

The polo-like protein kinases Fnk and Snk associate with a Ca^{2+} - and integrin-binding protein and are regulated dynamically with synaptic plasticity

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In order to stabilize changes in synaptic strength, neurons activate a program of gene expression that results in alterations of their molecular composition and structure. Here we demonstrate that *Fnk* and *Snk*, two members of the polo family of cell cycle associated kinases, are co-opted by the brain to serve in this program. Stimuli that produce synaptic plasticity, including those that evoke long-term potentiation (LTP), dramatically increase levels of both kinase mRNAs. Induced *Fnk* and *Snk* proteins are targeted to the dendrites of activated neurons, suggesting that they mediate phosphorylation of proteins in this compartment. Moreover, a conserved C-terminal domain in these kinases is shown to interact specifically with *Cib*, a Ca^{2+} - and integrin-binding protein. Together, these studies suggest a novel signal transduction mechanism in the stabilization of long-term synaptic plasticity.

Keywords: gene induction/hippocampus/integrin/LTP/seizure

Introduction

Activity-dependent alterations in synaptic efficacy are thought to underlie learning and memory, epileptogenesis, drug abuse and several neurological diseases (Nestler and Aghajanian, 1997; Kuhl and Skehel, 1998; Milner *et al.*, 1998). A primary cellular model for such synaptic plasticity is long-term potentiation (LTP; Bliss and Collingridge, 1993). Like memory, LTP can exist in both short- and long-lived forms. Short-lived forms of LTP rely on phosphorylation-dependent modifications of pre-existing

proteins. The activity of both serine/threonine- and tyrosine-specific protein kinases has been implicated in this process (Grant, 1994; Grant and Silva, 1994; Huang *et al.*, 1996; Roberson *et al.*, 1996). Moreover, it has been proposed that post-translational modifications altering the activity of kinases and kinase-regulating proteins may act in the transfer of a short-lived LTP to longer lasting potentiations (Schwartz, 1993; Lisman, 1994). However, enduring forms of LTP require alterations in the molecular composition and structure of neurons, and are dependent on RNA and protein synthesis (Goelet *et al.*, 1986; Curran and Morgan, 1987; Sheng and Greenberg, 1990; Kuhl, 1999). To understand the underlying genetic program, it will be necessary to identify the specific gene products that are increased in hippocampal neurons by plasticity-inducing neuronal activity. Currently, we know that the genomic response of synaptically activated neurons includes the induction of transcription factors (Morgan *et al.*, 1987; Saffen *et al.*, 1988), as well as proteins that may directly modify synaptic function (Nedivi *et al.*, 1993; Qian *et al.*, 1993; Yamagata *et al.*, 1993; Link *et al.*, 1995; Lyford *et al.*, 1995; Frey *et al.*, 1996; Brakeman *et al.*, 1997). To this latter class of proteins belongs the serine/threonine kinase Pim-1 which we discovered previously in a subtractive screen for activity-dependent genes (Konietzko and Kuhl, 1998; Konietzko *et al.*, 1999). Pim-1 is induced rapidly by plasticity-producing stimulation and is instrumental in the formation of enduring hippocampal LTP (Konietzko *et al.*, 1999).

The polo-like kinases are a family of serine/threonine-specific protein kinases that, like Pim-1, are induced as immediate early genes in non-neuronal cells (Glover *et al.*, 1998; Nigg, 1998). Guided by this observation and the concept that certain aspects of cell cycle regulation and differentiation might be co-opted by the brain to serve functional plasticity, we examined here the influence of neuronal activity on the expression of the polo-like kinases in brain. In mammals, this family consists of three members, Plk, Fnk and Snk. Whereas the function of Snk is less clear, Plk and Fnk have been implicated in the control of multiple stages of cell division (Glover *et al.*, 1998; Nigg, 1998). Our studies suggest a role for Fnk and Snk outside the cell cycle. Whereas we do not detect expression of Plk in the brain, both Fnk and Snk are constitutively expressed in post-mitotic neurons. Moreover, stimuli that induce seizures or LTP result in a dramatic increase in the synthesis of *Fnk* and *Snk* mRNA. This increase is reflected in a concomitant increase of Fnk and Snk protein in somata and dendrites of activated neurons. Both kinases interact with *Cib*, a protein previously shown to bind to Ca^{2+} and the cytoplasmic tail of integrin α IIb (Naik *et al.*, 1997). The interaction of Fnk and Snk with *Cib* is dependent on the presence of the polo-box, a conserved C-terminal domain in the kinase proteins whose function

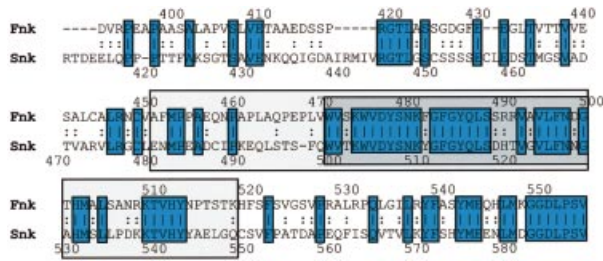


Fig. 1. Comparison of C-terminal amino acid sequences of rat Fnk and Snk. Amino acid residues of Fnk (393–556) and Snk (412–590) are aligned. Identical amino acids are highlighted in blue. The polo-box (Polo30) and a larger region (Polo70) used in the two-hybrid analyses are framed and shown in dark and light gray, respectively. The proteins share 76.6% sequence identity in the polo-box region (Polo30).

has not been determined. The association of Fnk and Snk with an integrin-binding protein suggests a specific role in the stabilization of LTP.

Results

The family of polo-like kinases

Three family members of the polo-like kinases, Plk, Fnk and Snk, have been described in mammals (Glover *et al.*, 1998; Nigg, 1998). In addition, the human *Prk* gene was cloned and classified as the fourth member of this family (Li *et al.*, 1996). All members of this family are characterized by the same domain topology, and alignment in a phylogenetic tree indicated that the polo-like kinases diverged before the subfamily of calmodulin kinase-related genes developed. As the first step of the present analysis, we cloned *Fnk* and *Snk* from rat brain (DDBJ/EMBL/GenBank accession Nos AF136584 and AF136583). Comparison of the deduced amino acid sequences of human *Prk*, rat *Fnk* and mouse *Fnk* (Donohue *et al.*, 1995) shows that the three polypeptides are ~90% identical except for a 17-amino-acid insertion present in rat *Fnk* and human *Prk*. However, *Prk* is lacking the most 5' sequences that constitute the N-terminus of the predicted rodent proteins. As this is a very GC-rich sequence stretch and consequently difficult to extend for processing enzymes used in cloning, we suggest that *Prk* represents a truncated human sequence of *Fnk* (see also Chase *et al.*, 1998). Moreover, sequences encoding the 17-amino-acid insertion of human and rat *Fnk* are also present in mouse mRNA from NIH-3T3 fibroblasts (DDBJ/EMBL/GenBank accession No. AF136586) and are flanked by two alternative 5' consensus splice sites in the mouse genomic sequence. This suggests that the published sequence of mouse *Fnk* most likely represents a splice variant. Rat *Fnk* shares ~50% sequence identity with rat *Snk* which is ~90% identical to the mouse homolog (Simmons *et al.*, 1992). The N-terminal half of *Fnk* and *Snk* harbors a serine/threonine-specific kinase domain including all 11 subdomains described to be specific for serine/threonine kinases (Hanks and Hunter, 1995). The C-terminal half contains a 30-amino-acid domain referred to as the polo-box which is highly conserved in all family members (Figure 1). This motif has not been described in any other protein and its function has not been determined.

