Identification and characterization of a novel sucrose-non-fermenting protein kinase/AMP-activated protein kinase-related protein kinase, SNARK

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Subtraction hybridization after the exposure of keratinocytes to ultraviolet radiation identified a differentially expressed cDNA that encodes a protein of 630 amino acid residues possessing significant similarity to the catalytic domain of the sucrose-non-fermenting protein kinase (SNF1)/AMP-activated protein kinase (AMPK) family of serine/threonine protein kinases. Northern blotting and reverse-transcriptase-mediated PCR demonstrated that mRNA transcripts for the SNF1/AMPK-related kinase (SNARK) were widely expressed in rodent tissues. The SNARK gene was localized to human chromosome 1q32 by fluorescent *in situ* hybridization. SNARK was translated *in vitro* to yield a single protein band of approx. 76 kDa; Western analysis of transfected baby hamster kidney (BHK) cells detected two SNARK-immunoreactive bands of approx. 76–80 kDa. SNARK was capable of autophosphorylation *in vitro*; immunoprecipitated SNARK exhibited phosphotransferase activity with the synthetic peptide substrate HMRSAMSGLHLVKRR (SAMS) as a kinase substrate. SNARK activity was significantly increased by AMP and 5-amino-4-imidazolecarboxamide riboside (AICAriboside) in rat keratinocyte cells, implying that SNARK might be activated by an AMPK kinase-dependent pathway. Furthermore, glucose deprivation increased SNARK activity 3-fold in BHK fibroblasts. These findings identify SNARK as a glucose- and AICAriboside-regulated member of the AMPK-related gene family that represents a new candidate mediator of the cellular response to metabolic stress.

Key words: AICAriboside, glucose, metabolism, signal transduction, stress.

INTRODUCTION

Protein kinase cascades are highly conserved between animals, fungi and plants. The sucrose-non-fermenting protein kinase (SNF1) from *Saccharomyces cerevisiae* and its mammalian counterpart, AMP-activated protein kinase (AMPK), form a family of serine/threonine kinases and are key components in yeast and mammalian stress response systems [1]. This family of kinases is commonly activated in response to cellular and environmental stresses such as nutrient deprivation. Yeast SNF1 responds to glucose deprivation by derepressing genes implicated in carbon source utilization and by modulating the transcription of glucose-regulated genes involved in gluconeogenesis, respiration, sporulation, thermotolerance, peroxisome biogenesis and cell cycle regulation [2]. AMPK is similarly activated by environmental stresses that result in an increase in the cellular AMP:ATP ratio. Activated AMPK switches off anabolic pathways (e.g. fatty acid and cholesterol synthesis) and induces ATP-generating catabolic pathways [1].

The SNF1/AMPK family of serine/threonine kinases has expanded rapidly with the identification of several SNF1/AMPK-related protein kinases in plants [3–11], *Plasmodium falciparum* [12], *Chlamydomonas* [13] and mammals [14–19]. These protein kinases have been assigned to the SNF1/AMPK family primarily on the basis of their structural similarity to the catalytic domains of SNF1 and AMPK. The available structural and functional data are consistent with the notion that SNF1, AMPK and related kinases represent components of signalling cascades that control metabolism, gene expression and perhaps cell proliferation in response to cellular, metabolic and environmental stresses [1].

To understand the cellular response of keratinocytes to the environmental stress of UVB radiation, we employed subtraction hybridization to identify UVB-induced genes in rat keratinocytes. Several of the cDNA species identified corresponded to known genes such as ferritin heavy chain, ribosomal subunits, keratin, elongation factor 1α, thioltransferase, cyclin G, cornifins, cellubrevins, poly(A)-binding protein and the cell cycle checkpoint regulator p21$^{CIP1/WAF1}$ [20]. In contrast, several differentially expressed UVB-induced cDNA species contained only 3′-untranslated sequences, precluding an initial definitive identification of their cognate proteins. We now report the cloning and characterization of a full-length cDNA for a UVB-induced mRNA transcript, now designated SNF1/AMPK-related kinase (SNARK), that encodes a new mammalian member of the SNF1/AMPK gene family.

Abbreviations used: AICAriboside, 5-amino-4-imidazolecarboxamide riboside; AICAribotide, 5-amino-4-imidazolecarboxamide ribotide; AMPK, AMP-activated protein kinase; BHK, baby hamster kidney cell line; ECL, enhanced chemiluminescence; MBP, myelin basic protein; NRKC, neonatal rat keratinocyte cell line; ORF, open reading frame; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; RT–PCR, reverse-transcriptase-mediated PCR; SAMS, synthetic peptide substrate HMRSAMSGLHLVKRR; SNARK, SNF1/AMPK-related kinase; SNF1, sucrose-non-fermenting protein kinase.

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EXPERIMENTAL

Materials

Radiochemicals were purchased from ICN Biomedicals (Irvine, CA, U.S.A.) ([α-^{32}P]dATP; more than 3000 Ci/mmol), Amersham Pharmacia Biotech (Baie D'Urfé, Quebec, Canada) ([γ-^{32}P]ATP; more than 5000 Ci/mmol) or NEN Life Sciences (Guelph, Ontario) ([^{35}S]methionine; more than 1000 Ci/mmol). Cell culture supplies and SuperScript preamplification system were obtained from Canadian Life Technologies (Burlington, Ontario, Canada). The TOPO TA cloning kit and pcDNA3.1 vector were purchased from Invitrogen (San Diego, CA, U.S.A.). Nylon and PVDF membranes, the T7-Sequencing kit, the glutathione S-transferase gene fusion system and Protein A–Sepharose CL4B were from Amersham Pharmacia Biotech. The TnT coupled reticulocyte lysate system was from Promega (Madison, WI, U.S.A.). The Bradford DC Protein assay kit was purchased from Bio-Rad (Mississauga, Ontario, Canada). The NEN Renaissance enhanced chemiluminescence (ECL) reagent plus kit and the Kodak BioMax MS and ML films were purchased from Mandel Scientific (Guelph, Ontario, Canada). Most of the chemicals and protease inhibitors were purchased from BioShop (Oakville, Ontario, Canada). Dephosphorylated myelin basic protein (MBP) was obtained from Upstate Biotechnology (New York, NY, U.S.A.). Dephosphorylated β-casein, whole histone and protamine sulphate were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) inhibitor peptides were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The SAMS peptide (HMRSAMSGLHLVKRR) was synthesized by the Protein Synthesis Facility (Hospital for Sick Children, Toronto, Ontario, Canada). AMPKα2 antibody was kindly provided by Dr Neil Ruderman (Boston, MA, U.S.A.).

Cell culture and treatments

Neonatal rat keratinocytes (NRKCs) and baby hamster kidney (BHK) cells were propagated as described previously [21,22]. Cells were seeded into 10 cm dishes at a density of 10^6 NRKC cells or 3×10^6 BHK cells per dish and were incubated for 2–3 days before experimentation. The medium was removed and replaced with low-serum medium, i.e. DMEM containing 0.1 % (v/v) newborn calf serum, 100 μg/ml streptomycin and 100 i.u./ml penicillin and incubated for either 18 h (BHK cells) or 1.5 h (NRKC cells) before each experiment. Glucose deprivation was performed as described previously [10] except that 25 mM glucose was used for control plates.

DNA and RNA analysis

Sequencing of SNARK cDNA species was performed with Sp6 and T7 primers with the use of the Sequenase T7 DNA polymerase kit or by the York University Core Sequencing Facility (Toronto, Ontario, Canada) with an Applied Biosystems Sequencer-Stretch Model and the *Taq* polymerase Dye Dioxy (Applied Biosystems) terminator cycle sequencing method. Clustal multiple sequence alignment was performed by using the MBS-Aligner program. RNA was isolated and analysed by Northern blotting and reverse-transcriptase-mediated PCR (RT–PCR) as described previously [23]. The full-length SNARK cDNA was labelled with [α-^{32}P]ATP with the use of a random priming technique [24] and used as a probe for Northern and Southern analyses as described previously [23].

RT–PCR

First-strand cDNA was generated with the SuperScript preamplification system and the primers 5′-CCGGATCCATGG-AGTCGGTGGCCTTACAC-3′ and 5′-CCGGATCCCTAAG-AGTTCCCCAGACTCA-3′ to amplify portions of SNARK transcripts. PCR was performed with the Perkin Elmer GeneAmp 2400 PCR system by using *Pfu* DNA polymerase in a final volume of 50 μl. The following conditions were used: denaturation at 94 °C for 5 min, 35 cycles consisting of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. Half of the PCR was loaded on a 1 % (w/v) agarose gel, immobilized on a nylon membrane and then probed as described above. PCR products were subcloned with the TOPO TA Cloning kit and sequenced to verify the identity of SNARK PCR products.

Human chromosomal mapping

After the isolation of a partial human SNARK genomic fragment, chromosomal localization was performed by FISH (fluorescence *in situ* hybridization) [25] to normal human lymphocyte chromosomes counterstained with propidium iodide and DAPI (4,6-diamidino-2-phenylindole) by the Canadian Genome Analysis and Technology Program, FISH Mapping Resource Centre (Hospital for Sick Children, Toronto, Ontario, Canada).

Phylogenies

Phylogenies of the SNF1/AMPK family of protein sequences similar to SNARK were estimated by the neighbour-joining method with PHYLIP [26]. Amino acid sequences were aligned with the Clustal program [27]; only those sites that could be aligned unambiguously were included for phylogenetic analysis. For neighbour-joining, distances between sequences were estimated by using the PROTDIST program with PAM distances [26]. Similar results were obtained with parsimony as implemented in PAUP* version 4.0b4 [28].

Cloning of SNARK cDNA species

The full-length clone containing the entire open reading frame (ORF) of SNARK was generated by using overlapping SNARK cDNA clones isolated from rat lung, kidney and keratinocytes. The complete ORF of SNARK was subcloned into the pcDNA3.1 vector.

Generation of antisera

A polypeptide containing 203 residues (corresponding to nt 858–1467) was expressed as a glutathione S-transferase fusion protein, purified by PAGE and used to immunize three rabbits (Cocalico Biologicals, Reamstown, PA, U.S.A.). Polyclonal anti-SNARK antiserum was collected and used for Western blot analysis and immunoprecipitations.

Transcription and translation *in vitro*

The SNARK/pcDNA3.1 plasmid was used as the template for transcription and translation *in vitro*. The TnT coupled reticulocyte lysate system was used in accordance with the manufacturer's protocol. Immunoprecipitation [29] of ^{35}S-SNARK and ^{35}S-luciferase (system control) was performed with 2 μl of the TnT reactions as described; 20 μl of 2 × SDS loading buffer [250 mM Tris/HCl (pH 6.8)/4 % (w/v) SDS/20 % (w/v) glycerol/0.04 % Bromophenol Blue] was added to the complexes before SDS/PAGE [10 % (w/v) gel]. The gel was dried and then exposed to Kodak BioMax MS film.

