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Induction of cell–cell detachment during glucose starvation through F-actin conversion by SNARK, the fourth member of the AMP-activated protein kinase catalytic subunit family☆

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Abstract

SNARK, the fourth member of the AMPK catalytic subunit family, was originally identified in a rat kidney cDNA library, and in this study we isolated its human homologue. A BLAST search analysis using rat SNARK protein yielded a single high homology clone, DKFZp434J037, isolated from human testis, and since its hypothetical protein showed 84% homology to rat SNARK protein, we assumed DKFZp434J037 to be the human SNARK cDNA. The human SNARK cDNA is 3443 bp long and encodes a 628 amino acid protein having an estimated molecular weight of 69 kDa, and its chromosomal localization had been assigned to 1q32.1. The same as other members of AMPK catalytic subunit family, human SNARK showed AMP-dependent GST-SAMS phosphorylation activity and enhanced HepG2 cell survival during glucose starvation. Human SNARK-overexpressing HepG2 cells (H/SNK) showed acute cell–cell detachment when exposed to glucose-free medium and the cell–cell detachment correlated well with the detection of G-actin. Deletion mutant analysis strongly suggested that the putative catalytic domain of SNARK is necessary for the cell–cell detachment, and Western blotting analysis showed that phosphorylation of FAK and PKC, which were dramatically increased by glucose starvation in HepG2 cells, was markedly suppressed by SNARK.

Keywords: Protein kinase; SNARK; AMPK; Glucose starvation; Cell–cell detachment

AMPK is a mammalian homologue of yeast protein kinase SNF-1 [1,2]. AMPK is activated by increases in the intracellular AMP:ATP ratio that is well documented in cells under metabolic stress, hypoxia, heat shock, and ischemia [2–4]. The SNF-1/AMPK family is highly conserved in several species and plays a critical role in glucose metabolism [1]. AMPK displays kinase activity as a result of hetero-trimer conformation and three isotypes are well known: the α-subunit as the catalytic subunit, and the β- and γ-subunits as the regulatory subunit [1,3,5]. Five members of AMPK catalytic subunit family have been identified: AMPK-α1, AMPK-α2 [3], MELK (from mouse; [6]), SNARK (from rat; [7]), and ARK5 (from human; [8]).

AMPK was originally investigated in regulation to exercise and diabetes, since AMPK has been reported to play a major role in glucose and cholesterol metabolism [2]. We recently demonstrated that AMPK also plays a critical role in tumorigenesis through its ability to respond to metabolic stress [9,10]. The cell growth and survival of human hepatoma are highly dependent on nutrients, especially glucose, supplied by the bloodstream, and the hepatoma cell lines undergo acute cell death when exposed to glucose-free medium [10,11]. The cell death induced by glucose starvation, however, is clearly prevented by AMPK [10] and glucose starvation-induced cell death is also prevented under hypoxic conditions [9,12]. Since we have demonstrated that the

☆ *Abbreviations:* SNF-1, sucrose-non-fermenting protein kinase; AMPK, AMP-activated protein kinase; SNARK, SNF-1/AMPK-related protein kinase; MELK, maternal embryonic leucine zipper kinase; ARK5, AMP-activated protein kinase family member 5; FAK, focal adhesion kinase; PKC, protein kinase C; GST, glutathione *S*-transferase.

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hypoxia-induced tolerance to cell death induced by glucose starvation is also mediated by AMPK [9,12], and the growth of tumor cells expressing antisense RNA-expression vector of AMPK has been shown to be suppressed in nude mice [10], AMPK also plays a major role in tumorigenesis.

