers at the left. B. NIH-3T3 cells (2 x 10⁶) were mock transfected (-) or transfected by electroporation with pcDNA-PKL12 and the pcDNA3-HA-GlcNAcK expression plasmids. Whole extracts were prepared 24 h after transfection and analyzed directly (total protein) or after immunoprecipitation with the anti-HA antibody (1:200) (IP protein),
in western blot using the anti-PKL12 antibody. The 35 kDa band corresponding to PKL12 was observed only in western blot analysis of immunoprecipitates obtained using anti-HA antibody, in extracts of pcDNA3-PKL12/pcDNA3-HA-GlcNAcK-transfected cells.

**Figure 3. A. Evaluation of a specific anti-GlcNAcK antiserum.** A total of 20 µg of whole extracts of bacterial clones overexpressing GST-GlcNAcK were analyzed in western blot (-) using a rabbit antiserum obtained against recombinant GlcNAcK or a specific anti-GST antibody as primary antibodies. (+) Antisera were preincubated with 100 µg of GlcNAcK. The anti-GlcNAcK antiserum (1:2000) recognizes two specific bands of approximately 50 and 58 kDa (arrows) in whole extracts (20 µg) of bacterial clones overexpressing the GST-GlcNAcK protein (-), but not in negative control bacteria (not shown).

**B. Expression analysis of GlcNAcK in various cell lines.** Whole cell extracts (50 µg) were analyzed in western blot using the specific anti-GlcNAcK antibody. Murine cell lines were B cells at several maturation stages (FL5.12, WEHI, A.20), a T cell line (EL4), normal (NIH-3T3) or transformed fibroblasts (BALB3T3-SV40), and an endothelial cell line (ST2). Cos-1 (COS) was a monkey cell line.

**Figure 4. Intracellular localization of endogenous and overexpressed GlcNAcK protein in adherent cells.** A. NIH-3T3 cells were plated on coverslips, fixed, and immunostained using rabbit anti-GlcNAcK antiserum (1:160) or, when indicated (PKL12), the anti-PKL12 antiserum (1:200). As secondary antibody, Cy3-goat anti-rabbit IgG (red) was used. B. NIH-3T3 cells (2 x 10^6) were transfected by electroporation with pcDNA-PKL12 and pcDNA3-HA-GlcNAcK expression plasmids, plated on coverslips, fixed and immunostained using rabbit anti-GlcNAcK antibody (1:160) or, when indicated, the anti-PKL12 antiserum (1:160). Cy3-goat anti-rabbit IgG (red) or Cy2-anti-mouse IgG (green) were used as secondary antibody. The images were superimposed to visualize co-localization (overlay, yellow).

**Figure 5. In vitro analysis of GlcNAcK as a substrate for PKL12 ser/thr kinase activity.** Purified his-PKL12 (0.5 µg) or the corresponding control fraction (his-control) were used in *in vitro* kinase assays with a combination of 10 mM Mg^{2+} and 10 mM Mn^{2+} (as activating metals), and several protein fractions (GST-SP, 10 µg; GST-GlcNAcK, 20 µg and GST, 10 µg) as
PKL12-GlcNAcK interaction

substrate. The GST-SP fraction contains a positive control and GST was used as the negative substrate control. After the kinase reaction, samples were separated by SDS-PAGE. The gel was stained with Coomassie blue and photographed (panel B), then dried and exposed. The figure shows the autoradiograph of the dried gel. The molecular weight size markers are indicated on the left. The mobility of the purified proteins included in the kinase assays, visualised after Coomassie blue staining of the gel (Fig. 5B), are indicated in the central part of the figure. A. Autoradiography; B. Coomassie blue staining of the gel in panel A, used to confirm mobility and mass loading of the purified proteins included in the kinase assay.

**Figure 6. Influence of GlcNAcK on the PKL12 in vitro kinase activity.** *In vitro* kinase assays were performed using the indicated amounts of purified his-PKL12 protein (0.25 and 0.1 µg), alone or with the positive control protein (GST-SP; 1 µg), and with GST-SIP16Δ (4 µg; Part A) or GST-GlcNAcK (4 µg; Part B). In all cases, his-PKL12 and GST-GlcNAcK proteins were pre-incubated (30°C, 5 min) before being added to the reaction sample. After the kinase reaction, samples were separated by SDS-PAGE. The gel was stained with Coomassie blue and photographed (lower panels), then dried and exposed for autoradiography (upper panels). Molecular weight size markers are indicated on the left. The mobility of the purified proteins included in the kinase assays, visualised after Coomassie blue staining, are indicated at the right.

**Figure 7. Influence of PKL12 on GlcNAcK activity and expression in different cell lines.** Cells were transfected with either the pcDNA3 (MOCK) or the pcDNA3-PKL12 plasmid. BA/F3 cells display stable transfection; NIH-3T3 cells and Cos-1 cells were transiently transfected. Upper panel, GlcNAcK activity, determined as described in Experimental Procedures. Values are mean ± SD of three independent experiments. Lower panel, GlcNAcK and PKL12 expression, detected by western blot.
ACKNOWLEDGEMENTS

We would like to thank Drs. Santos Mañes, Isabel Mérida and Miguel Aracil for critical reading of the manuscript and Rosanna García for excellent technical assistance. We also thank Coral Bastos and Catherine Mark for secretarial and editorial support, respectively. This work was partially supported by grants 07/057/96 and 08.6/0021/1997 from the Consejería de Cultura de la Comunidad Autónoma de Madrid, SAF98-0008-CO4-O3 del Plan Nacional de Salud y Farmacia, CICYT, and BIO4-CT95-0284 from the Biotechnology Program, EU to A.B. The Department of Immunology and Oncology was founded and is supported by the Spanish Council for Scientific Research (CSIC) and by the Pharmacia Corporation.
Figure 2

### A

| kDa | HIS-PKL12 | HIS-control |
|-----|-----------|-------------|
|     | GST SIP-16 | GST SIP-16 | GST |
| 47.5|           |            |     |
| 32.5|           |            |     |
| 25  |           |            |     |

PKL12

### B

| Total protein | IP protein |
|---------------|------------|
| kDa           | +PKL12     | +PKL12     |
|               | +HA-GlcNAcK| +HA-GlcNAcK|

32.5

PKL12
Figure 3

A

kDa

-GlcNAcK

- +

62

47.5

-GST

- +

GST-GlcNAcK

GST-GlcNAcK

B

kDa

kDa

NIH3T3  SV40  ST2  FL5.12  BA/F3  A.20  WEHI  EL4  COS

47.5

32.5

GlcNacK
Figure 4
Figure 5
|          | -GST-SIP16 | +GST-SIP16 |          | -GST-SIP16 | +GST-SIP16 |          | -GST-SIP16 | +GST-SIP16 |
|----------|------------|------------|----------|------------|------------|----------|------------|------------|
| PKL12 (g)| 0.25       | 0.25       | 0.25     | 0.1        | 0.25       | 0.1      | 0.25       | 0.1        |

**Figure 6**
GlcNAc kinase activity (mU/mg)

Figure 7
Functional interaction between the ser/thr kinase PKL12 and GlcNAc kinase, a prominent enzyme implicated in the salvage pathway for GlcNAc recycling
Jose Manuel Ligos, Teresa Laín de Lera, Stephan Hinderlich, Bárbara Guinea, Luís Sánchez, Ramón Roca, Alfonso Valencia and Antonio Bernad

J. Biol. Chem. published online December 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105766200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts