GAK: a cyclin G associated kinase contains a tensin/auxilin-like domain

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Abstract We have cloned a cDNA encoding a novel association partner of cyclin G by West-Western blotting. The cDNA encodes a protein that harbors a Ser/Thr protein kinase-like catalytic domain at the N-terminal. Hence, we named it GAK (cyclin G-associated kinase). The long C-terminal extension shares homology with tensin and auxilin, and contains a leucine zipper region. Co-immunoprecipitation and Western blotting showed that GAK and cyclin G associate together in vivo. GAK also co-precipitated with CDK5, and CDK5 was found to be associated with cyclin G. We also showed by BIAcore analysis that the GAK-cyclin G interaction was direct.

Key words: Cyclin G; CDK5; Tensin; Auxilin; Kinase

1. Introduction

The cell cycle of eukaryotes is regulated by cyclins and cyclin-dependent protein kinases (CDKs) [1–3]. Several species of cyclin-CDK complexes have been identified and shown to regulate different transitions in the cell cycle. Previously, we found a new cyclin (cyclin G) [4] that is homologous to human cyclin A and fission yeast Cig1 [5,6]. The recent discovery that cyclin G is a direct transcriptional target of the product of the p53 tumor suppressor gene highlighted its potential importance as a possible mediator between apoptosis and the cell cycle [7,8]. Transcription of cyclin G is induced within a few hours of growth factor stimulation of quiescent cells [4]. A retroviral vector-mediated gene transfer of antisense cyclin G inhibited proliferation of human osteogenic sarcoma cells [9], suggesting a pivotal role for cyclin G in growth regulation of mammalian cells. Otherwise, neither the biochemical regulatory mechanism nor the physiological function of cyclin G has been defined. In order to investigate the physiological role of cyclin G, we have searched for the proteins that associate with cyclin G by screening West-Western blots. Here we report the cloning of a cDNA encoding a novel Ser/Thr kinase which we call GAK, and demonstrate that it directly associates with cyclin G using two different techniques.

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The accession number of GAK in the DDBJ/EMBL/GenBank is D38560.

2. Materials and methods

2.1. Antibodies

Anti-GAK monoclonal antibodies (1E1 and 1C2) were produced by immunization with the rat GST-GAK fusion protein according to standard methods [10]. Anti-cyclin G (pGpep and pCyG) and anti-GAK (pGAK) polyclonal antibodies were raised by standard techniques in New Zealand White rabbits with the synthetic peptide of rat cyclin G (CTLPFERRNDLNFERL) and rat GST-GAK as antigens, respectively. Anti-CDC2 (MBL, Nagoya), anti-CDK2 (MBL), and anti-CDK5 (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibodies, and anti-cyclin D1 (MBL), anti-cyclin B1 (Oncogene Science), anti-cyclin E (UBI, New York), anti-CDK6, anti-CDK7 (Santa Cruz Biotechnology), and anti-CDK4 (UBI) polyclonal antibodies were purchased from the specified companies.

2.2. Molecular cloning of GAK

A rat fibroblast (3Y1) cDNA library was prepared using the λZapII vector (Stratagene, San Diego, CA), lysed and transferred to nitrocellulose filters. The filters were incubated with the GST-cyclin G fusion protein for several hours at 4°C in TBST buffer. The filters were screened using our anti-cyclin G polyclonal antibody, which was raised against the synthetic peptide of rat cyclin G (CTLPFERRNDLNFERL). Subsequently, the filters were incubated with anti-rabbit Ig antibody conjugated to alkaline phosphatase, which enabled the positive clones to be visualized by reaction with NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indoyl phosphate). λphage clones yielding duplicate positive signals were turned into plasmid forms by the SOLR system (Stratagene) and were subjected to further analysis. The DNA sequence of both strands was determined by the chain termination reaction.

2.3. Binding ability of anti-cyclin G and anti-GAK antibodies to GST fusion proteins

GST fusion proteins (1 μg) and relevant antibodies (1 μg) were mixed in PBS supplemented with protease inhibitors (100 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin A). The mixture was rotated at 4°C for 2 h, and 40 μl of 50% glutathione-Sepharose 4B was added. After further rotation at 4°C for 2 h, the complex of GST fusion protein and antibody was precipitated with glutathione beads. The beads were washed 3 times with 500 μl of PBS supplemented with protease inhibitors and mixed with 20 μl of SDS-PAGE sample buffer. After boiling for 5 min, 5 μl of each sample was loaded on a 10% SDS-PAGE gel and subjected to Western blotting as described below. Precipitated and blotted antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG plus anti-rabbit IgG.

2.4. Immunoprecipitation and Western blotting

Immunoprecipitation was done by collecting and lysing subconfluent NRK cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% NP40) supplemented with protease inhibitors and phosphatase inhibitors (10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM sodium vanadate and 1 mM EGTA). After clarifying the extract by centrifugation at 10000×*g* for 5 min, aliquots of the supernatant were immunoprecipitated by protein A-Sepharose alone. The precleared lysates were subsequently immunoprecipitated by the relevant antibodies. For Western blot analysis, equal quantities of fresh or immunoprecipitated cell extract were adsorbed to protein A-Sepharose, separated by 10% SDS-PAGE, transferred onto Immobilon mem-

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brane (Millipore) and probed with indicated antibodies in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20). Immunoreactive protein bands were visualized by ECL (Amersham, UK) or RENAISSENSE (Dupont, Boston, MA) chemiluminescence reagents. The prestained size marker was purchased from BioRad.