The fourth member of the AMPK catalytic subunit family, SNARK, was originally isolated in a rat kidney cDNA [7] and rat SNARK cDNA is 2929 bp long and encodes 630 amino acids [7]. High expression of *SNARK* was detected in the kidney and its enzymatic activity was stimulated by glucose starvation [7]. In this study we identified the DKFZp434J037 isolated from a human testis cDNA library by BLAST search analysis as the human homologue of SNARK, cloned it from testis 1st strand cDNA by PCR technology, and characterized it and its functional role during glucose starvation in human hepatoma cell line HepG2. The results demonstrated that human SNARK displays the typical characteristics of the AMPK catalytic subunit family: AMP-dependent GST-SAMS phosphorylation and induction of cell survival during glucose starvation, and that human SNARK induces acute cell–cell detachment due to its kinase activity during glucose starvation. These observations are important, because they show that AMPK plays a role in regulating cell–cell and cell–matrix adhesion as well as in regulating energy metabolism.

Materials and methods

Construction of human FLAG-tagged SNARK expression vector. Human FLAG-tagged SNARK cDNA was constructed from testicular first-strand cDNA (Biochain) by LA PCR (Takara Biomedicals, Japan). Thirty cycles of LA PCR were performed with the up-stream primer: 5′-GAATTCATGGATTATAAAGATGATGATGATAAAG AGTCGCTGGTTTTCGCG-3′ and the down-stream primer 5′-CT CGAGTCAGGTGAGCTTTGAGCAGACCCTCAGTGCCTGTCG -3′. The PCR product was ligated into pT7-Blue T vector for subcloning, and the insert cDNA was digested with *Eco*RI and *Xho*I and then re-ligated into pcDNA3.1(+) expression vector.

Antibodies. Antibodies against human α-catenin, β-catenin, total FAK (Transduction Laboratories), phosphorylated c-Src (Tyr 416), phosphorylated pan-PKC (Cell Signaling Technology), total c-Src, phosphorylated FAK (Tyr 397), total pan-PKC, and vinculin (Upstate Biotechnology) were purchased.

Cell line and transfection. Human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS: Sigma–Aldrich Japan).

For transfection, cells were seeded into a 6-well plate at 2.5×10^5/well and transfection was performed with TransFast Transfection Reagent (5 μg DNA/well: Promega) with some modifications. After 48 h, G418 selection was performed at 800 μg/ml to prepare a human FLAG-SNARK-expressing cell line.

Preparation of anti-human SNARK antibody. Antibody to human SNARK was raised in rabbits by immunizing them with a peptide based on the predicted amino acid sequence of human SNARK (KKPRQRESGYYSSPEPS). The peptide was synthesized and coupled to keyhole-limpet hemocyanin (KLH) at the C-terminus. Japanese White rabbits were immunized with 0.5 mg of peptide conjugate by initially injecting it in 50% Freund's complete adjuvant (Sigma) and in 50% Freund's incomplete adjuvant (Sigma) for subsequent immunizations. One week after the booster injection of 0.5 mg of peptide conjugate, polyclonal anti-human SNARK antiserum was collected and used for Western blotting analysis.

Western blotting analysis. Proteins were prepared for Western blotting analysis by lysing cells for 30 min with 1% NP-40 containing buffer. All procedures were carried out at 4 °C. Proteins were collected by centrifugation at 15,000 rpm for 15 min and their protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as the standard.

Sample proteins separated by SDS–PAGE were transferred onto nitrocellulose membranes by a semi-dry blotting system. The membranes were blocked for 1 h at room temperature with PBS containing 5% (w/v) skimmed milk (Becton–Dickinson), then washed with a mixture of PBS and 0.05% Tween 20 (Sigma, Tween–PBS), and incubated overnight at room temperature with antibody diluted with PBS. After washing with Tween–PBS, the membranes were incubated with a 2000-fold diluted biotinylated anti-rabbit IgG antibody (Vector Lab.), then washed with Tween–PBS, and incubated with avidin-HRP (Vector Lab.) at room temperature for 30 min. The membranes were again washed with Tween–PBS and then developed with the ECL system (Amersham–Pharmacia Biotech K.K., UK).