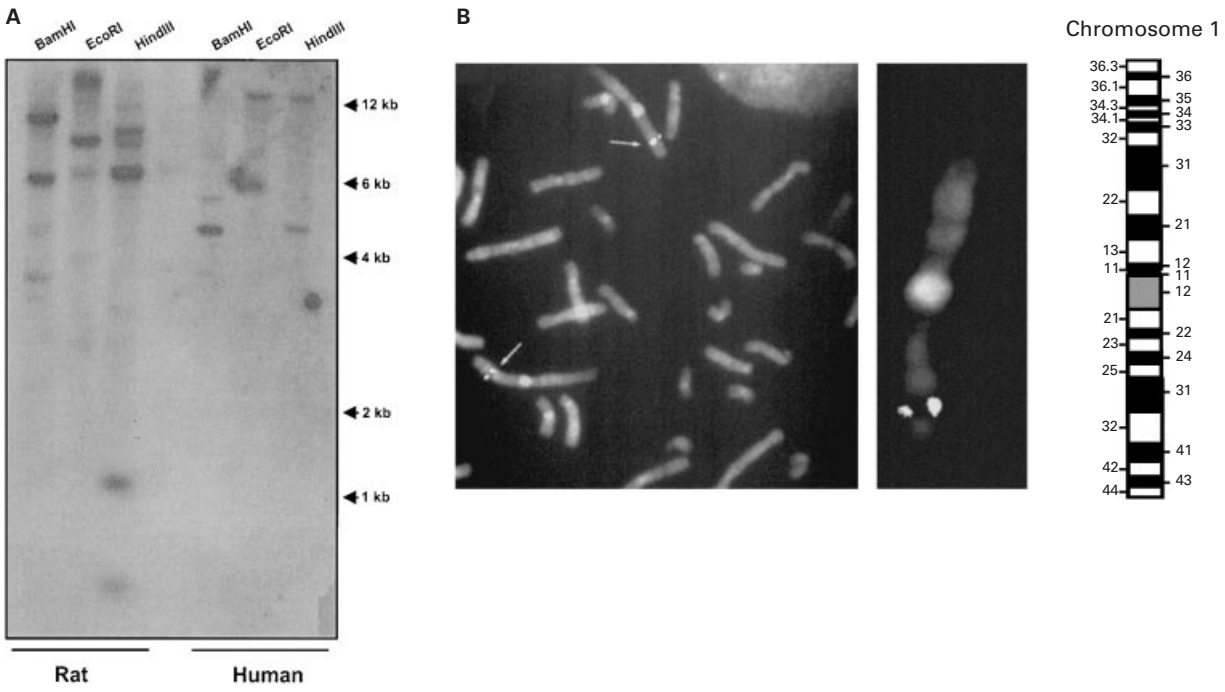

Figure 1 Analysis of SNARK in the rat and human genomes

(**A**) Southern blot analysis of genomic DNA isolated from rat liver and human lymphocytes. Genomic DNA (15 μg) was digested with one of the restriction endonucleases *Bam*HI, *Eco*RI or *Hin*dIII. The blot was hybridized with a 1.5 kb fragment (nt 1400–2929) corresponding largely to the 3′ end of the SNARK cDNA. The origin of the DNA is indicated at the bottom. The approximate positions of the DNA size markers are indicated at the right. (**B**) Chromosomal localization of human SNARK. A human SNARK genomic clone was used to map SNARK to human chromosome 1q32. Positive hybridization signals at 1q32 (seen as bright spots on the chromosome) were indicated by arrows on both alleles in more than 90% of the cells. A schematic representation of human chromosome 1 is shown at the right.

Protein extracts, immunoprecipitation and Western blot analysis

Cell cultures were scraped and homogenized in lysis buffer as described previously [30]. The total protein concentration was assayed by Bradford DC protein assay. Immunoprecipitation [29] was performed with 500 μg of total protein as described previously [10], except that the final concentration of NaCl in the immunoprecipitation reaction was 150 mM. Western blot analysis of immune complexes was performed by SDS/PAGE [8% (w/v) gel] followed by transfer and immobilization of proteins on a PVDF membrane. Membranes were blocked for 2 h with 2% (w/v) gelatin in Tris-buffered saline (0.5 M Tris/HCl/1.5 M NaCl) containing 0.1% (v/v) Tween 20 (TBST), then incubated with SNARK antiserum (1:1600 dilution in TBST) for 4 h and incubated with a secondary antibody of horseradish peroxidase-linked anti-rabbit IgG (Amersham Pharmacia Biotech) for 1 h. After extensive washing, the membrane was developed with ECL for 60 s and exposed to Kodak Biomax ML film. Equal loading of protein was verified by staining with Ponceau S.

Kinase assay

After immunoprecipitation with antiserum, SNARK activity was analysed by performing kinase assays with immunoprecipitated SNARK and a variety of substrates (MBP, β-casein, whole histone fraction, protamine sulphate and the SAMS peptide). Kinase assays with the SAMS peptide were performed as described [31,32], except that the three washes were done with immunoprecipitation buffer containing 500 mM NaCl followed by a final wash in kinase assay buffer. For kinase assays performed to determine substrate specificity (with substrates other than the SAMS peptide), reactions included 30 μg of substrate, 500 nM PKA inhibitor peptide and 1 μM PKC inhibitor peptide. Activities were calculated as fmol of phosphate incorporated into the SAMS peptide/min per milligram of lysate subjected to immunoprecipitation, minus the activity obtained with a blank reaction (cell lysate and Protein A–Sepharose only).

Cell transfections

Stable SNARK-transfected cell lines were generated with the calcium phosphate precipitation method of DNA transfer as described previously, with the aminoglycoside Geneticin (G418) to select for successful transfectants [33].

RESULTS

Mapping novel rat clone to human chromosome

Southern blot analysis demonstrated a simple pattern of hybridizing bands in both rat and human genomic DNA samples, which is consistent with a single copy of SNARK in the mammalian genome (Figure 1A). The human chromosomal localization of SNARK was determined by hybridizing an isolated rat SNARK cDNA fragment with a human P1-derived artificial chromosome (PAC) library. This experiment identified a single hybridizing genomic PAC clone that localized the human SNARK homologue to human chromosome 1q32 (Figure 1B).

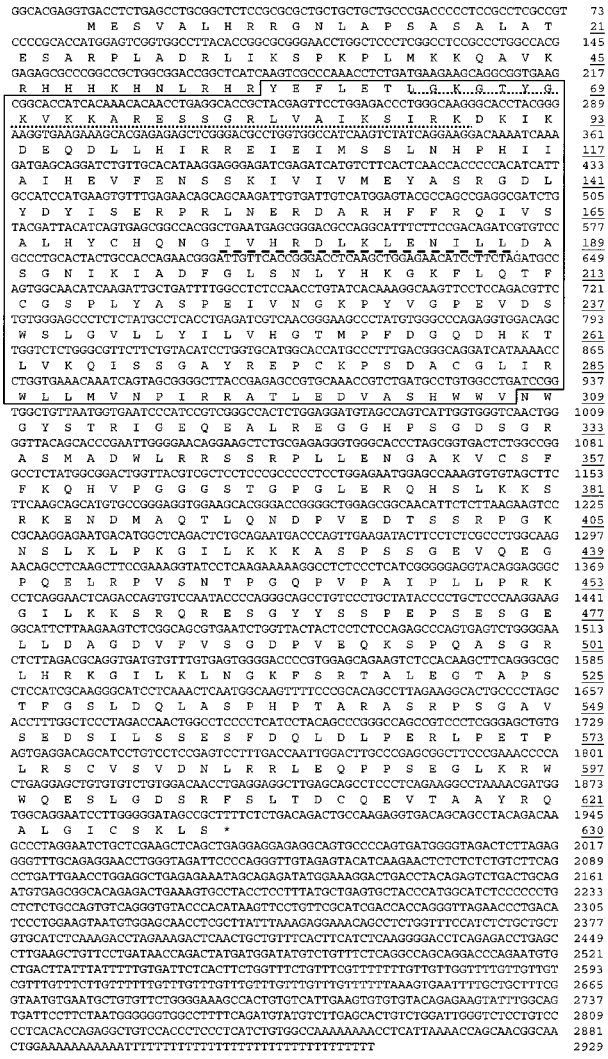

Figure 2 Nucleotide and deduced amino acid sequences of SNARK

The deduced amino acid sequence of SNARK is shown in single-letter code above the respective coding nucleotide sequence. Nucleotide number assignment is shown at the right and amino acid number assignment is shown as underlined numbers at the right. The protein serine/threonine kinase catalytic domain is boxed. The protein kinase ATP-binding region signature is underscored with a dotted line. The serine/threonine kinase active-site signature is underlined with a broken line. The asterisk indicates termination of the protein-coding region.