2.5. Preparation of Myc-tagged cyclin G and transformation of COS7 cells

The *myc*-tagged cyclin G cDNA was synthesized by PCR (with *Pfu* DNA polymerase) using the 5′ primer 5′-ACGCGTAAGATGGAACAAAAGCTTATTTCTGAAGAAGACTTGATAGAAGTACTGACAACTGAC-3′ (where the underlined sequence is the sense sequence coding for a C-terminal 9E10 *myc* epitope) and the 3′ primer 5′-CGTCGAATTCTAACCCATGGTTTCGGG-3′ with rat cyclin G cDNA as a template. The PCR product generated was digested with *Pst*I/*Eco*RI, and inserted into the *Pst*I/*Eco*RI site of the pAP3neo vector (Nojima, unpublished). The plasmid DNA was transfected into COS7 cells using lipofectamine (Gibco-BRL, Gaithersburg, MD). Anti-*myc* monoclonal antibody was purchased from Oncogene Science.

2.6. Association of GAK with cyclin G in vitro as assessed by BIAcore

In order to perform analysis with a surface plasmon resonance biosensor (BIAcore, Pharmacia), polyclonal antibody against GST (Pharmacia) was immobilized onto a dextran flow cell matrix (Sensor chip CM5, Pharmacia) at an amount corresponding to 1000 R ($\sim$1.0 ng/mm^2). 50 μl of GST-GAK (1 μM) was injected and trapped by the antibody via the GST portion of the molecule. Subsequently, 40 μl of affinity purified cyclin G (0.5–2.0 μM) or Hacl (1 μM) was injected to detect any interactions. All sensograms were recorded at a flow rate of 5 μl/min at 20°C in HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium azide, 0.05% Tween-20). At the end of each measurement, immobilized GST antibody was regenerated using 0.1 M glycine-HCl (pH 2.5), and the same amount of a new GST-GAK sample was injected to monitor separate interactions. Kinetic data were analyzed using standard kinetic equations [11] implemented in a software package (Kinetics Evaluation) supplied by Pharmacia Biosensor and run on a Compaq PC.

3. Results

3.1. Molecular cloning of GAK

To search for the cDNA encoding an association partner of cyclin G, we prepared a rat fibroblast (3Y1) cDNA library with the λZapII vector and screened it by West-Western blotting using an anti-cyclin G polyclonal antibody. Of about a million phages, seven λphage clones showed positive signals. The DNA sequence, as determined by the chain termination reaction, revealed that one of the clones encoded a novel Ser/Thr protein kinase (1305 amino acids), which we named GAK, an acronym for cyclin G-associated kinase. The details of the other clones will be published elsewhere.

The amino acid sequence of the cDNA, as deduced from the DNA sequence, indicated that the structure of the kinase domain closely resembles that of Nek1 [12], CDK2 [2,13,14], Plk [14] and Tsk-1 [15] kinases (Fig. 1A,B). As GAK lacks a PSTAIR motif, the amino acid sequence found in all CDKs, it cannot be considered a member of the CDK family. In the middle part of the molecule (Fig. 1C), GAK resembles both tensin, an actin-binding component of adhesion plaques (also called focal contacts) and other submembranous cytoskeletal structures [16], and auxilin, a coat component of brain clathrin-coated vesicles [17,18]. The domain homologous to tensin and auxilin (we call it the TAG domain hereafter) occupies more than half of the whole molecule and contains, in addition, a leucine zipper region [19] in the C-terminal portion of this domain. In the C-terminal domain of the molecule (132 amino acids), GAK and auxilin are up to 80% identical, and this region is very rich in serine and proline residues (14% and 18% respectively). A phosphorylation recognition site for a tyrosine kinase is located at position 403–410 (KGDLDISY) [20–22] at the left end of the TAG domain. This recognition site is also found in auxilin (KGDLDFTY at position 58–65) but the sequence is less conserved in tensin (SCELDLVY at position 64–71). The kinase domain of GAK harbors two putative nucleotide binding sequences (GXGXXG) at positions 13–18 and 337–342 [23]. One (KKLS at position 90–93), 19 and 24 potential phosphorylation sites for cAMP-dependent protein kinase, protein kinase C and casein kinase II protein kinase, respectively, were also found (locations are not shown). A hydropathy plot [24] suggests that the overall structure of GAK is hydrophilic and no conspicuous cluster of hydrophobic regions could be found (data not shown).

A Northern blot showed that GAK mRNA occurs as a single band in NRK cells (data not shown), whose size (about 4.4 kb) is similar to that of the cDNA we cloned. Although auxilin is transcribed specifically in neural cells, GAK mRNA was ubiquitous and was expressed at variable levels in all the tissues we examined (data not shown).

3.2. Characterization of the antibodies

We prepared six kinds of antibodies, namely two anti-cyclin G (pGpep and pCyG) polyclonal antibodies, one anti-GAK polyclonal antibody (pGAK) and three anti-GAK monoclonal antibodies (1E1, 1C2 and 3H9). pGpep antibody, which we used for West-Western screening, binds to native GST-cyclin G and was useful for West-Western screening, GST pull-down experiment (Fig. 2A, lane 3) and immunoprecipitation (Fig. 3C, lane 12). The positive signal obtained by West-Western screening was not due to cross-reactivity of the antibody to GAK, because the GST-GAK fusion protein was not recognized by this antibody (lane 2). It was found that pGpep antibody did not effectively detect the cyclin G band on Western blots. Therefore, we purchased anti-cyclin G antibody from Santa Cruz Biotechnology to perform Western analysis. This antibody was also effective for immunoprecipitation as judged by the GST pull-down experiment (lanes 5 and 6) compared to the negative control (lanes 2 and 7). We did not use pCyG (lane 4) since it showed similar properties to pGpep.