Measurement of enzyme activity. We used anti FLAG antibody-conjugated Sepharose to prepare human SNARK immunoprecipitates from cells incubated (1 h) in the presence and absence of glucose. After incubation, cells were lysed with PBS containing 0.1% NP-40 and immunoprecipitates were collected. Immunoprecipitates were washed six times and used to assay phosphorylation of GST-SAMS. Enzyme assay was performed at 30 °C in buffer containing 15 mM Hepes (pH 7.0), 200 μM of 5′-AMP, 200 μM GST-SAMS, cell lysate, 0.01% Briji 35, 0.3 mM DTT, 15 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (10 μCi). After incubation, the fusion protein was purified with glutathione–Sepharose (Amersham–Pharmacia) and counted in a scintillation counter (Beckman–Coulter).

Cell survival assay. Cell survival was assessed by the Hoechst 33342/PI staining procedure, as previously described [13]. Hoechst 33342 and PI were purchased from Molecular Probes. After incubation, cells were collected and stained with Hoechst 33342 and PI, and then examined by fluorescence microscopy. The cell death induction ratio was calculated as the ratio of the number of cells containing PI-stained nuclei to the number of total cells (approximately 500 cells).

Fluorescence staining of actin. Cells were seeded on an 8-well chamber slide (Nalge Nunc International) and incubated in medium containing and not containing glucose. The cells were then fixed with formaldehyde/Triton X-100 mixture and stained for actin with FITC-conjugated phalloidin.

Results and discussion

Identification of the DKFZp434J037 clone as human SNARK

A BLAST search analysis (rat SNARK protein vs. human cDNA clones listed in the US National Cancer Institute, USA) yielded a single high homology clone, DKFZp434J037 (Gene Bank No./ID: NM-030952/13569921), isolated from human testis as the putative human SNARK. The hypothetical protein of DKFZp434J037 showed 84% homology to rat SNARK protein and we concluded that DKFZp434J037 is human SNARK. Human SNARK cDNA is 3443 bp long and encodes 628 amino acids, and its chromosomal localization was mapped to 1q32.1 by Human Genome

BLAST Analysis (Fig. 1A). A homology search analysis showed 45.9%, 41.2%, 41.3%, and 55.3% homology to AMPK-α1, AMPK-α2, MELK, and ARK5, respectively (Fig. 1A). Since our previous studies had demonstrated that activation of the AMPK catalytic subunit family induced survival of HepG2 cells during glucose starvation [8–10,12,14], the effect of SNARK overexpression in HepG2 cells during glucose starvation was assessed as a means of characterizing human SNARK as a member of the AMPK catalytic subunit family. As shown in Fig. 1B, 24 h glucose starvation dramatically induced the death of HepG2 cells, the same as in our previous studies [8–10,12,14], however, partial prevention (about 60% cell survival) of the cell death induced by glucose starvation was observed in human SNARK-overexpressing HepG2 (H/SNK) cells (Fig. 1B), suggesting that human SNARK has an effect similar to those of AMPK catalytic subunits. Its biochemical properties were then examined to further characterize human SNARK. Phosphorylation of SAMS by the AMPK catalytic subunit family is well known as the common biochemical property of members of the AMPK catalytic subunit family [4,15]. Rat SNARK has also been found to phosphorylate SAMS and its activity is enhanced by glucose starvation for 90 min [7]. Since we prepared FLAG-tagged SNARK, immunoprecipitates formed with anti-FLAG antibody-conjugated Sepharose were used for the kinase assay. Slight phosphorylation of SAMS was observed in immunoprecipitates from H/SNK cells in this study, but not from HepG2 cells (Fig. 1C). Moreover, the FLAG-SNARK was collected from H/SNK cells exposed to glucose-free medium for 1 h phosphorylated more SAMS, and the phosphorylation was further accelerated when SAMS phosphorylation by FLAG-SNARK was assessed in the presence of 200 μM AMP (Fig. 1C). Based on the results of the functional and biochemical analysis, the hypothetical protein of the DKFZp434J037 clone has been clearly identified as the human homologue of rat SNARK.