Positive hybridization signals at 1q32 were noted in more than 90 % of the metaphasic cells.

SNARK is a member of the AMPK/SNF1 family of protein kinases

With the original partial SNARK cDNA [20] as a probe, we identified a full-length cDNA clone containing 2929 nt with a single, uninterrupted ORF of 1893 nt, beginning at nt 83 and terminating at position 1975 (Figure 2). The ORF encoded a putative protein of 630 residues with a predicted molecular mass of 69.95 kDa and a theoretical pI of 9.35. Comparison of the deduced amino acid sequence of the N-terminal region of the protein (residues 57–308) with other known proteins revealed 48 % and 50 % identity (68 % similarity by including conservative substitutions scored by the BLOSUM62 matrix) within the catalytic domain of SNF1 protein kinase [34] and AMPK [35] respectively, prompting the designation of the protein as SNF1/AMPK-related kinase, abbreviated SNARK (Figure 3). SNARK contains all 11 catalytic subdomains conserved in serine/threonine protein kinases [36] (Figure 3). Analysis of the catalytic domain of SNARK with the Prosite program revealed a protein kinase ATP-binding region signature (residues 63–89) and a serine/threonine protein kinase active-site signature (residues 175–187) (Figure 2). The sequences at the C-terminus of SNARK were distinct and not well conserved with C-terminal sequences of other SNF1/AMPK family members. The instability index was computed to be 58.40 with the Protparam Tool program, classifying SNARK as an unstable protein.

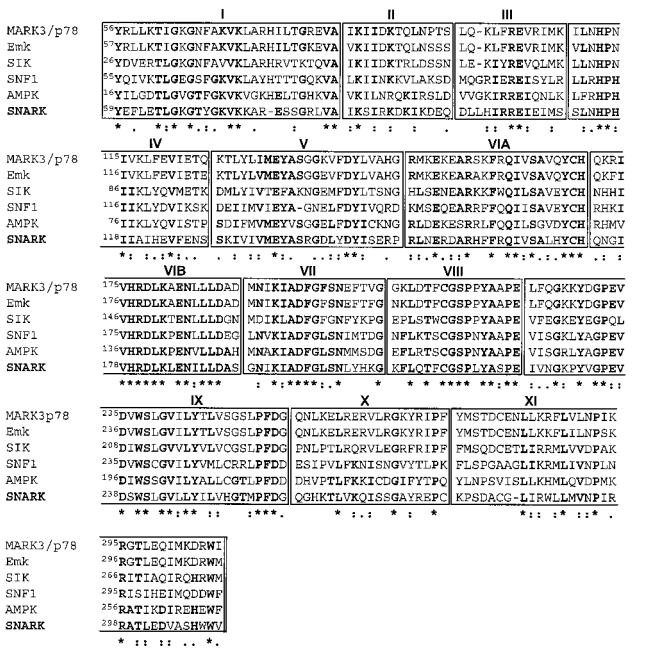

Figure 3 Alignment of the deduced amino acid sequence of rat SNARK and other members of the SNF1/AMPK family by using the CLUSTAL W algorithm

The protein kinase catalytic domains are boxed and numbered above in roman numerals. Positions with a single, fully conserved residue are indicated by asterisks; conservative substitutions are indicated by dots under the sequences (two dots indicates substitution with a strong group, score more than 0.5; one dot indicates a substitution with a weak group, score 0.5 or less). The amino acid residues are numbered in italics at the left. Accession numbers of the respective sequences are as follows: MARK3/p78, NP_002367; Emk, CAB06295; SIK, BAA82673; SNF1, P06782; AMPK, A53621.

A phylogenetic tree illustrating the relationship of the SNARK catalytic subdomains I–XI to other SNF-1/AMPK family members (Figure 4) demonstrates that SNARK originated very early in eukaryotic evolution, diverging before the divergence of yeast and humans. On the basis of the phylogeny of the catalytic subdomains, SNARK is no more closely related to SNF1 than it is to AMPK and represents a new branch of the SNF1/AMPK family of protein kinases.

Tissue distribution of SNARK mRNA species

Northern blot analysis demonstrated that SNARK RNA transcripts were most abundant in rat kidney (Figure 5A). RT–PCR detected two SNARK cDNA products in RNA from rat heart, skin, spleen, lung, uterus, liver and a neonatal rat keratinocyte cell line, NRKC (Figure 5B). The two different SNARK RT–PCR products were cloned from several tissues, sequenced and found to encode either authentic SNARK

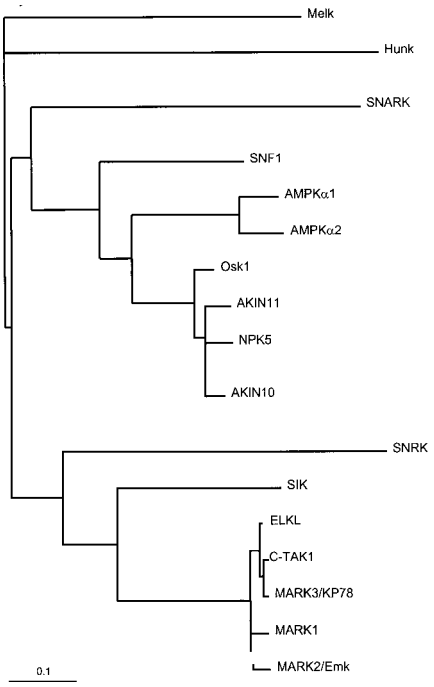

Figure 4 Phylogenetic tree of SNARK and SNF1/AMPK family members

The relationship between the catalytic subdomains I–XI of SNARK and SNF1/AMPK family members is shown. Database accession numbers used are as follows: Melk, X95351; Hunk, AF167987; SNF1, P06782; AMPKα1, P54645; AMPK α2, Q09137; Osk1, BAA36298; AKIN11, X99279; NPK5, A56009; AKIN10, Q38997; SNRK, X89383; SIK, BAA82673; ELKL, AAF64455; C-TAK1, 3089349; MARK3/KP78, NP_002367; MARK1, Z83868; MARK2/Emk, CAB06295.