All of the anti-GAK antibodies seem to recognize the denatured form of GST-GAK, which was largely degraded during the purification process (Fig. 2B, lanes 2, 4, 6 and 8). Anti-GAK polyclonal antibody (pGAK) was useful for immuno-

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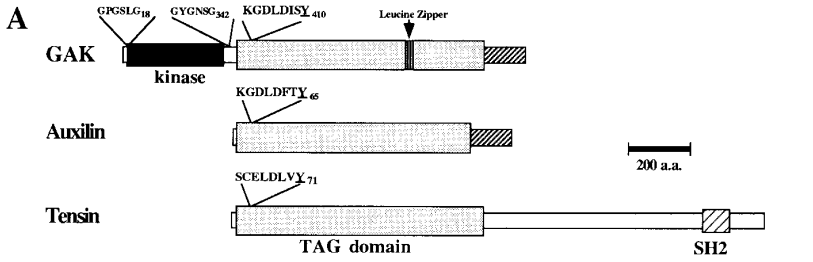
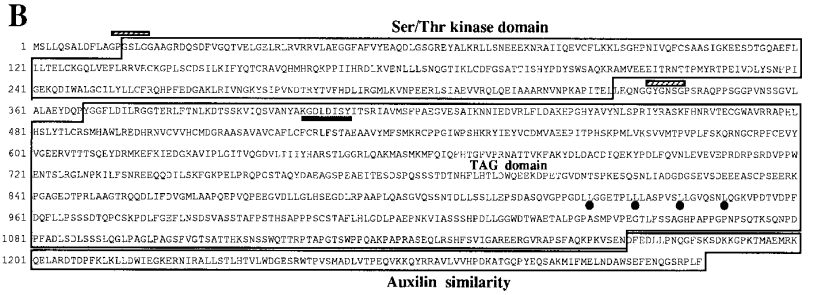
Fig. 1. Structure of GAK. A: Schematic presentation of the structure of rat GAK, tensin [16] and auxilin [17]. The consensus domain for Ser/Thr kinases (filled box), the conserved domains between GAK and auxilin (shaded box), and homologous regions between GAK, tensin and auxilin (TAG domain as stippled box) are indicated. The locations of the nucleotide binding sequences (GXGXXG), the phosphorylation site for tyrosine kinase (KGDLDISY), and the leucine zipper are also shown. B: Amino acid sequence of rat GAK. The Ser/Thr kinase domain, TAG domain and the region homologous to auxilin are encircled. The locations of the phosphorylation site for tyrosine kinase (filled bar), and for the leucine zipper (filled circles) are denoted. C: Comparison of the amino acid sequences of GAK, auxilin and tensin. Shaded boxes represent identical amino acids.





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GAK       Y-GGFLDILRGGTERLFT--------NLKDTSSKVIQSVANYAKGDLDTSYITSRIAVMS   419
Auxilin   YGGGLFDMVKGGAGRLFSNLKDNLKDTLKDTSSRVIQSVTSYTKGDLDFTYVTSRIIVMS    74
Tensin    ELPSPGQSITKQVDTPDATRSPRGGQAHCKASRSMSVTAAMESSCELDLVYITERIIAVS    80

          FFAEGVES-AIKNNIEDVRLFLDAKHPGHYAVYNLSPRIYRASKFHNRVTRCGWAVRRAP   478
          FPLDSVDI-GFRNQVDDIRSFLDSRHLDHYTVYNLSSKSYRTAKFHSRVSEGSWEIRQAP   133
          YSSTAEEPRSFRSNLREVAHMLKSKHGQNYVLFNLSERRHDISKLHPKVLDFGWPDLHTP   140

          HLHSLYTLCRSMHAWLREDHRNVCVVHCMDGRAASAVAVCAFLCFCRLFSTAEAAVYMFS   538
          SLHNEFAVCRNMYNWLLQNPKNVCVVHCLDGRAASSILVGAMFIFCNIYSIPGPAVRLLY   193
          AIEKICSICKAMDTWLNAAAHNVVVLHNKGNRGRLGVVVAAYMHYSNISASADQALDRFA   200

          MKR-----CPTGIWFSHKPYIPYVCDMVAEEPITPHSKFMLVKSVVMTEVEFFSKQRNGC   593
          AKR-----PGIGLSFSHFPYLGYMCDLLADKPYRPHFKFLTIKSITVSFVEFFNKQRNGC   248
          MKRFYEDKVVPVGQPSQKRYIHYFSGLLS-GSIKMNNKPLFLHHVIMHGIPNFES-KGGC   258

          RPFCVYVGEERVTTSQEYDEMKEFKIEDGKAVIPLGITVQGDVLTIIYHARSTLGGRL   653
          RPYCDVLIGETKIYTTCADFERMKEYRVQDGKIFIPLSITVQSDVVVSMYHLRSTIGSRL   308
          RPFLKIYQAMQPVYTSG-IYNVQGDSQTGICITIEP-GLLLKGLILKCYH------KKF   310

          QAKMASMKMFQIQFHTGFVPRNATTVKFAKYDLDACDIQEKYPDLFQVNLEVEVEPRDRP   713
          QAKVTNTQIFQLQFHTGFIPLDTTVLKFTKPELDACDVPEKYPQLFQVTLDVELQPHDKV   368
          RSPTRDV-IFRVQFHTCAV-HDLDIV-FGKPDLDEAFRDERFFEYGKVEFVFSYGFEKIQ   367

          SRDVPPWENTSLRGLNPKILFSNREEQQDI-ASKFGKPELPRQPGSTAQYDAEAGSPEAE   772
          MELTPPWEHYCTKDVNFSILFSSHQEHQDT-LVLGGQAPIDIPPDNPRHFGQGGFFSTLC   427
          GMEHLENGPSVSVDYNTSDPLIRWDSYENFNIQREDSAEGTWAEPALPGKHLEKEVGHTQ   427