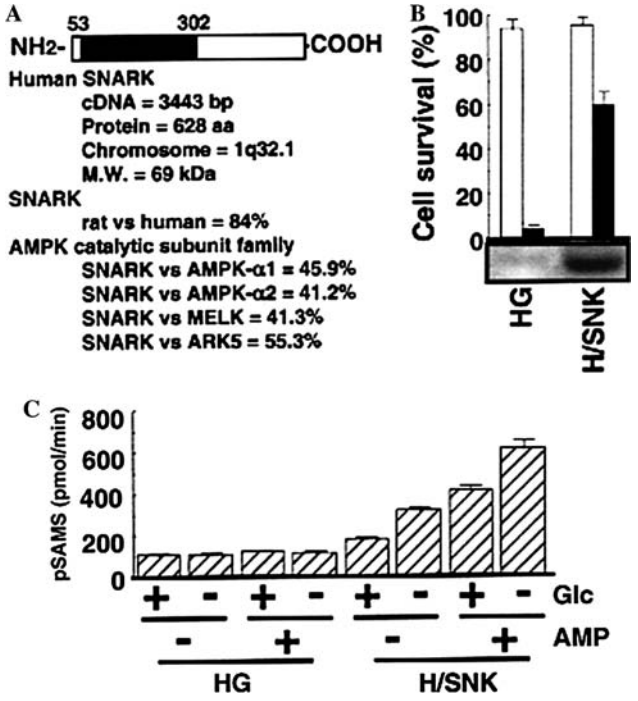

Fig. 1. Identification of human SNARK. (A) Physical properties of human SNARK and results of the homology analysis. The closed box in the schematic model of human SNARK represents the putative catalytic domain and the numbers are amino acid numbers. (B) Proteins were extracted from HepG2 (HG) cells and human SNARK-overexpressing HepG2 (H/SNK) cells, and Western blotting was performed with polyclonal antibody against human SNARK. HG cells and H/SNK cells were exposed for 24 h to medium containing (open) or not containing (closed) glucose, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is shown as means of data from three experiments and the bars represent SE values. (C) The kinase activity of human SNARK was measured as phosphorylation of GST-SAMS using immunoprecipitates with anti-FLAG antibody-conjugated Sepharose from HG cells and H/SNK cells exposed for 1 h to medium containing or not containing glucose (Glc) in the presence or absence of 200 μM AMP.

Acute cell–cell detachment induced by SNARK during glucose starvation

The results of the current study demonstrated human SNARK-induced tolerance of HepG2 cells to cell death by glucose starvation. The investigation of SNARK-induced tolerance revealed a unique function of SNARK. As shown in Fig. 2A, H/SNK cells, but not HepG2 cells, showed acute cell–cell detachment as early as 5 h after the start of glucose starvation. No cell death was induced in HepG2 cells by exposure to glucose starvation for 5 h, and Hoechst 33342/PI staining revealed that H/SNK cells rounded by exposure to 5 h glucose starvation did not exhibit any cell death phenotype (Fig. 2A). Thus, the rounding of H/SNK cells exposed to glucose starvation is attributable to cell–cell detachment rather than being an effect of induction of cell death. Fluorescence staining of actin fibers which are required to maintain cell shape was performed to further investigate the cell–cell detachment induced by SNARK. A fluorescence study with FITC-phalloidin revealed that parental HepG2 cells did not undergo either cell–cell dissociation or F-actin conversion during up to 60 min of glucose starvation (Fig. 2B). However, the presence of G-actin was observed in H/SNK cells as early as 30 min after the start of glucose starvation, and longer glucose starvation (60 min) induced increased F-actin conversion to G-actin. Since the conversion of F-actin to G-actin preceded cell–cell detachment, the results suggested that the cell–cell dissociation observed at the cell morphology level after glucose starvation is related to structural F-actin conversion. The conversion of F-actin in H/SNK cells after glucose starvation was also observed in the presence of actinomycin D, which