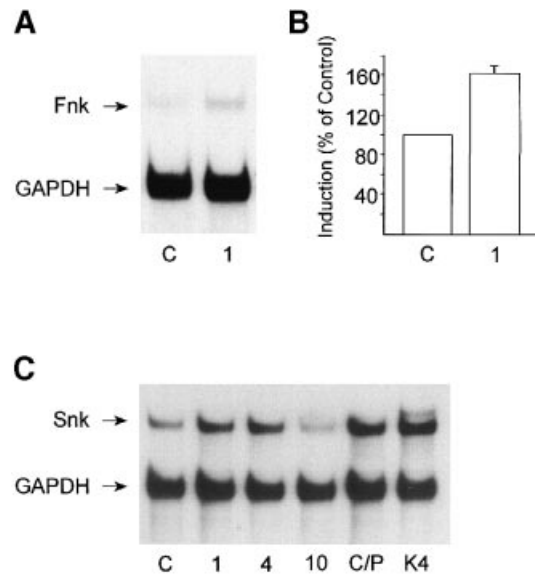


Fig. 2. Regulation of *Fnk* and *Snk* mRNA levels in the hippocampus. (A) Autoradiograph of Northern blot analysis of *Fnk*-specific transcripts. A 2 μ g aliquot of poly(A)⁺ RNA was loaded per lane. The blot was hybridized to a probe specific for *Fnk*. Hybridization to a probe specific for GAPDH was used as a loading control. Lane C, mRNA from saline-injected animals. Lane 1, mRNA isolated 1 h after PTZ-induced seizures. (B) Quantification of *Fnk* Northern blots given in bar diagrams. Error bars indicate SEMs ($n = 3$). Abbreviations are as in (A). (C) Autoradiograph of Northern blot analysis of *Snk*-specific transcripts. A 5 μ g aliquot of total hippocampal RNA was loaded per lane. The blot was hybridized to probes specific for *Snk* and GAPDH. Lane C, RNA from saline-injected animals. Lanes 1, 4 and 10, the numbers indicate the time in hours after the onset of PTZ-induced seizures. Lane C/P, RNA isolated 4 h after the onset of PTZ-induced seizures in the presence of CHX. Lane K4, RNA isolated 4 h after the onset of KA-induced seizures.

Levels of *Fnk* and *Snk* mRNA are regulated by neuronal activity

Expression of *Fnk* and *Snk* in the brain of untreated rats and rats that had undergone a pentylenetetrazole (PTZ)-induced seizure was assayed by Northern blot analysis (Figure 2). The corresponding transcripts had a size of 2.4 (*Fnk*) and 2.9 (*Snk*) kb. Constitutive expression of *Fnk* mRNA was low but was induced 1.6-fold by seizure activity (Figure 2A and B). By comparison, basal expression of *Snk* was higher but was induced similarly by seizures. Induction was independent of new protein synthesis as it occurred in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Figure 2C). Using *in situ* hybridization, we examined further the distribution and time course of basal and seizure-induced *Fnk* and *Snk* mRNA expression. In agreement with the Northern blot analysis, we observed low constitutive levels of *Fnk* mRNA in the hippocampus and cortex (Figure 3A). Higher constitutive levels were observed for *Snk* mRNA. In tissue of control rats, *Snk* mRNA levels were high in layers II, III, IV and VI of the occipital, parietal and temporal cortex. Regions with detectable basal expression also included the dentate gyrus, hippocampus proper, medial habenula, amygdala and putamen (Figure 3B). Hybridization to white matter was essentially the same as in sense control tissue (not shown). Following a PTZ-induced seizure, *Fnk* was strongly induced in the dentate gyrus and to a lesser extent in fields CA1 and CA3 of the

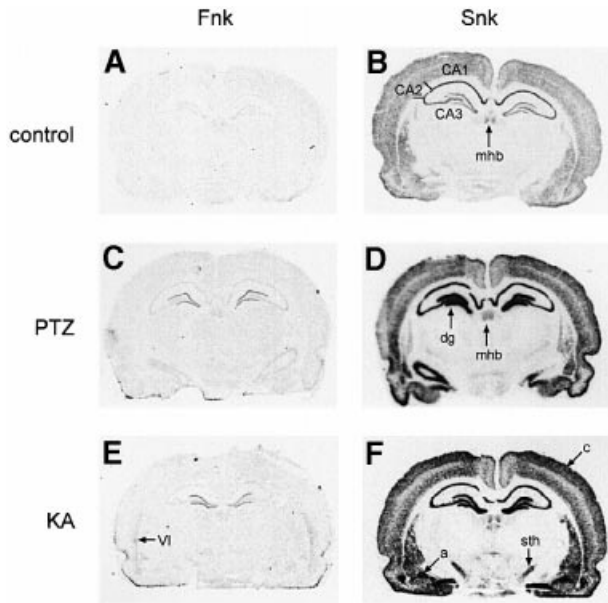


Fig. 3. Comparative analysis of brain *Fnk* and *Snk* mRNA levels before and after seizure. Coronal sections were analyzed for *Fnk* mRNA (A, C and E) and *Snk* mRNA (B, D and F) using *in situ* hybridization with gene-specific antisense probes. (A and B) Control rat; (C and D) rat sacrificed 4 h after PTZ-induced seizure; (E and F) rat sacrificed 4 h after KA-induced seizures. a, amygdala; c, cortex; CA1–3, fields CA1–3 of the hippocampus; dg, dentate gyrus; mhb, medial habenula; sth, subthalamic nucleus; VI, layer VI of the cortex.

hippocampus (Figure 3C). *Snk* was induced more broadly, with increased mRNA levels observable in cortical layers, fields CA1 and CA3 of the hippocampus and in the dentate gyrus (Figure 3D). In contrast to the effects of PTZ, kainic acid (KA)-induced seizures develop more slowly and recur for several hours (Ben-Ari *et al.*, 1981). This stronger seizure episode resulted in additional increases in *Fnk* mRNA levels in layer VI of the temporal cortex (Figure 3E); *Snk* mRNA levels were elevated further in neocortex, hippocampal CA2 stratum pyramidale, striatum, amygdala and subthalamus (Figure 3F). Neither PTZ nor KA affected *Plk* expression, which was undetectable in brain (not shown). Seizure effects on *Fnk* and *Snk* kinase expression were not influenced by adrenalectomy (not shown), thereby demonstrating that corticosterone released during the seizure episode (Sun *et al.*, 1993) does not account for the changes in gene expression described here.