          ITESDSPQSSTDTNHFLHTLDWQEEKDPETGVDNTSPKESQSNLIADGDGSEVSDEEEA   832
          WQDQKSEKSFCEEDHAAEVNQESEQSDQELLTLSSPHGNANGDKPHAARKPSKKQQE---   484
          GPL-DGSLYAKVKKKDSLH--GSIGAVNTARLPLSAAPNHVEHTSSVSSDSGNSTASTKT   484

          SCPSEERK------PGAGEDTPRLAAGTRQQDLIFDVGMLAAPQEPVQPEEGVDLLGLHS   886
          ----------------------------------------PAAPAPPED-VDLLGLEG   501
          DRTDEPGA------PGAPTGHAVLSPEEKRDVDRLLVGFGLESAAPMHNHAPGPAPARLP   538

          EGDLPAAPLQASGVQSSNTDLLSSLLEPSDASQVGPFGDLLGGETPLLIASFVSLLGV-   945
          -SAVSKNFS--SPAAPPSNSELLSDLFGGGGAAGPVQSGQ-SGVDDVFHPSGPTSTQSTP   557
          AGPGKHVVPAQVH-VNGAGTPLLAE--RETDILDDELPNQ--DGHSVGSLGTLSSLDGT-   592

          -QSNLQGKVPDTVDPFDQFLLFSSSDTQPCSKPDLFGFFLNSDSVASST-AFPSTHSAPP   1003
          RRSATSTSASPTLPVGEGATFDPFGAPSKPSGQDLLGSFLNTAS-ASSD-PF-----FQF   610
          -TTASEAGFHEAPRVGSLSSLFNGPASYNGAEKMLKEGLYEAEPLSNGAYPYSNQNTEMG   651

          PSCSTAFLHLGDLPAEPNKVIASSSHPDLLGWDTWAFTALPGPASMPVPEGTLFSSAGH   1063
          TRSPSPTVH-------ASSTPAVNIQPDVSGAWD-W-HTK-PGGFGM-GSKSAATSPTG-   658
          HHLRDPLAHL-RPSRSAQEHIAGYPQRQPASASPAWLQPPVPQPYLYGYDLPSAHRSQSF   710

          PAPPGPNPSQTKSQNPDPFADLSDL-SSSLQ---GLPAGLPAGSFVGTSATTHKSNSSWQ   1119
          -SSHQTPTHQNRPQTLDPFADLGTLGGSSFASKPSTPTGLG-GGFPPLSSPQKASPQPMG   716

          TTRPTAPGTSW-PPQAKP---APRASEQLRSHFSV-----IGAREEPGVRAPSFAQKPKV   1170
          GGWQQGGGYNWQQTQSKPQSSMPHSSPQNRPNYNVSFSSMPGGQNERGKAAANLEGKQK-   775

          SENDFEDLLPNQGF-SKSDKKGPKTMAEMRKQRLARDTDPFKLKLLDWIEGKERNIRALL   1229
          -AADFEDLLSGQGFNAHKDKKGPRTIAEMRKEEMAKEMDPEKLKILEWIEGKERNIRALL   834

          STLHTVLWDGESRWTPVSMADLVTPEQVKKQYRRAVLVVHPDKATGQPYEQSAKMIPMEL   1289
          STMATVLWAGETKWKPVGMADLVTPEQVKKVIRKAVLVVHPDKATGQPYEQYAKMIPMEL   894

          NDAWSEFENQGSRPLF                                               1305
          NDAWSEFENQGQKPLY                                               910
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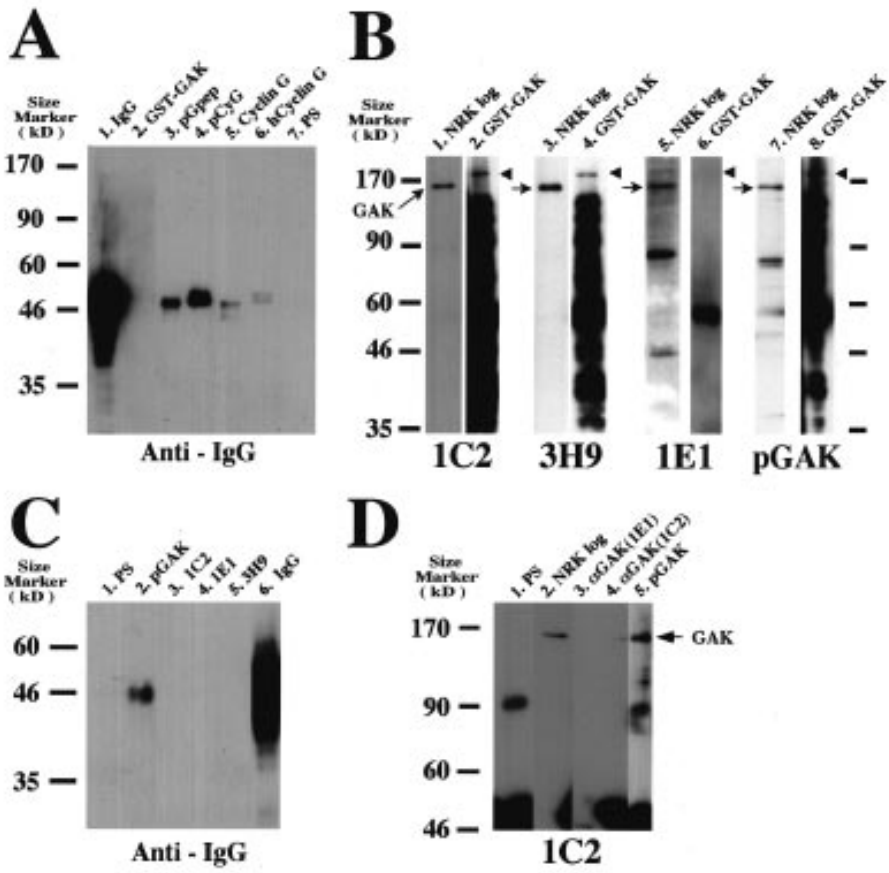