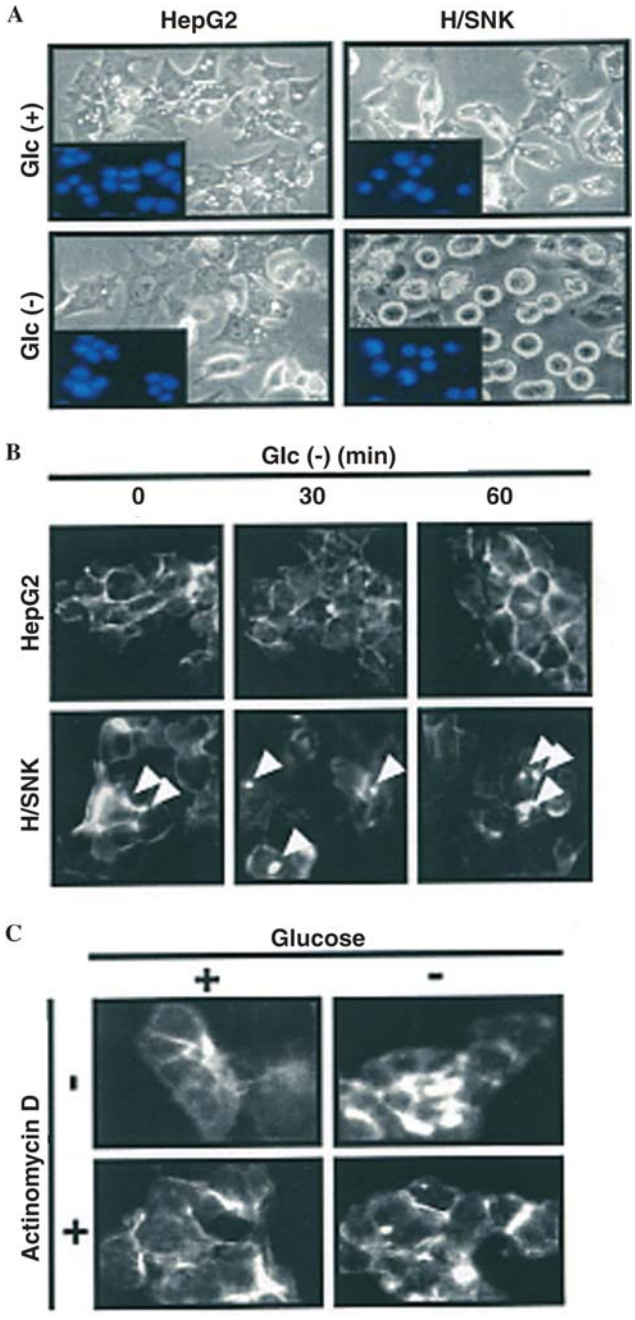

Fig. 2. Cell–cell detachment and formation of G-actin induced by SNARK during glucose starvation. (A) HepG2 and H/SNK cell lines were exposed for 5 h to medium containing (Glc (+)) or not containing (Glc (−)) glucose, and cell morphology (large photograph) was observed under a phase-contrast microscope. Cells were also stained with Hoechst 33342/PI after subjection and their nuclear morphology (small photograph) was observed under a fluorescence microscope. (B) HepG2 and H/SNK cells were exposed to glucose-free medium for the times indicated and actin was fluorescence stained with FITC-phalloidin. Arrowheads indicate G-actin. (C) H/SNK cells were exposed for 60 min to medium containing or not containing glucose in the presence or absence of actinomycin D, and actin was fluorescence stained with FITC-phalloidin.

suppresses a de novo mRNA synthesis (Fig. 2C), suggesting that the F-actin conversion is triggered by SNARK alone.