To determine the specificity of induction of *Fnk* and *Snk* mRNA by neuronal activity, we examined the effects of electrical stimulation and the induction of LTP. LTP can be induced at synapses within the hippocampus by high-frequency orthodromic stimulation (Bliss and Collingridge, 1993). Induction of LTP was accompanied by increases in *Fnk* and *Snk* mRNA levels (Figure 4). Granule cells of the adult hippocampus were stimulated synaptically by activating their major afferent projection from the entorhinal cortex using a chronically implanted stimulating electrode (Staubli and Scafidi, 1997). Stimulation of the perforant path at the intensity required to produce a population spike, when administered at low

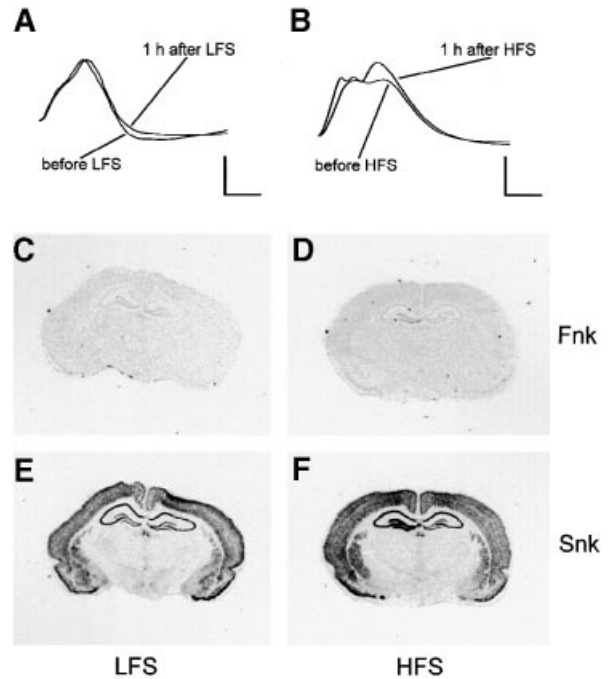


Fig. 4. Induction of LTP induces *Fnk* and *Snk* mRNAs in dentate gyrus granule cells in freely moving rats. Coronal sections were assayed for *Fnk* and *Snk* mRNA using *in situ* hybridization with gene-specific antisense probes. (A and B) Superimposed field potentials before and 1 h after (A) low-frequency stimulation (LFS) and (B) high-frequency stimulation (HFS) showing the induction of LTP with the latter. (C and D) *Fnk* mRNA levels 1 h after unilateral application of (C) LFS or (D) HFS. (E and F) *Snk* mRNA levels 1 h after unilateral application of (E) LFS or (F) HFS. The scale bar in (A) and (B) is 5 mV/2 ms.

frequency (0.2 Hz), did not result in LTP or an increase in *Fnk* or *Snk* mRNA levels (Figure 4A, C and E). By contrast, when LTP was evoked in the granule cells by delivering the same intensity stimuli at high frequency (400 Hz), *Fnk* and *Snk* were induced consistently in the ipsilateral dentate gyrus (Figure 4B, D and F). *Fnk* and *Snk* mRNAs were induced in each of four rats sacrificed 1 h after the LTP stimulation. These results demonstrate that of the three mammalian *Plk* genes, *Fnk* and *Snk* were expressed in the brain in overlapping but distinct populations of neurons and are regulated similarly by seizures and by electrical stimulation.

***Fnk* and *Snk* proteins are induced rapidly and enriched in somata and dendrites of activated neurons**

We next determined if the strong induction of *Fnk* and *Snk* mRNA by neuronal activity results in a corresponding increase in *Fnk* and *Snk* protein levels. In the hippocampal formation of control rats, immunoreactivity for both kinase proteins was localized to the somata and, at low basal levels, in the dendritic layers of the hippocampus proper and the dentate gyrus (Figure 5A and B). The distribution of *Fnk* and *Snk* immunostaining reflected patterns of mRNA expression; however, the relative levels of expressed kinase proteins differed from the mRNA levels observed in *in situ* hybridization and Northern blot analyses. Specifically, *Fnk* protein was detected at higher