Fig. 2. Characterization of cellular GAK protein and anti-cyclin G or anti-GAK antibodies. A: Binding ability of anti-cyclin G antibodies to GST-cyclin G. GST-cyclin G was mixed with the anti-cyclin G peptide (pGpep; lane 3) used for West-Western screening, anti-GST-cyclin G (pCyG; lane 4), anti-rat cyclin G of Santa Cruz (cyclin G; lane 5), anti-human cyclin G of Santa Cruz (hCyclin G; lane 6), or preimmune serum (PS; lane 7). Subsequently, these samples were precipitated with glutathione-Sepharose 4B, precipitated, Western-blotted and probed with anti-IgG antibody. To show the absence of cross-reaction of the antibodies with the GST portion of GST-cyclin G, pGpep was mixed with GST-GAK (lane 2). As a positive control for the IgG band, 1 μg of normal rabbit IgG was loaded on the same gel (lane 1). Because all of the polyclonal antibodies were prepared by immunizing rabbits, the affinity of the HRP-conjugated secondary anti-rabbit IgG antibody to the samples in lanes 3–7 are expected to be equal. B: Quality of anti-GAK antibodies used in the experiments. Lysates (20 μg) of NRK cells growing in log phase (lanes 1, 3, 5 and 7), or affinity-purified GST-GAK (100 μg; filled triangles denote full-length GST-GAK in lanes 2, 4, 6 and 8) were loaded, Western-blotted and probed with monoclonal (1C2, 3H9 or 1E1) or polyclonal (pGAK) anti-GAK antibodies. C: Binding ability of anti-GAK antibodies to GST-GAK was assessed as described in A by mixing GST-GAK with preimmune serum (lane 1), anti-GAK polyclonal antibody (lane 2), anti-GAK monoclonal antibodies (lanes 3, 4 and 5). Normal rabbit IgG was loaded on the gel (lane 6). D: Immunoprecipitation of GAK in NRK cell lysates with anti-GAK antibodies. NRK cell extract (500 μg) was immunoprecipitated with preimmune serum (lane 1), 1E1 (lane 3), 1C2 (lane 4) and pGAK (lane 5). NRK cell extract (20 μg) was also electrophoresed (lane 2) to assign the GAK band.

precipitation (Fig. 2C, lane 2; Fig. 2D, lane 5) but detected bands other than the intact GAK band when used for Western analysis (Fig. 2B, lane 7). An anti-GAK monoclonal antibody (1E1) was useful for immunostaining (data not shown) and Western blotting, although it also detected extra bands (Fig. 2B, lane 5). On the other hand, two anti-GAK monoclonal antibodies (1C2 and 3H9) displayed essentially a single band for GAK with high sensitivity in Western blotting (Fig. 2B, lanes 1 and 3). However, these monoclonal antibodies did not recognize the native form of GST-GAK (Fig. 2C, lane 3, 4 and 5), and for that reason the native form of GAK was only inefficiently immunoprecipitated from the NRK extract (Fig. 2D, lanes 3 and 4). Therefore, we primarily used pGAK for immunoprecipitation, pGAK or 1E1 for immunostaining (data not shown), and 1E1, 1C2 or 3H9 for Western blot analysis.

3.3. Association of GAK with cyclin G in vivo

We demonstrated an association of GAK with cyclin G in vivo by performing immunoprecipitation of NRK (normal rat fibroblast) cell extracts with an antibody directed against rat cyclin G (Santa Cruz), followed by Western blotting the immunoprecipitates with an anti-GAK monoclonal antibody (1C2) (Fig. 3A-i, lane 3). We also demonstrated their association by reciprocal immunoprecipitation (with pGAK) and Western blotting (Fig. 3A-ii, lane 1). In both cases, the immunoprecipitates with preimmune serum (PS) yielded no band and were used as negative controls (Fig. 3A-i, lane 2 and Fig. 3A-ii, lane 4). Pre-treatment of anti-GAK antibody with affinity-purified GST-fused GAK protein weakened the intensity of the band at 33kD (Fig. 3A-ii, lanes 2 and 3). This result suggests that the binding of anti-cyclin G antibody to GAK was competitive. It remains to be investigated whether the