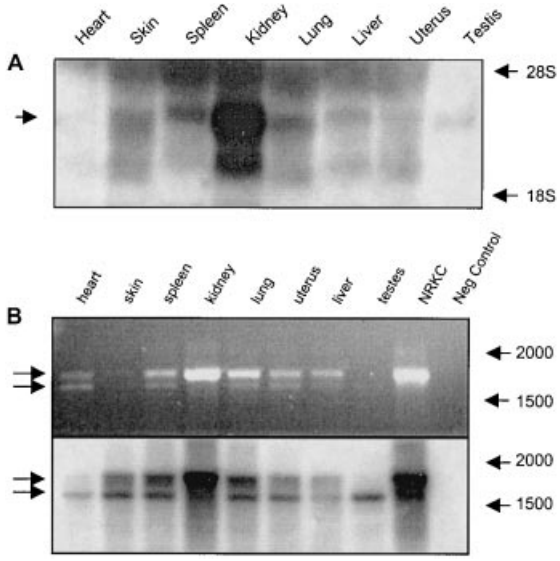

Figure 5 Tissue distribution of SNARK gene expression

(**A**) Northern blot analysis of rat tissues showing the tissue distribution of SNARK mRNA. Total RNA (10 μg) isolated from each rat tissue (as indicated above the lanes) was subjected to electrophoresis, transferred to a nylon membrane and probed with a fragment of the SNARK cDNA corresponding to the protein coding region (nt 0–1975), then exposed to film. The position of the SNARK mRNA is indicated by an arrow at the left; positions of the 28 S and 18 S rRNA species are indicated at the right. (**B**) RT–PCR analysis of SNARK in various rat tissues. The upper panel shows an ethidium-bromide-stained gel of RT–PCR products resulting from the PCR amplification of first-strand cDNA species prepared from a variety of rat tissues and NRKC cells by using the primers detailed in the Experimental section. A negative control reaction containing no first-strand cDNA was included to verify the specificity of primer products (neg control). The lower panel shows a Southern blot analysis of the RT–PCR products resulting from each tissue type. The Southern blot was hybridized with an internal fragment of the SNARK cDNA. Positions of migration of the SNARK mRNA species are indicated by arrows at the left; DNA size markers are shown (in nucleotides) at the right.

(1437 bp) or an internally deleted SNARK transcript (SNARK-Δ, 1247 bp). Whereas rat kidney contained predominantly the intact SNARK transcript and testes expressed only the 1247 bp SNARK-Δ transcript, both intact and SNARK-Δ transcripts were detected in skin, spleen, lung, uterus and liver. The SNARK-Δ transcript contained a 57 bp in-frame deletion, spanning parts of kinase domains I and II, and a 133 bp out-of-frame deletion in kinase domains IX–XI, including the invariant lysine residue involved in maximal enzyme activity. Translation of the SNARK-Δ transcript is predicted to give rise to a prematurely terminated protein of approx. 415 amino acids. Internally deleted rat AMPK transcripts have also been reported [37].

Immunoprecipitation of SNARK in transfected BHK cells

Transcription and translation *in vitro* with the full-length SNARK cDNA template in the presence of [^{35}S]methionine resulted in a major protein product of approx. 76 kDa (Figure 6A, lane 1) that was immunoprecipitated by SNARK antiserum (lane 5). A clearly detectable protein doublet, with a size of approx. 76–80 kDa, was detected in two separate clones of SNARK-transfected BHK cells (BHK + 1 and BHK + 11) with SNARK antiserum (Figure 6B, lanes 1 and 3) but not with non-immune serum (lane 2).

SNARK autophosphorylation

To assess whether SNARK was capable of autophosphorylation, immunoprecipitated SNARK was incubated with [γ-^{32}P]ATP and reaction products were examined by SDS/PAGE (Figure 7).

Although no autophosphorylated products were detected in samples of immunoprecipitated endogenous SNARK from wild-type BHK cells (Figure 7, lane 1), one major phosphorylated band, possibly a protein doublet, was detected in the immunoprecipitates from SNARK-transfected BHK cells (lanes 2 and 4). The size of the phosphorylated band(s) corresponds to the size of SNARK detected in these cell lines by Western blot analysis (approx. 76–80 kDa). Furthermore, no phosphorylated proteins were observed in cell extracts after immunoprecipitation with non-immune serum (Figure 7, lanes 3 and 5) or in samples containing extract but no antiserum (lane 6). These results demonstrate that SNARK is a protein kinase capable of autophosphorylation *in vitro*.

SNARK displays preferential substrate specificity

To determine whether immunoprecipitated SNARK protein possessed the ability to phosphorylate protein substrates *in vitro*, we performed kinase assays with candidate substrates including dephosphorylated MBP, dephosphorylated β-casein, whole histone fraction, protamine sulphate and the SAMS peptide (a well-established AMPK substrate corresponding to the site in rat acetyl-CoA carboxylase phosphorylated by AMPK [31]). Peptide inhibitors of PKA and PKC were included in these reactions to eliminate the phosphorylation of these substrates by these enzymes. Kinase assays performed on immunoprecipitated SNARK from NRKC cell lysates showed that, whereas SNARK was able to phosphorylate the SAMS peptide, its ability to