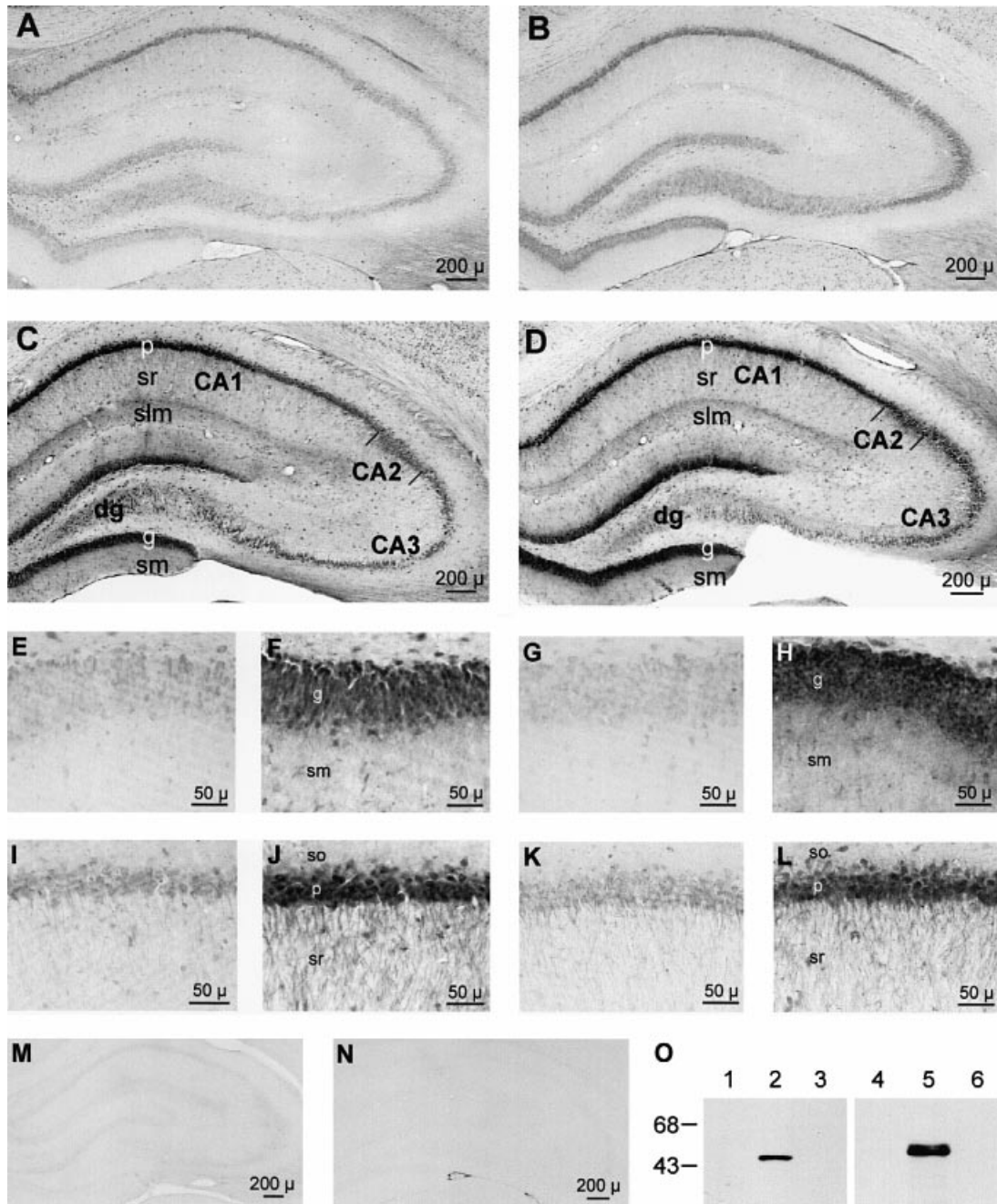


Fig. 5. Fnk and Snk proteins are localized to neuronal somata and dendrites of stimulated hippocampal neurons. Sections through hippocampi of control and KA-stimulated rat brain (4 h survival) were analyzed for Fnk protein (A, C, E, F, I and J) or Snk protein (B, D, G, H, K and L) using affinity-purified antisera. (A and B) Control hippocampus, only weak Fnk (A) and Snk (B) immunoreactivity is detected within the dentate gyrus and fields CA1–3. (C and D) After KA-induced seizures, Fnk (C) and Snk (D) immunoreactivity is increased in the granular and molecular layer of the dentate gyrus and in region CA1 with prominent staining of the dendritic processes. (E–H) High-power views of granule cells of the dentate gyrus before (E and G) and after KA-induced seizures (F and H). (I–L) High-power views of hippocampal field CA1 before (I and K) and after (J and L) KA-induced seizures. Sections from the same animal incubated with a serum depleted of either Fnk (M) or Snk (N) antibodies had no staining. (O) Immunoblots demonstrating the specific binding of the antisera to the corresponding recombinant kinase protein and that no cross-reactivity was observed. Recombinant Fnk protein (100 ng) was reacted with immunodepleted Fnk-specific antisera (lane 1), Fnk-specific antisera (lane 2) and Snk-specific antisera (lane 6). Recombinant Snk protein (100 ng) was reacted with immunodepleted Snk-specific antisera (lane 4), Snk-specific antisera (lane 5) and Fnk-specific antisera (lane 3). CA1–3, hippocampal fields CA1–3; dg, dentate gyrus; g, granular cell layer; p, pyramidal cell layer; slm, stratum lacunosum moleculare; sm, stratum moleculare; so, stratum oriens; sr, stratum radiatum.

levels than the corresponding mRNA. This is most likely due to different affinities of the antisera for the corresponding protein; alternatively, the translation efficacy of the two kinase mRNAs might differ. However, as observed

in the analysis of mRNA, recurrent KA-induced seizures markedly increased the immunoreactivity of both kinases (Figure 5C and D). The increase was most pronounced in the dentate gyrus granule cell somata and dendrites

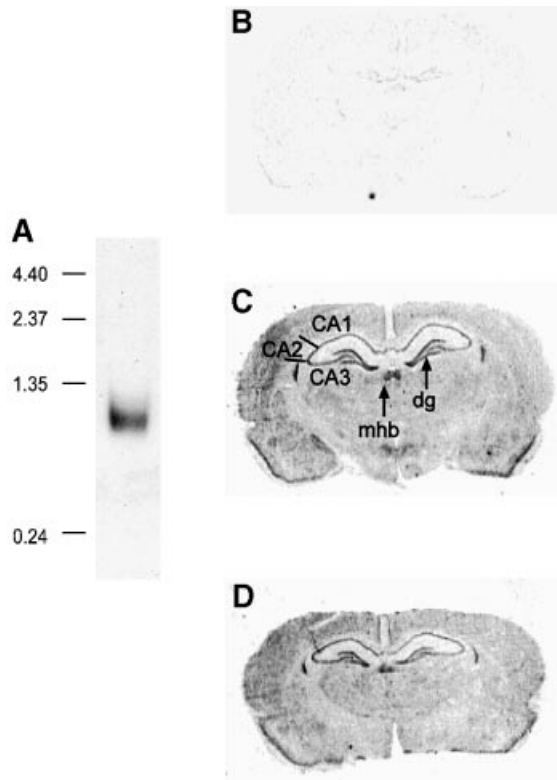


Fig. 6. *Cib* mRNA is expressed in rat brain. Autoradiograph of Northern blot analysis of RNA extracted from cortex (A) and *in situ* hybridizations of coronal sections with gene-specific sense (B) and antisense (C and D) probes for *Cib*. (A) A 2 μ g aliquot of poly(A)⁺ RNA was loaded. The blot was hybridized to a probe specific for *Cib*. Size markers in kilobases are indicated on the left. (B and C) Control rat, (D) rat sacrificed 4 h after PTZ-induced seizure. CA1–3, hippocampal fields CA1–3 of the hippocampus; dg, dentate gyrus; mhb, medial habenula.

(Figure 5E–H), and in the pyramidal cell somata of field CA1 and their dendrites in the stratum radiatum (Figure 5I–L).

The Ca²⁺- and integrin-binding protein *Cib* specifically interacts with *Fnk* and *Snk*

Cib had previously been isolated in a two-hybrid screen as a Ca²⁺-binding protein that interacts with the cytoplasmic tail of the integrin α IIB (Naik *et al.*, 1997) and, in adult brain, integrins are concentrated in regions of synaptic contact (Einheber *et al.*, 1996; Bahr *et al.*, 1997). Importantly, a database entry (O.Yuan, DDBJ/EMBL/GenBank accession No. U83236) suggested that *Cib* might interact with *Snk*. We found that *Cib* is expressed constitutively in the brain: Northern analysis of hippocampal RNA identified a single band of ~900 bp (Figure 6A). Using *in situ* hybridization, we examined the distribution of *Cib* mRNA expression. We observed constitutive levels of *Cib* mRNA in the hippocampus and cortex (Figure 6C) that were unaffected by seizure activity (Figure 6D). We further determined whether *Cib* protein shows an expression similar to that observed for *Fnk* and *Snk* proteins. Figure 7 shows that *Cib* immunoreactivity is localized to the somata and in the dendritic layers of the hippocampus proper and the dentate gyrus. As *Cib* was expressed in the hippocampus with an identical subcellular distribution to *Fnk* and *Snk* (compare Figures 7 and 5),