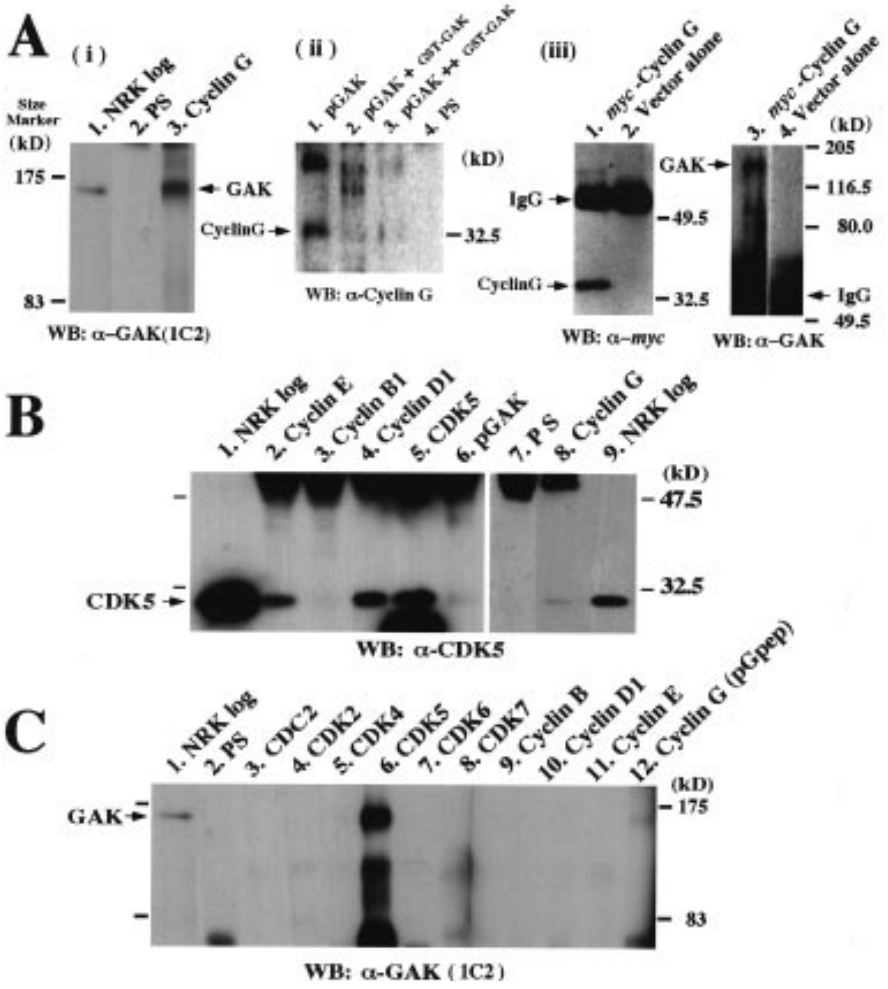

Fig. 3. Association of GAK with cyclin G and CDK5 in vivo. A: Association of GAK with cyclin G in vivo. (i) 10 μg of NRK cell extract was electrophoresed (lane 1) to assign the GAK band. 500 μg of NRK cell extract was immunoprecipitated with preimmune serum (lane 2) or with anti-cyclin G polyclonal antibody (Santa Cruz; lane 3) and immunoblotted with anti-GAK monoclonal antibody (1C2). The band for GAK observed in lanes 1 and 3 but not in lane 2 is denoted by an arrow. (ii) NRK cell extract (500 μg) was immunoprecipitated with anti-GAK polyclonal antibody (lanes 1–3), or preimmune serum (lane 4) and immunoblotted with anti-cyclin G polyclonal antibody. 10 μg (lane 2) or 3.5 μg (lane 3) of affinity-purified GST-GAK protein was preadsorbed to the anti-GAK polyclonal antibody (pGAK) to conduct competition experiments. (iii) COS7 cell extracts transfected by the plasmid DNA with (lanes 1 and 3) or without (lanes 2 and 4) *myc*-tagged cyclin G were immunoprecipitated and immunoblotted with either anti-*myc* (lanes 1 and 2) or anti-GAK (lanes 3 and 4) antibodies. Arrows denote the band for *myc*-tagged cyclin G, indicating successful expression, and for GAK, demonstrating a stable in vivo association with *myc*-tagged cyclin G. B: Association of CDK5 with either GAK (lane 6) or cyclin G (lane 8), demonstrated by immunoprecipitation with corresponding antibodies and Western analysis with anti-CDK5 (DC17) monoclonal antibody. C: Association of GAK with either CDK5 (lane 6) or cyclin G (lane 12), demonstrated by immunoprecipitation with corresponding antibodies and Western analysis with anti-GAK monoclonal antibody (1C2). Western analysis was performed on NRK extracts (denoted as NRK log) and on immunoprecipitates obtained with anti-cyclin E, anti-cyclin B1, anti-cyclin D1, anti-cyclin G (pGpep), anti-CDK4, anti-CDK6, anti-CDK7 or anti-GAK polyclonal antibodies or with anti-CDC2 (2A10), anti-CDK2 (8A12) or anti-CDK5 (DC17) monoclonal antibodies. Rabbit preimmune serum (PS) was also used for immunoprecipitation as a negative control.

unidentified upper bands observed in lanes 2–4 of Fig. 3A-ii are modified forms of cyclin G or contaminant proteins. Association of GAK with cyclin G in vivo was further confirmed by immunoprecipitation of a COS7 cell extract bearing exogenously expressed *myc*-tagged cyclin G with anti-*myc* monoclonal antibody, followed by Western blotting with an anti-GAK monoclonal antibody (1E1) (Fig. 3A-iii, lane 3). Successful expression of *myc*-tagged cyclin G was demonstrated by Western analysis of the blot with an anti-*myc* antibody (Fig. 3A-iii, lane 1). The *myc*-tagged cyclin G migrated to a slightly different location compared to the native cyclin G because of the presence of additional *myc* peptides at the N-terminus.

To examine whether GAK is the only cyclin G-associated kinase, association of cyclin G with other known CDKs was examined by immunoprecipitation and Western blotting. No association was detected when an NRK extract was immunoprecipitated by anti-cyclin G antibody and Western blotted with antibodies directed against CDC2, CDK2, CDK4 and CDK6 (data not shown). On the other hand, association of cyclin G with CDK5 was demonstrated when anti-cyclin G immunoprecipitates were subjected to Western analysis and probed with anti-CDK5 antibody as shown in Fig. 3B (lane 8), although the intensity of the band was very faint. It remains to be established whether the weak intensity of the band is due to inefficient immunoprecipitation with anti-cyclin