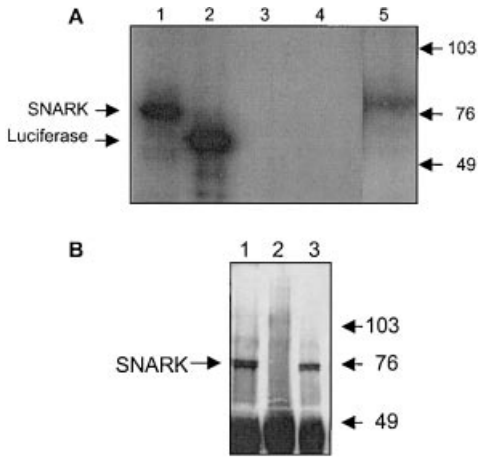

Figure 6 Biochemical analysis of SNARK protein

(**A**) Analysis of SNARK protein transcribed and translated in rabbit reticulocyte lysate. In lanes 1 and 2, one-tenth of the TNT reaction was loaded directly on an SDS/8% (w/v) polyacrylamide gel. Lanes 3–5, size-fractionated immunoprecipitations [29]: lanes 3 and 4, control immunoprecipitation reactions in which no TNT products or T7-luciferase TNT products were incubated with SNARK antiserum, respectively; lane 5, SNARK TNT immunoprecipitated with SNARK antiserum. After gel electrophoresis, the SDS/polyacrylamide gel was fixed, dried and exposed for 4 h to BioMax MS film with an intensifying screen. (**B**) Western blot analysis of SNARK protein in stably transformed BHK cells. Protein extract (500 μg) from BHK + 1 (lanes 1 and 2) and BHK + 11 (lane 3) were immunoprecipitated with SNARK antiserum (lanes 1 and 3) or with non-immune serum (lane 2), subjected to SDS/PAGE [8% (w/v) gel] and transferred to a PVDF membrane. The membrane was probed with the SNARK antiserum and a horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody, followed by development with ECL and exposure to film. The positions of the protein standards are shown (in kDa) at the right. The position of the SNARK protein is indicated at the left.

phosphorylate MBP, β-casein, whole histone fraction and protamine sulphate was minimal under these conditions (Table 1). The ability of SNARK to phosphorylate the SAMS peptide substrate was unaffected by the presence of PKA and PKC inhibitors (results not shown), indicating that the observed kinase activity was not due to the phosphorylation of SAMS peptide by PKA or PKC.

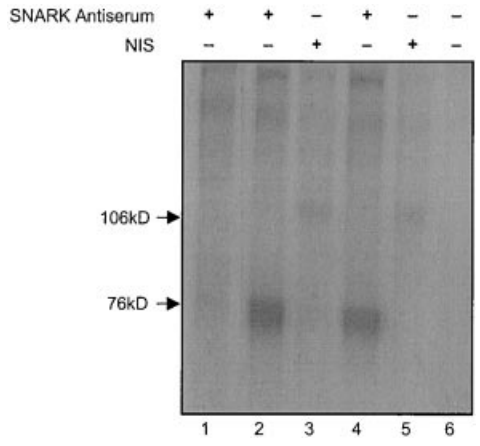

Figure 7 Autophosphorylation of SNARK

SNARK was immunoprecipitated from 500 μg of wild-type BHK (lane 1), BHK + 1 (lanes 2, 3 and 6) and BHK + 11 (lanes 4 and 5) cell lysates with either SNARK antiserum (lanes 1, 2 and 4), non-immune serum (lanes 3 and 5) or no antiserum (lane 6; negative immunoprecipitation control). Immunoprecipitations were incubated with [γ-^{32}P]ATP at 30 °C for 30 min and then stopped by the addition of 2 × SDS loading buffer and boiling for 5 min. Samples were subjected to SDS/PAGE [8% (w/v) gel]; the gel was dried and exposed to film.

Table 1 Substrate specificity of the SNARK protein

Immunoprecipitation of 500 μg of NRKC cell lysate with SNARK antiserum was performed as described in the Experimental section. Phosphotransferase activity was assayed with either SAMS peptide (250 μM) or 30 μg of dephosphorylated MBP, dephosphorylated β-casein, whole histone fraction or protamine sulphate in the presence of 1 μM PKC inhibitor and 500 nM PKA inhibitor peptides. Phosphotransferase activity is expressed in fmol of phosphate transferred to the SAMS peptide/min at 30 °C per mg of protein subjected to immunoprecipitation. Results are means ± S.E.M. for two individual assays with at least eight samples per group. $^*P < 0.01$ compared with all other substrates.

Substrate	Activity (fmol/min per mg)
SAMS peptide	145 ± 46*
MBP	31 ± 11
β-Casein	37 ± 12
Whole histone fraction	19 ± 6
Protamine sulphate	7 ± 2

SNARK exhibits AMPK-like phosphotransferase activity

To establish the level of basal SNARK phosphotransferase activity in various cell lines, kinase assays were performed with immunoprecipitated SNARK and the SAMS peptide. Wild-type BHK cells exhibited a low basal level of SAMS phosphotransferase activity (Figure 8A). The basal SNARK phosphotransferase activities detected in SNARK-transfected BHK cells (BHK + 1) and in NRKC cells were 3.4-fold ($P < 0.001$) and 2-fold ($P < 0.05$) higher respectively than the levels found in wild-type BHK cells (Figure 8A, filled columns). Because AMPKα2 antibodies immunoprecipitated 4-fold less SAMS phosphotransferase activity than the SNARK antiserum in SNARK-transfected BHK cells (results not shown), the phosphotransferase activity detected in our assay system seemed to be specific for SNARK kinase activity.

SNARK activity is increased by AMP and 5-amino-4-imidazolecarboxamide riboside (AICAriboside)

AMPK is activated by environmental stresses that lead to the depletion of cellular ATP and the elevation of AMP concentration [1]. To evaluate the effect of cellular stress on SNARK activity, we examined the effects of AMP on SNARK phosphotransferase activity in wild-type BHK, SNARK-transfected BHK and NRKC cell lines. Although no significant change in SNARK phosphotransferase activity was observed when wild-type and SNARK-transfected BHK cell lysates were assayed in the presence of 200 μM AMP, SNARK phosphotransferase activity increased by 1.7-fold ($P < 0.01$) in NRKC cells (Figure 8A, hatched columns).

The adenosine analogue AICAriboside provides a method of stimulating AMPK activity in whole cells, even in the presence of high glucose concentrations, and mimics the effects of AMP on the AMPK cascade [38,39]. Intriguingly, SNARK activity was induced 2.8-fold ($P < 0.05$) in NRKC when treated with 1 mM AICAriboside for 1 h (Figure 8B).