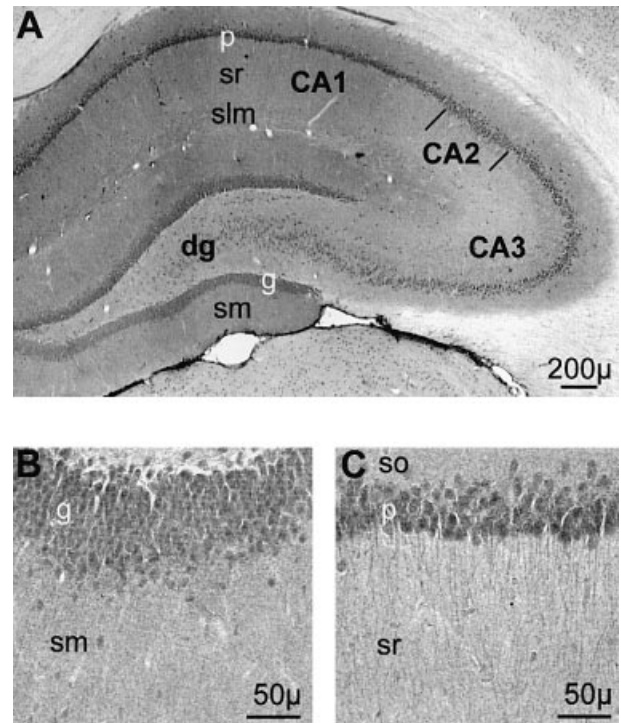


Fig. 7. *Cib* protein is localized to neuronal somata and dendrites of hippocampal neurons. Sections through hippocampi of rat brain were analyzed for *Cib* protein using a monoclonal mouse anti-*Cib* antibody (A–C). Immunoreactivity is detected within the dentate gyrus and fields CA1–3 of hippocampus (A). (B and C) High-power views show immunoreactivity in the granular and molecular layer of the dentate gyrus (B) and region CA1 with prominent staining of the dendritic processes (C). CA1–3, hippocampal fields CA1–3; dg, dentate gyrus; g, granular cell layer; p, pyramidal cell layer; slm, stratum lacunosum moleculare; sm, stratum moleculare; so, stratum oriens; sr, stratum radiatum.

we next determined if *Snk* and *Fnk* can interact with *Cib*. We cloned rat *Cib* from brain (DDBJ/EMBL/GenBank accession No. AF136585) and conducted a yeast two-hybrid analysis. Full-length *Snk* and *Fnk* cDNAs were expressed as GAL4 activator fusion proteins, and their interaction with *Cib* expressed as a GAL4 DNA-binding domain fusion protein was tested. Yeast co-transformed with *Cib* and either kinase expressed β -galactosidase. No β -galactosidase activity was observed when either protein was expressed alone or in the presence of unrelated proteins that were expressed as GAL4 activation or DNA-binding domain fusions. These findings were quantified in liquid β -galactosidase assays and are shown in Figure 8A and B. To determine the specific domain in *Snk* and *Fnk* responsible for the interaction, various segments of *Snk* and *Fnk* were co-expressed with *Cib*. Interaction was only observed in the presence of C-terminal fragments of *Snk* or *Fnk*; moreover, no interaction with *Cib* was seen with N-terminal fragments that encode the kinase domain. Figure 8A and B shows that 30 amino acids of the polo-box of *Snk* or *Fnk* were sufficient to confer binding to *Cib*. These interactions were of a strength similar to that observed for *Cib* and the full-length kinase proteins, and for *Snf4* and *Snf1* frequently used as positive controls in yeast interaction studies (Fields and Song, 1989).

To determine whether the interaction between *Cib* and the kinases was direct, we analyzed their interaction using

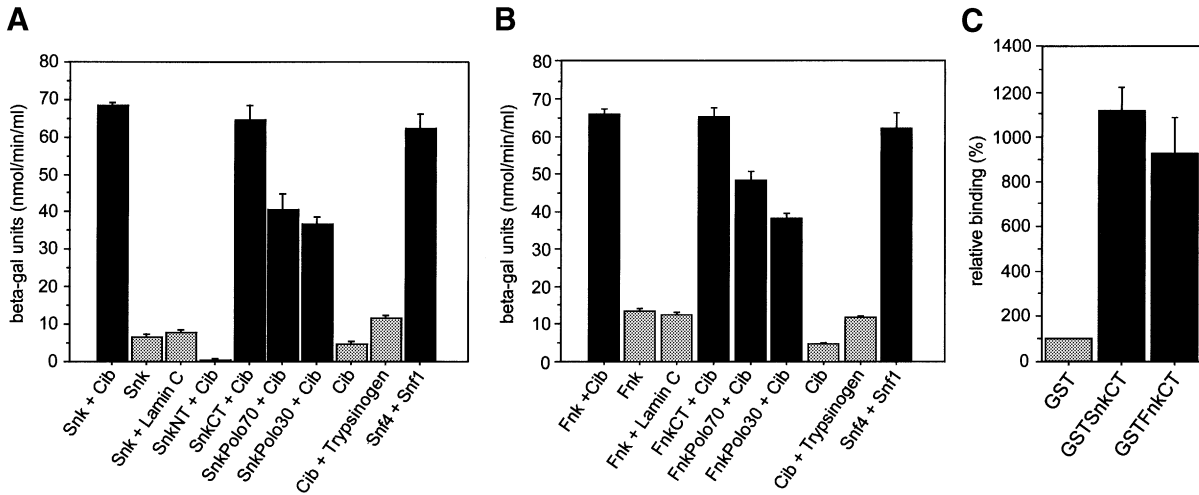


Fig. 8. Analysis of interactions between Snk and Cib, and Fnk and Cib. (A) Yeast two-hybrid interaction analysis for Snk and Cib. (B) Yeast two-hybrid interaction analysis for Fnk and Cib. Assays in (A) and (B) were performed in liquid culture and β -galactosidase activity was quantified. The enzymatic assays shown represent the average of three independent co-transformants. Interaction was only observed between Cib and either the full-length kinase proteins, C-terminal fragments or polo-box domains. No interaction was seen between full-length kinases and laminin C, between Cib and an N-terminal Snk fragment, and between Cib and trypsinogen which was used as an additional negative control. Snf4 and Snf1 served as positive controls. Cib, complete coding region of Cib; Fnk, complete coding region of Fnk; FnkCT, C-terminal 313 amino acids of Fnk; FnkPolo30, 30 amino acids of the polo-box of Fnk; FnkPolo70, 70 amino acids of the polo-box domain of Fnk; Snk, complete coding region of Snk; SnkCT, C-terminal 331 amino acids of Snk; SnkNT, N-terminal 352 amino acids of Snk; SnkPolo30, 30 amino acids of the polo-box of Snk; SnkPolo70, 70 amino acids of the polo-box domain of Snk. (C) *In vitro* binding analysis of the interaction between Snk and Cib, and between Fnk and Cib. Myc-tagged GST protein (GST), Myc-tagged Snk C-terminal fusion protein (GSTSnkCT) and Myc-tagged Fnk C-terminal fusion protein (GSTFnkCT) were bound to HA-tagged GST-Cib fusion protein. The amount of bound Cib was quantified using a monoclonal mouse anti-HA antibody and a secondary peroxidase-conjugated mouse antibody. Relative binding data were: GST, 100%; GSTFnkCT, 925%; GSTSnkCT, 1114%.

in vitro binding assays. As shown in Figure 8C, the C-terminal polypeptides of Snk and Fnk interacted with bacterially expressed glutathione *S*-transferase (GST)-Cib protein; there were no interactions with GST alone.