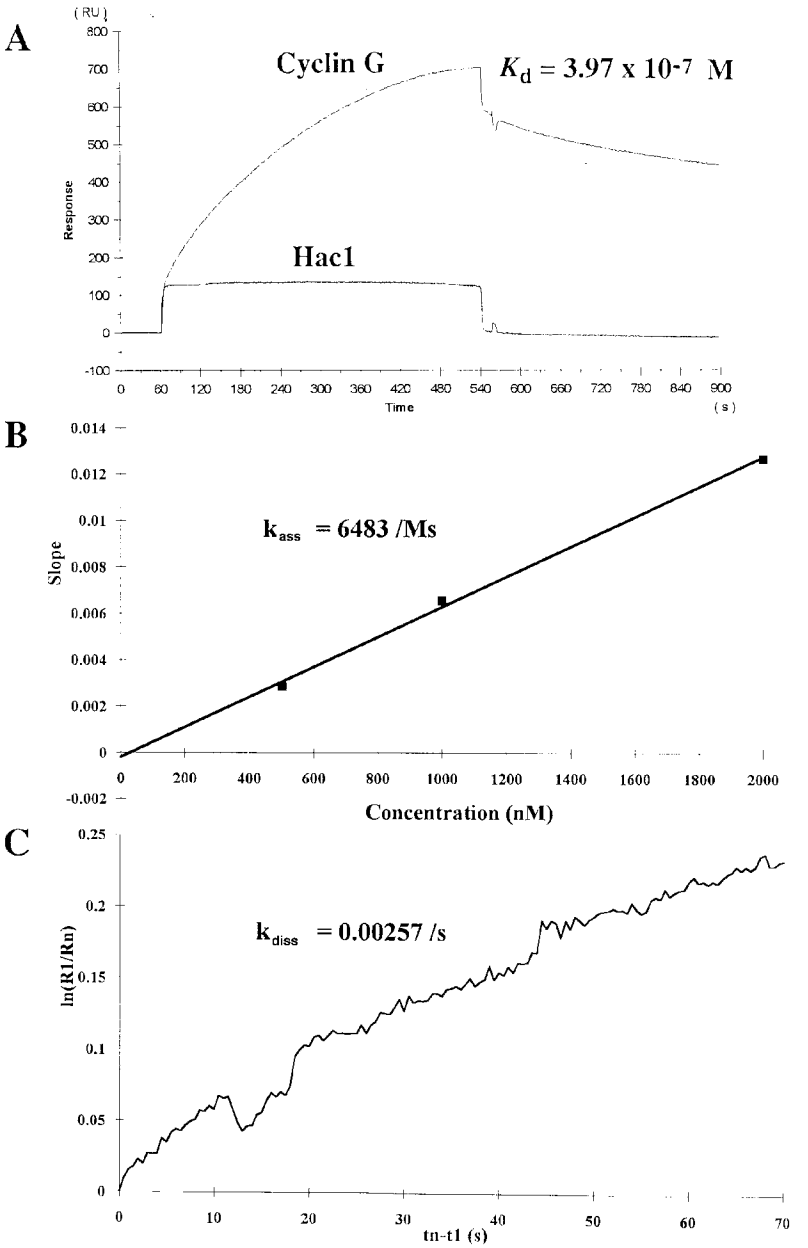

Fig. 4. Association of the affinity-purified GST-GAK fusion protein with cyclin G in vitro. A: Overlay presentation of the sensograms for the association of GST-GAK/cyclin G and GST-GAK/Hac1. B: The slope of the interaction curve at each cyclin G concentration was used for kinetic analysis. Purified cyclin G protein was added at various concentrations ranging from 0.5 μM to 2 μM in HBS buffer. The association constant k_{ass} was obtained from the slope of the fitted line. C: Dissociation of GAK-GST and cyclin G under continuous buffer flow. The dissociation rate (k_{diss}) was obtained from the slope of the ln(R1/Rn) versus tn−t1 plot. The affinity constant of the GST-GAK/cyclin G interaction was calculated from $K_d = k_{diss}/k_{ass}$.

G antibody, or to weak association of cyclin G with CDK5 in vivo. A Western blot analysis also detected association of GAK with CDK5 in vivo, but here again this interaction appeared weak (lane 6). Association of cyclin D and cyclin E with CDK5 was also detected (lanes 2 and 4) as reported previously in human fibroblast cells (WI38) [25] and mouse neural cell extracts [26].

To investigate whether GAK associates with other components of the cyclin-CDK complex, the immunoprecipitates obtained with relevant antibodies were analyzed by Western blotting using an anti-GAK monoclonal antibody (1C2) (Fig. 3C). Among the antibodies tested, GAK seems to associate only with CDK5 (lane 6) and cyclin G (lane 12). The band for CDK5 (lane 6) is very strong, probably as a result of the high quality of the anti-CDK5 monoclonal antibody. Association of GAK with cyclin G was also confirmed by the detection of the GAK band in an immunoprecipitate obtained with the anti-cyclin G polyclonal antibody, pGpep (lane 12). The association of GAK with other components of cyclin/CDK complexes, such as cyclin A, cyclin C, cyclin D2, cyclin D3, cyclin F, cyclin H or cyclin I, whose antibodies were not available for us, remains to be tested.

3.4. Analysis of the cyclin G-GAK interaction using the BIA-core system

To analyze the kinetics of the cyclin G-GAK interaction in vitro, the GST fusion proteins of cyclin G and GAK were purified to homogeneity. GAK and cyclin G proteins without the GST portion were also prepared by digestion of GST-GAK or GST-cyclin G with thrombin. GST-GAK is susceptible to protease attack in *E. coli* cells as shown in Fig. 2B. For this reason, we primarily obtained a truncated form of ~60 kDa (full size GST-GAK is ~190 kDa) that retained, nonetheless, histone H1 kinase activity as will be described later. Therefore, the kinase domain seems to be conserved. On the other hand, the affinity-purified cyclin G, after removal of the GST portion by treatment with thrombin, displayed a single band at the expected size on SDS-PAGE (data not shown). Analysis by surface plasmon resonance biosensor technology [11,27–29] suggested that GAK directly associates with GST-cyclin G, probably via the kinase domain at the N-terminus of the molecule. In this experiment, anti-GST antibody was covalently attached to the dextran matrix of a BIAcore sensor chip to assess the association at a neutral pH value. The binding of the GST-GAK fusion protein to the antibody and the association of subsequently applied cyclin G with GST-GAK were monitored as a change in refractive index. The resonance signal during the injection of cyclin G and the displacement of the baseline during the subsequent rinsing process indicate a stable association between GAK and cyclin G. In the negative control experiment, in which a yeast DNA binding protein, Hac1 [30], was used, no binding of Hac1 to GAK was observed, as shown by an overlay presentation of the resonance signal (Fig. 4A). Analysis of the association and dissociation phases of the interaction revealed an association rate of 6483 M^{-1} s^{-1} and a dissociation rate of 2.57×10^{-3} s^{-1} (Fig. 4B,C). The K_d value calculated from these rate constants was 397 nM (Fig. 4C), suggesting that GAK and cyclin G form a stable complex in vivo.