SNARK activity is increased by glucose deprivation

The concentration of glucose in culture medium is an important modulator of both SNF1 activity in yeast cells [40,41] and

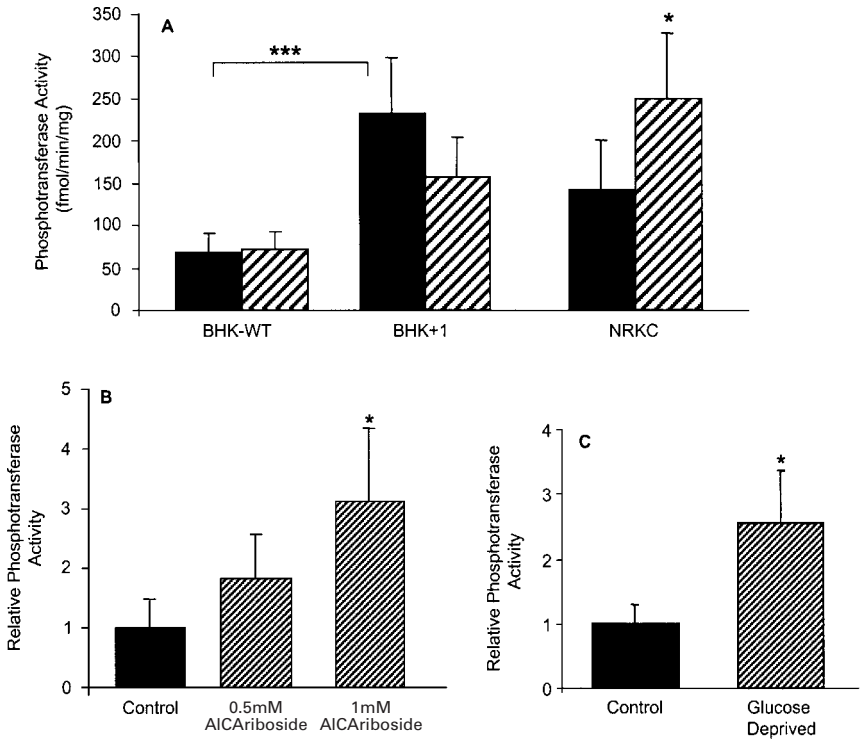

Figure 8 SNARK phosphotransferase activity is differentially regulated by AMP and cellular stresses

(**A**) SNARK activation is AMP-dependent. SNARK protein was immunoprecipitated from 500 μg of cell lysate from wild-type BHK, SNARK-transfected BHK (BHK+1) and NRKC cells. Kinase assays were performed, as described previously, in the presence or absence of 200 μM AMP with the SAMS peptide. Basal phosphotransferase activities assayed in the absence of AMP are shown as filled columns and activities assayed in the presence of 200 μM AMP are shown as hatched columns. Results are means $\pm$ S.E.M. for two individual experiments with at least 10 samples per group. $^{*}P < 0.05$, compared with NRKC without AMP; $^{***}P < 0.001$ compared with BHK-WT with or without AMP. For comparative purposes, AMPKα2 activity was assayed in wild-type BHK cells and found to be equivalent to the SNARK activity levels measured under basal growth conditions in these cells, whereas AMPKα2 activity was 4-fold lower and 1.5-fold lower than SNARK activity levels in SNARK-transfected BHK and NRKC cell lines respectively (results not shown). (**B**) Activation of SNARK in NRKC cells in response to treatment with AICAriboside. NRKC cells were treated with 0 (control), 0.5 or 1 mM AICAriboside for 1 h. SNARK protein was then immunoprecipitated from 500 μg of NRKC cell lysate with SNARK antiserum; kinase assays were performed in the presence of 200 μM AMP with the SAMS peptide. Results are expressed as phosphotransferase activity relative to control values (control = 1) and are means $\pm$ S.E.M. for two individual experiments with at least 10 samples per group per experiment. $^{*}P < 0.05$ compared with control values. With the same assay conditions, basal AMPKα2 activity was found to be 1.5-fold lower than SNARK activity and increased at least 2-fold on treatment with 1 mM AICAriboside in NRKC cells (results not shown). Basal AMPKα1 activity was found to be 88-fold higher than basal SNARK activity but was not stimulated by treatment with AICAriboside (results not shown). (**C**) Activation of SNARK in wild-type BHK cells resulting from glucose deprivation. Wild-type BHK cells were exposed to glucose-free medium for 0 (control) or 90 min as described previously [10]. SNARK was immunoprecipitated from 500 μg of cell lysate and kinase assays were performed with the SAMS peptide as substrate in the presence of AMP. Results are expressed as phosphotransferase activity relative to control values (control = 1) and are mean $\pm$ S.E.M. for two individual experiments with at least 10 samples per group per experiment. $^{*}P < 0.03$ compared with control values. Basal AMPKα2 activity levels measured in wild-type BHK cells were comparable with basal SNARK activities detected in these cells (results not shown).

AMPK activity in pancreatic β-cells [10]. SNARK activity was increased 2.6-fold in glucose-deprived BHK cells ($P < 0.03$; Figure 8C). These findings demonstrate that SNARK activity responds to glucose deprivation in a manner similar to that described for yeast SNF1 and rat AMPK [10,41]. Furthermore, immunoreactive SNARK was localized to the exocrine and endocrine compartments of the human pancreas (Figure 9A). Consistent with this finding was our Western analysis of cell lysates from the rat INS-1, mouse αTC and rat InR1G9 cell lines, which revealed SNARK-immunoreactive proteins in the INS-1 and αTC cell lines corresponding in size to that found in SNARK-transfected BHK cells (i.e. approx. 80 kDa) (Figure 9B). Although no 80 kDa SNARK protein was detected in the hamster InR1G9 cell line, a protein migrating at approx. 106 kDa was detected in both the InR1G9 and SNARK-transfected BHK cells. This larger protein might represent a form of SNARK that undergoes differential post-translational modification and requires further characterization.

DISCUSSION

This study describes the cloning of a new member of the SNF1/AMPK family of serine/threonine kinases, localized to human chromosome 1q32. The 3.5 kb SNARK mRNA encodes for a 76–80 kDa protein containing amino acid motifs characteristic of serine/threonine kinases. SNARK mRNA transcripts were detectable by Northern blotting and RT–PCR in almost all tissues examined; hence, like AMPK [31,37], SNARK is not a cell-specific kinase. The detection of two SNARK RNA isoforms, including the SNARK ($-\Delta$) transcript that is predicted to give rise to a non-functional protein, highlights the importance of using probes or primers specific for the detection of full-length SNARK in future studies of SNARK expression in cell types.