Cell–cell detachment requires the catalytic domain of SNARK

The results of this study demonstrated that SNARK induced cell–cell detachment in HepG2 cells during glucose starvation, and SNARK induced acute conversion to G-actin in cells during glucose starvation. Since F-actin, but not G-actin, plays a dominant role in maintaining cell shape and cell–cell adhesion in concert with E-cadherin, α- and β-catenin, vinculin, and actinin [16–19], we investigated how SNARK induces the conversion of F-actin to G-actin during glucose starvation. In the current study we prepared the three deletion mutants of SNARK shown in Fig. 3A: deletion mutant-1 (Δ-I) contains the catalytic domain, deletion mutant-2 (Δ-II) contains the non-catalytic domain, and deletion mutant-3 (Δ-III) lacks 100 C-terminal amino acid. Then the effect of these SNARK mutants on the induction of tolerance to glucose starvation was examined. As shown in Fig. 3B, tolerance to glucose starvation was observed in HepG2 cells overexpressing full-length SNARK and in the Δ-I and Δ-III SNARK mutant HepG2 cells, but not in the Δ-II mutant cells. The enzyme activity and AMP-dependency of the deletion mutants were also examined. As shown in Fig. 3C, the cells expressing full-length, Δ-I, and Δ-III SNARK exhibited GST-SMAS phosphorylation activity, and the activity was greatly increased in the presence of AMP, whereas the phosphorylation activity of the Δ-II mutant was very low even in the presence of AMP (Fig. 3C). These results strongly suggest that the tolerance of HepG2 cells to glucose starvation as a result of overexpression of SNARK is dependent on its enzyme activity, and that the Δ-I and Δ-III mutants, but not the Δ-II mutant, have sufficient activity. These findings are highly consistent with our previous findings that AMPK activity is necessary for tolerance to glucose starvation [8–10,12,14]. We then used these active and inactive mutants of SNARK to investigate glucose starvation-induced conversion of F-actin to G-actin in HepG2 cells. As shown in Fig. 3D, G-actin was detected in HepG2 cells overexpressing full-length, the Δ-I mutants, and the Δ-II mutant SNARK, during 60 min of glucose starvation, whereas no G-actin was observed in HepG2 cells transfected with the Δ-II mutant or the control vector (empty vector) even 60 min after glucose starvation (Fig. 3D). The Δ-II mutant did not induce G-actin formation during longer exposure (2–5 h) to glucose starvation (data not shown). These results strongly suggest that the cell–cell detachment caused by the conversion of F-actin to G-actin is dependent on the SNARK activity.

Glucose starvation-induced phosphorylation of FAK and PKC is inhibited by SNARK

Several factors promoting cell adhesion, including cell–cell and cell–matrix adhesion, have been identified,