Direct association of immobilized GST-CDK5 with GST-free cyclin G was also examined by BIAcore system, but we could not detect any stable association between these two molecules (data not shown). It remains to be established whether the association between the affinity-purified cyclin G and CDK5 proteins that were prepared by expression in *E. coli* cells requires some modifications such as phosphorylation, or their association is merely indirect.

4. Discussion

Here we report the existence of a novel association partner of the cyclin-CDK complex that we refer to as GAK. We have demonstrated and confirmed the association of cyclin G with GAK in vivo by reciprocal immunoprecipitation and Western blot analysis in four different experiments as shown in Fig. 3A-i (lane 3), Fig. 3A-ii (lane 1), Fig. 3A-iii (lane 3) and Fig. 3C (lane 12). We also demonstrated in vitro, using the BIAcore system, that the interaction was direct (Fig. 4). Furthermore, we found that GAK also associates with the CDK5 complex (Fig. 3B,C, lanes 6) by reciprocal immunoprecipitation and Western blot analysis. GAK migrated to a position corresponding to a molecular mass of 160 kDa in SDS-PAGE, a size that coincided with one of the unidentified association partners of CDK5 reported previously [31]. It is feasible that GAK corresponds to their 180 kDa protein. In fact, we were able to detect a single band of 160 kDa in silver-stained SDS-PAGE gels in immunoprecipitates of NRK cell extracts obtained with either anti-CDK5 or anti-GAK antibodies (data not shown).

It has been reported previously that p35, a cyclin-like protein, is the primary association partner of CDK5 in neural tissues [31,32]. As CDK5 is ubiquitous, one would expect that cyclins other than p35 function in non-neural tissues such as NRK cells as the regulatory subunit of CDK5. We demonstrated here by immunoprecipitation and Western blot analysis that cyclin G associates with the CDK5 complex in NRK cells. However, the BIAcore analysis suggested that their association is not stable at least under the conditions we used here. Therefore, cyclin G may not be the authentic CDK5 regulatory subunit of CDK5 in NRK cells. In fact, our preliminary kinase assays using histone H1 as a substrate demonstrated that cyclin G constitutes only a part of the kinase activity of CDK5 (data not shown). This property of cyclin G is similar to that of cyclin D and cyclin E, with which CDK5 associates [25,26], but no regulatory action on the kinase activity was reported so far.

GAK harbors several noticeable amino acid sequences of potential interest. Firstly, it possesses a protein kinase domain which resembles that of Nek1, CDK2, Plk and Tsk-1. Considering the absence of the PSTAIR motif in GAK, it is evident that GAK does not belong to the CDK family. Secondly, it harbors two putative dinucleotide-binding motifs in the kinase domain suggesting that GAK activity is somehow modulated by dinucleotides such as ADP or GDP. Thirdly, a phosphorylation recognition site for a tyrosine kinase is located at position 403–410 (KGDLDISY) [20–22] at the left end of the TAG domain. Fourthly, it contains a leucine zipper motif in the middle of the molecule whose physiological meaning is unknown at this stage. Fifthly, the amino acid sequence in the middle part of GAK resembles tensin and auxilin.

Tensin is an actin-binding component of the focal adhesion plaque, which is an anchorage point of the plasma membrane to the substratum. The focal adhesion plaque is usually associated with cytoplasmic microfilament bundles, and stress fibers, composed of bundles of actin filaments, are often anchored to these regions via an association with tensin [33]. Tensin includes both phosphorylated tyrosine residues and a Src homology 2 (SH2) domain, which is shared by a number of signal transduction proteins [34]. It is suggested that tensin may link signal transduction pathways with the cytoskeleton by possessing both actin-binding and phosphotyrosine-binding properties and being itself a target for tyrosine kinases [35]. Since GAK also has tensin-like properties such as an actin-binding domain and a target site for tyrosine kinases, it is conceivable that GAK plays a similar role in the cytoskeletal signal transduction pathway. Absence of the SH2 domain in GAK and differences in the recognized amino acid sequences around the target tyrosine residue indicate that the physiological roles of tensin and GAK as signal transducers may be somewhat distinct. The presence of a Ser/Thr kinase domain at the N-terminal portion of the molecule suggests that GAK plays a more positive role as a modulator of signal transduction, by relaying the phosphorylation signal from an unidentified tyrosine kinase to unidentified GAK phosphorylation targets. The examination of these possibilities will be the subject of our future experiments.

Auxilin is a coat protein of brain clathrin-coated vesicles,

which directly interacts with the heavy chain of clathrin, supporting its assembly into regular cages, and is involved in the regulation of nerve-cell-specific functions [17]. The putative consensus sequence for the SH3 domain (PXXXPPXXP) [36] found in auxilin was not detected either in GAK or in tensin. The C-terminal portion of GAK (132 residues) closely resembles auxilin and the secondary structure of this region is shown to be predominantly α-helical [17]. Inasmuch as the main body of GAK is similar to auxilin, it is feasible that GAK actually binds to cytoskeletal membranous structures. It is hoped that our future investigations will reveal the physiological significance of these structural resemblances in relation to the role of cyclin G.

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