Within its catalytic domain, SNARK is most closely related to the SNF1/AMPK family of protein kinases, possessing a high degree of identity at the amino acid level (Figure 3). Members of the SNF1/AMPK protein kinase family have been highly

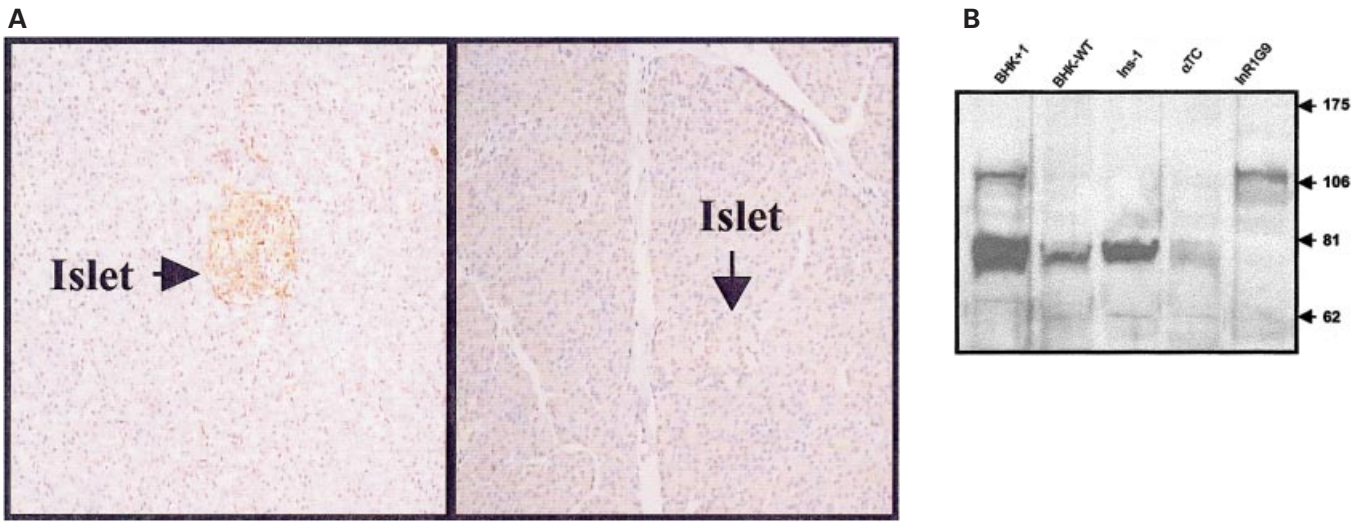

Figure 9 Detection of SNARK protein in pancreatic islet cells

(**A**) Immunohistochemical detection of SNARK protein in human pancreas. Immunohistochemistry was performed on sections of human pancreas. Right panel: staining of the pancreatic section with non-immune serum (1:800 dilution). Left panel: staining of the section with the SNARK antiserum (1:800 dilution). The pancreatic islet in each section is indicated. (**B**) Western analysis of BHK and pancreatic cell lines. Cell lysates (100 μg) from BHK + 1, wild-type BHK, rat INS-1 (insulinoma), mouse αTC (glucagonoma) and hamster InR1G9 (glucagonoma) cell lines were subjected to SDS/PAGE [8% (w/v) gel] and then transferred to a PVDF membrane. Membranes were incubated with the SNARK antiserum and an anti-rabbit IgG-HRP secondary antibody, followed by development with ECL and exposure to film. The positions of protein size markers are shown (in kDa) at the right.

conserved throughout evolution and the hallmark members of this family, SNF1 and AMPK, are generally thought to represent key metabolic sensors in stress response systems. AMPK is activated by environmental, metabolic and cellular stresses [1], including exercise and glucose deprivation [10,42]. These stresses deplete cellular ATP and, via the adenylate kinase reaction, elevate the AMP concentration, which serves as a switch to enhance AMPK activity.

On the basis of the structural similarity with mammalian AMPK, we suspected that SNARK and AMPK might exhibit common features, both in the regulation of kinase activity and in their specificity for downstream targets. Consistent with this hypothesis was our demonstration that SNARK, like AMPK, phosphorylates the synthetic SAMS peptide substrate, derived from the site on acetyl Co-A carboxylase that is phosphorylated by AMPK [31]. In contrast, minimal SNARK kinase activity was observed with substrates such as MBP, casein, whole histone fraction and protamine sulphate. Importantly, the antisera used in our studies for the analysis of SNARK autophosphorylation and kinase activity was directed against peptide sequences in the C-terminal region of SNARK that exhibit no similarity to AMPK or other known members of this kinase family. Taken together, the structural and functional data establish SNARK as a new mammalian member of the SNF1/AMPK family of kinases.

We originally isolated a partial SNARK cDNA encoding 3′-untranslated sequences after UVB irradiation of rat keratinocytes [20]. Although keratinocyte SNARK RNA transcripts are induced by UVB [20], we have not detected the induction of SNARK phosphotransferase activity in keratinocytes by UVB (results not shown). In contrast, glucose deprivation significantly activated SNARK activity in BHK fibroblasts, which is consistent with experiments demonstrating that AMPK activity in pancreatic β-cells is modulated in response to the extracellular glucose concentration [10]. Glucose deprivation of pancreatic β-cell lines resulted in a more than 5-fold activation of AMPK activity within 30 min of glucose removal [10]. AMPK activation was associated with a large increase in the cellular AMP-to-ATP ratio resulting from low levels of extracellular glucose; AMPK activity was inversely correlated with insulin secretion. Conversely, AMPK activity was inhibited with increasing glucose concentrations in MIN6 β cells and immunoneutralization of the AMPK complex diminished glucose-regulated gene transcription *in vitro* [43]. Because SNARK immunoreactivity is also localized to human islets and rodent islet cell lines (Figure 9), it seems reasonable to postulate that SNARK, like AMPK, is a candidate mediator of the islet cell response to metabolic stress.

Analysis of AMPK activity has relied extensively on the use of AICAriboside, a nucleoside taken up by cells and converted by adenosine kinase to the monophosphorylated form, 5-aminoimidazole-4-carboxamide ribotide (AICAribotide). Although AICAribotide mimics many of the effects of AMP, previous studies have suggested that it is not a completely specific AMPK activator [44,45]. For instance, AICAribotide has been reported to inhibit gluconeogenesis by inhibiting fructose-1,6-bisphosphatase [44] and to activate glycogenolysis by activating glycogen phosphorylase [45]. Our finding that both AMP and AICAriboside activate SNARK kinase activity extends the spectrum of AICAriboside action and raises the possibility that one or more activities previously attributed to the AMPK signalling cascade might in fact partly reflect SNARK activation. It remains to be determined whether AICAriboside or AMP activates SNARK directly or whether it does so indirectly via AMPK activation. The identification of SNARK as a new glucose-regulated member of the SNF1/AMPK-related kinase family suggests that future studies of the potential role of SNARK in the cellular response to metabolic stress are clearly warranted.

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