Further evidence for the interaction of Cib with Snk came from the analysis of their subcellular localization. COS cells as well as the neuronal cell line, Neuro2A (N2A), were transfected with expression plasmids encoding green fluorescent protein (GFP)-Snk fusion protein and hemagglutinin (HA)-tagged Cib. Figure 9 shows that in COS and N2A cells expressing only Snk, Snk was observed mainly in the cytoplasm (Figure 9A and G, respectively). In contrast, in the two cell lines transfected with *Cib* only, Cib was distributed within the cytoplasm but there were also significant levels in the nucleus (Figure 9B and H). In ~10% of COS cells transfected with *Cib* alone, the protein was observed exclusively in the nucleus (Figure 9C). Following double transfections of *Cib* and *Snk*, expression of Cib became restricted to the cytoplasm in COS as well as N2A cells (Figure 9E and K) and the localization of Cib resembled that seen for Snk (Figure 9D and J). Superposition of Snk and Cib immunostaining patterns indicated that their cytoplasmic distributions are largely identical (Figure 9F and C). Thus, a significant portion of Snk and Cib proteins are found in the same compartment (similar results were obtained for Fnk and Cib; data not shown). The results are consistent with the notion that Cib binds to Snk, although it is likely that Cib has additional roles in the nucleus where Snk is absent, at least in these transfected cells.

Localization of Cib and Snk in hippocampal neurons

The experiments above indicate the biochemical association of Cib and Snk, and demonstrate that these proteins

are co-localized extensively in double-transfected non-neuronal and neuronal cell lines overexpressing both proteins. Are Cib and Snk similarly localized under physiological conditions in the brain? This question was addressed by double-labeled immunofluorescence staining to visualize Snk and Cib protein expression in the hippocampal neurons of control and KA-treated rats. Under control conditions, immunoreactivity for Snk and Cib was detected in the subplasmalemmal cortex of the neuronal cytoplasm and distributed along dendrites of neurons. The Snk staining matched closely that of Cib (Figure 10A, B and C). In contrast to single transfected tissue culture cells expressing only Cib, where immunoreactivity was found in the cytoplasm and the nucleus, there was no significant Cib immunoreactivity observable in the nucleus of neurons which express both Cib and Snk. After KA-induced seizures, immunoreactivity of Snk was increased (Figure 10D) whereas that of Cib remained unchanged (Figure 10E). Figure 10F shows that immunolabeling of Cib and Snk overlapped spatially in seizure-activated hippocampal neurons.

Discussion

The family of polo-like kinases has previously been implicated in the control of the cell cycle. Specifically, Plk functions during M phase and has been suggested to be critically involved in a variety of processes, including cytokinesis, the promotion of spindle formation and the activation of a ubiquitin-protein ligase responsible for the degradation of mitotic cyclins (Glover *et al.*, 1998; Nigg, 1998). The yeast homolog of *Plk*, *Cdc5*, is essential for cell cycling in yeast (Hartwell, 1991). Remarkably, both *Plk* and *Prk/Fnk* can complement *Cdc5* mutations, suggesting that these kinases might fulfill similar functions