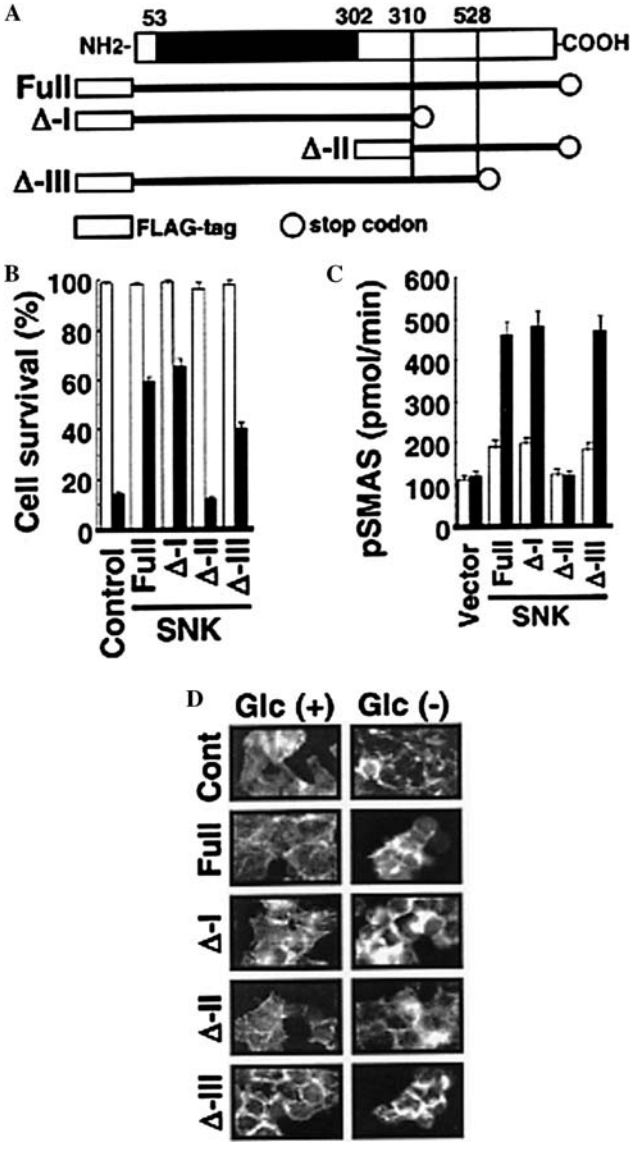

Fig. 3. Cell–cell detachment cross-linking to the enzyme activity. (A) Schematic model of the SNARK deletion mutants. The closed box in the schematic model of human SNARK represents the putative catalytic domain and the numbers are the amino acid number. (B) HepG2 cells and HepG2 cells overexpressing full-length (Full) or each of the deletion mutants (Δ-I–III) were exposed for 24 h to medium containing (open) or not containing (closed) glucose, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is shown as the means of data from three experiments and the bars represent SE values. (C) The kinase activity of full-length SNARK and deletion mutants of SNARK was measured as phosphorylation of GST-SAMS using immunoprecipitates with anti-FLAG antibody-conjugated Sepharose from HepG2 cells transfected with expression vector containing or not containing (Vector) full-length (Full) SNARK or each of the deletion mutants (Δ-I–III) in the presence (closed) or absence (open) of 200 μM AMP. (D) HepG2 cells overexpressing full-length (Full) SNARK or each of the deletion mutants (Δ-I–III) were exposed for 60 min to glucose-free medium, and actin was fluorescence stained with FITC-phalloidin.

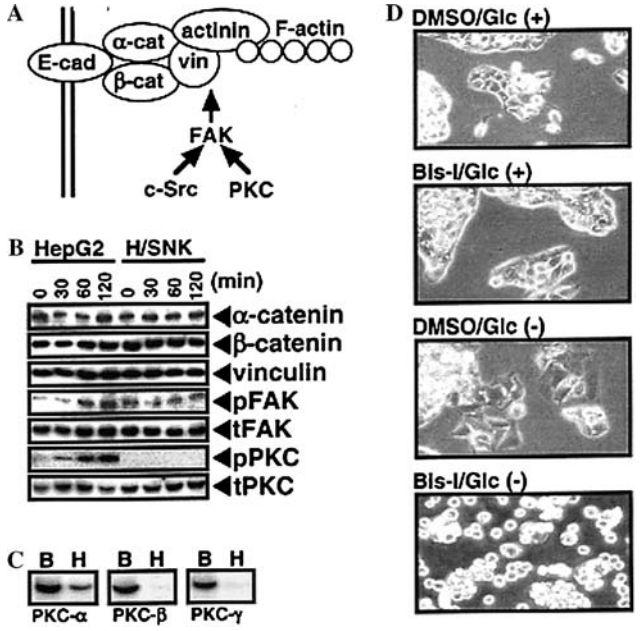

Fig. 4. Involvement of PKC and FAK in cell–cell adhesion. (A) A schematic model of cell–cell adhesion is shown. E-cad, E-cadherin; α-cat, α-catenin; β-cat, β-catenin; and vin, vinculin. (B) Western blotting was performed of cell extracts from the HepG2 cells and the H/SNK cells exposed to glucose-free medium for the times indicated. (C) Expression of cPKC isozymes. Western blotting of cell extracts from rat brain (B) or HepG2 cells (H) was performed with antibody against PKC-α, PKC-β, or PKC-γ. (D) HepG2 cells pretreated with DMSO or 20 μM bisindolylmaleimide-I (Bis-I) were exposed for 5 h to medium containing (+) or not containing (−) glucose. Cell morphology was observed under a phase-contrast microscope.