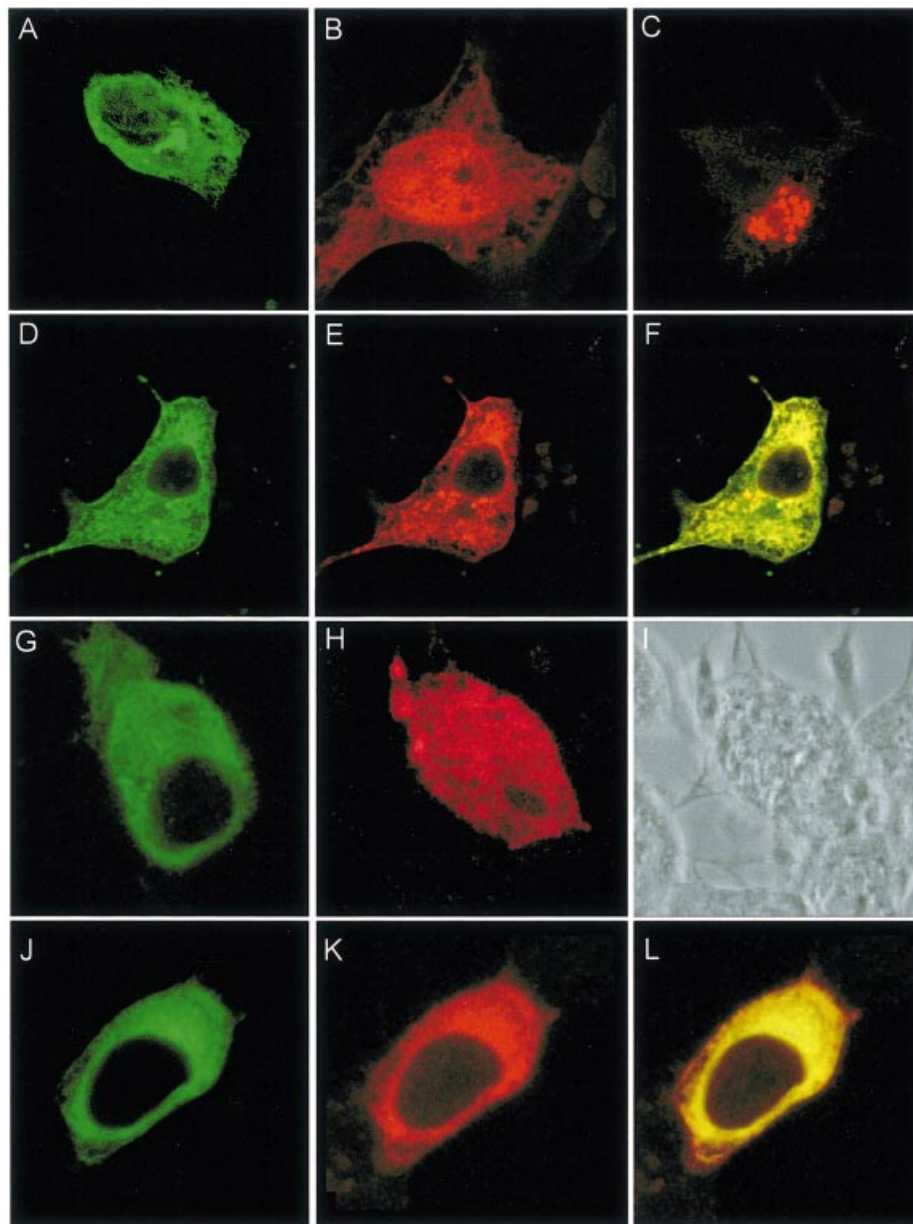


Fig. 9. Snk and Cib show identical subcellular localization when expressed together. Optical sections of single- and double-transfected COS and N2A cells were analyzed using confocal microscopy for the expression of GFP-Snk (green) or HA-tagged Cib detected by a Cy3-labeled secondary antibody (red). (A) Single transfected COS cell expressing GFP-Snk fusion protein. (B) Single transfected COS cell expressing HA-tagged Cib fusion protein. (C) Single transfected COS cell expressing HA-tagged Cib fusion protein with exclusive nuclear localization. (D) Double transfected COS cell expressing GFP-Snk fusion protein and HA-tagged Cib fusion protein. The localization of GFP-Snk fusion protein is shown. (E) The same cell as in (D). The localization of Cib fusion protein is shown. (F) Superposition of images shown in (D) and (E). (G) Single transfected N2A cell expressing GFP-Snk fusion protein. (H) Single transfected N2A cell expressing HA-tagged Cib fusion protein. (I) Phase-contrast image of the field containing the Cib-expressing cell shown in (H). In the field shown, there are several non-Cib-expressing cells which have no background staining as can be seen in (H). (J) Double transfected N2A cell expressing GFP-Snk fusion protein and HA-tagged Cib fusion protein. The localization of GFP-Snk fusion protein is shown. (K) Same cell as in (J). The localization of Cib fusion protein is shown. (L) Superposition of images shown in (J) and (K).

at least in a heterologous system (Lee and Erikson, 1997; Ouyang *et al.*, 1997). However, the function of *Fnk* and *Snk* is less clear. Their original identification as growth factor-stimulated genes has been taken to indicate that they might act at earlier cell cycle transitions (Simmons *et al.*, 1992; Donohue *et al.*, 1995; Chase *et al.*, 1998). Although proliferation might occur in a small subpopulation of neurons (Kempermann *et al.*, 1997; Parent *et al.*, 1997), the majority of neurons in which we detect *Fnk*

and *Snk* in the brain are post-mitotic, indicating that *Fnk* and *Snk* have a function outside the cell cycle.

The present study demonstrates that *Fnk* and *Snk* are expressed in the adult mammalian brain. In tissue from control rats, *Snk* is widely expressed at levels higher than those for *Fnk*. The expression of both genes is induced by the intense activity associated with seizures. Following induction, both protein kinases translocate into the dendrites of activated neurons. Furthermore, both *Fnk* and *Snk*

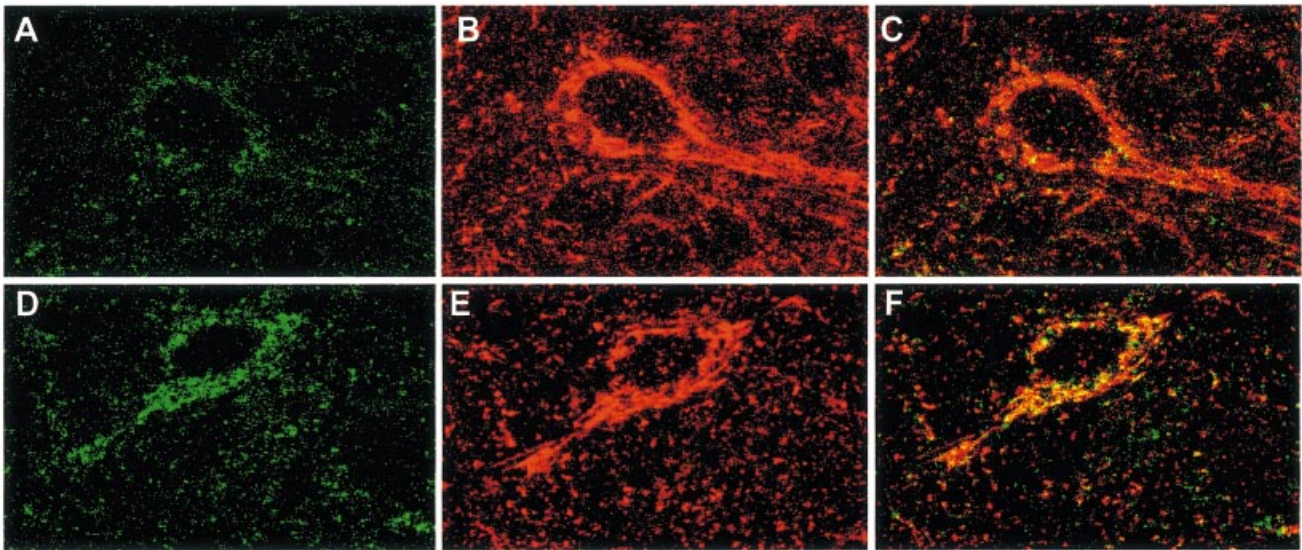


Fig. 10. Snk and Cib proteins co-localize in the somata and dendrites of hippocampal neurons. Sections through hippocampal region CA1 of control and KA-stimulated rat brain (4 h survival) were analyzed using confocal microscopy for the expression of Snk protein (Cy2, green) (A and D) and Cib protein (Cy3, red) (B and E). Images were superimposed in (C) and (F). (A and B) Control hippocampus; Snk (A) and Cib (B) immunoreactivity is detected in the subplasmalemmal cortex of the neuronal cytoplasm and dendritic processes. (D and E) After KA-induced seizures, immunoreactivity of Snk (D) is increased whereas that of Cib remains unchanged (E). (C) Superposition of images shown in (A) and (B), and (F) images shown in (D) and (E), demonstrates extensive co-localization of Cib and Snk in the cytoplasm and dendritic processes.

were induced consistently with LTP-producing stimulation, suggesting that they play a role in this plasticity. To gain insights into a possible function of Snk and Fnk, we analyzed the interaction of these kinases with the Ca^{2+} - and integrin-binding protein, Cib. We have shown that both kinases interact specifically with Cib via their polo-box domain. It has been reported that a mutation of the polo-box disrupts both mitotic function and localization of the other polo family member Plk (Lee *et al.*, 1998). The association and transfection experiments reported here not only indicate that Cib and Snk bind to each other and co-localize, but also suggest that the polo-box might be responsible for recruiting Cib from the nuclear into the cytoplasmic compartment. Cib might have specific functions in the nucleus and might there associate with other proteins. One such protein might be the catalytic domain of the DNA-dependent kinase (DNA-PKcs), a eukaryotic kinase activated by DNA ends and presumed to play a role in the repair of double-strand breaks (Wu and Lieber, 1997). In a two-hybrid screen, DNA-PKcs was found to interact specifically with Cib (in this study termed Kip for kinase-interacting protein) (Wu and Lieber, 1997). It remains to be seen whether the interaction indeed takes place in the nucleus and which motif within the DNA-PKcs is responsible for binding. In brain neurons, Cib immunoreactivity was not observed in the nucleus but co-localized extensively with the cytoplasmic distribution of the kinases in the somata and dendrites.