and one model for cell adhesion is schematically shown in Fig. 4A based on the previously proposed hypothesis [16–19]. E-cadherin, which recruits α-catenin, β-catenin, vinculin, and actinin, plays a critical role in cell–cell adhesion, and actin anchors on actinin resulting in its polymerization and the formation of fibers called F-actin. This cell-adhesion complex formation is regulated by focal adhesion kinase (FAK) activity and the FAK activation is in turn regulated by c-Src [19] or PKC [17]. Therefore, in the present study we investigated whether SNARK overexpression influences these cell adhesion factors. HepG2 or H/SNK cell lines were exposed to glucose-free medium for up to 120 min and Western blotting analysis was performed. As shown in Fig. 4B, the amounts of α-catenin, β-catenin, vinculin, total FAK, and total cPKC (α, βI, βII, and γ) in HepG2 cells did not change during glucose starvation, but marked changes in the phosphorylation of FAK and cPKC were observed. The phosphorylation of FAK and cPKC was very weak in HepG2 cells cultured in ordinary medium (DMEM), but their phosphorylation had increased after 30 min of glucose starvation, and increased further from 60 to 120 min (Fig. 4B). By contrast, no increase in phosphorylation of cPKC was observed during glucose starvation of H/SNK cells. More phosphorylated FAK was observed in ordinary medium than in HepG2 cells, but no significant change was observed during glucose starvation (Fig. 4B). Although c-Src is one of the key

factors regulating FAK [16,19], no changes in c-Src phosphorylation were observed during glucose starvation (data not shown). Three isotypes, PKC-α, PKC-β, and PKC-γ, have been identified as members of the cPKC family, and we attempted to determine which of these isotypes is phosphorylated during glucose starvation. As shown in Fig. 4C, Western blotting analysis revealed expression of PKC-α, but not the other two isotypes, in HepG2 cells, suggesting that PKC-α responded to glucose starvation. On the other hand, cPKC, which has also been reported to be an FAK activator during cell adhesion [17], was highly phosphorylated during glucose starvation. Investigation of the effect of bisindolylmaleimide-1, which specifically inhibits PKC, to confirm PKC involvement in regulating cell adhesion during glucose starvation revealed that it induced cell–cell detachment in HepG2 cells exposed to glucose starvation for 5 h (Fig. 4D), suggesting that the blockage of PKC activation is closely related to the cell–cell detachment. Western blotting demonstrated that cPKC was phosphorylated at the C-terminal serine residue (657th serine of PKC-α) during glucose starvation, and the phosphorylation was attributable to autophosphorylation in the presence of increased calcium ion and diacylglycerol concentrations [20]. Diacylglycerol is well known to be a cPKC activator [21] and suppression of diacylglycerol production by AMPK has been reported [22,23]. Thus, it is likely that the suppression of cPKC phosphorylation in H/SNK cells exposed to glucose starvation is due to inhibition of diacylglycerol production by SNARK.

Discussion of the biological significance of SNARK

Since SNARK, the fourth member of the AMPK catalytic subunit family, was originally identified as the response gene to DNA damage induced by ultraviolet irradiation [7], SNARK may be involved in cellular adaptation to DNA damage-induced stress. Although AMPK-α1 and α2 have been reported to be localized in the cytoplasm and nucleus, respectively [24], SNARK translocates from the cytoplasm into the nucleus in response to cellular stress (to be published elsewhere). In the current study SNARK was found to induce cell–cell detachment during glucose starvation through conversion of actin structure, which is obviously a cytoplasmic event. Actually, the SNARK Δ-I mutant exhibited the conversion of actin structure, but the mutant protein did not translocate into the nucleus during glucose starvation (to be published elsewhere). Nor did actinomycin D inhibit glucose starvation-induced SNARK-dependent actin conversion. Thus, SNARK appears to have biological roles both in the cytoplasm and the nucleus, however, the role of SNARK in the nucleus has not yet been clearly elucidated.

Acknowledgments

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