Cib shares >27% amino acid identity with calcineurin B, the regulatory subunit of calcineurin (phosphatase 2B), and has a similar identity to calmodulin (Naik *et al.*, 1997). Like calcineurin B and calmodulin, Cib contains EF-hand motifs responsible for Ca^{2+} binding. The structural similarity of Cib to calmodulin and to calcineurin B raises the possibility that Cib might be a regulatory subunit of Snk or Fnk or, alternatively, the regulatory subunit of a phosphatase whose catalytic subunit has not yet been

identified. In this case, the association of a kinase-phosphatase complex could facilitate rapid cycles of phosphorylation or result in an autocatalytic loop of kinase-phosphatase activation (Hunter, 1995). The importance of both the Ca^{2+} -calmodulin-regulated kinases (CaMKs) and calcineurin in synaptic plasticity has been demonstrated, and the regulation of *N*-methyl-D-aspartate (NMDA) receptor function is thought to be the basis for the proposed role of calcineurin and CaMKs in LTP and memory (Mayford *et al.*, 1995; Mansuy *et al.*, 1998; Snyder *et al.*, 1998; Winder *et al.*, 1998).

Similar functional analyses of Fnk, Snk and Cib have not yet been carried out. It remains to be determined whether the kinase activities of Fnk and Snk are increased with the induction of LTP and what specific role Cib has in modulating these processes. To address this issue, an important first goal for future research will be to identify a substrate which could be used to construct an assay. However, it is intriguing to speculate that these proteins might participate in integrin-mediated signaling during plastic events in the brain. Recent studies indicate that a wide array of α and β integrins are expressed in brain and that regional differences in the expression of specific integrin receptors might underlie differences in synaptic plasticity across brain systems (Pinkstaff *et al.*, 1999). Hippocampal slice experiments have indicated that blocking ligand binding by a major subclass of integrins prevents the stabilization of LTP (Staubli *et al.*, 1990, 1998; Xiao *et al.*, 1991; Bahr *et al.*, 1997; Grooms and Jones, 1997). Although integrin involvement in mammalian learning has not been investigated, a mutation of a synapse-associated integrin in *Drosophila* results in memory deficits (Grotewiel *et al.*, 1998). Integrins are membrane-spanning, non-covalently-bound heterodimers that form transmembrane links between the cellular matrix and the actin cytoskeleton (Howe *et al.*, 1998; Schoenwaelder and Burridge, 1999). They are frequently

clustered into specialized adhesive structures, in which numerous signaling components are concentrated (Clark and Brugge, 1995; Hemler, 1998). However, the molecular identity of such signaling centers in the mature nervous system is not known. Further information will be required to determine if Fnk, Snk and Cib are indeed components of such centers and if they mediate signaling in response to Ca²⁺ influx during NMDA receptor-mediated synaptic plasticity. The dramatic increase in Fnk and Snk following specific patterns of synaptic activity, the translocation of the kinase proteins to dendritic arbors and their association with a Ca²⁺- and integrin-binding protein suggest that this might be the case.

Materials and methods

Animal preparation and treatments

Adult male Sprague–Dawley rats (250 g, Charles River) were used. Drugs were administered by intraperitoneal injection: CHX in dimethyl-sulfoxide (DMSO), 120 mg/kg; PTZ in phosphate-buffered saline (PBS), 50 mg/kg; and KA in PBS, 10 mg/kg. Animals that received PTZ and CHX were injected with CHX 0.5 h prior to PTZ. Control animals were injected with similar volumes of isotonic saline (PBS). Of PTZ- and KA-injected rats, only those exhibiting motor seizures were included in experiments. All animals were sacrificed by decapitation at appropriate times. For the isolation of RNA, the brain was removed after decapitation and transferred into ice-cold PBS. Cortices and hippocampi were dissected, frozen in liquid nitrogen and stored at –75°C. For *in situ* hybridization, the brains were removed, frozen on dry ice and stored at –75°C. For immunohistochemistry, animals under deep anesthesia were perfused transcardially with 4% paraformaldehyde. Brains were dissected out and stored in the same fixative overnight.

Chronic electrophysiology

Adult male Sprague–Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (55 mg/kg) and stereotaxically implanted with a monopolar stimulating electrode in the perforant path and a monopolar recording electrode in the hilus of the dentate gyrus. Physiological recordings were used to find and maximize the evoked positive-going field potential (maximum spike-free amplitude range 7–12 mV). The electrodes were mounted onto the skull using dental cement and their leads connected to a permanently affixed head stage. The wound was sutured and analgesics administered, and the animals recovered for at least 7 days before being transferred to a chronic recording cage for the actual experiment involving standard *in vivo* electrophysiological techniques (Staubli and Scafidì, 1997). During the experimental sessions, the current intensity was adjusted (20–70 µA, pulse width 150 µs, biphasic) to produce a baseline response with a minimal population spike of 0.5–1 mV. Test pulses were delivered at 0.07 Hz for at least 20 min to establish stable baseline potentials, after which either high- or low-frequency stimulation (HFS or LFS) was initiated randomly. The HFS paradigm consisted of five 25-ms-long trains at 400 Hz, delivered 1 s apart, and repeated 50 times with a 1 min interval between repetitions. The same number of pulses was delivered for the LFS treatment, but at a rate of 0.2 Hz. Potentials were then monitored at 0.07 Hz for an hour, following which the rats were decapitated, and the brains removed and placed on dry ice.

PCR primers

The PCR primers used are listed in Table I.

Molecular cloning of rat Fnk, Snk and Cib

A rat cDNA fragment of *Fnk* (DDBJ/EMBL/GenBank accession No. AF136584) was obtained in an RT–PCR using PTZ/CHX-induced rat hippocampal RNA, primers Fnk1 and Fnk2 according to the published mouse *Fnk* cDNA sequence (Donohue *et al.*, 1995) and the Pre-amplification System (Gibco). The 1846 bp amplification product was cloned into pCR2.1 (Invitrogen) to generate pCR2.1-Fnk. This fragment contained 51 nucleotides of coding sequence not present in the published mouse sequence but was contained in a 366 bp RT–PCR product (DDBJ/EMBL/GenBank accession No. AF136586) using primers Fnk3 and Fnk4 and mouse 3T3 fibroblast RNA. As we were unable to amplify the entire coding region of *Fnk*, presumably due to a GC-rich stretch

located in the 5' region, a chimeric full-length mouse/rat *Fnk* clone was constructed. A 12 kb genomic *XhoI* fragment of *Fnk* was isolated from a λ mouse genomic library. A 396 bp *XbaI*–*Bsa*HI subfragment, containing the coding sequence for the N-terminal 31 amino acids missing in the rat *Fnk* clone, was ligated with a 1801 bp *Bsa*HI–*Eco*RI fragment from pCR2.1-Fnk and cloned into the *XbaI*–*Eco*RI sites of pSPORT2 (Gibco) to generate chimeric pSPORT2-Fnk. A 1767 bp rat cDNA fragment of *Snk* was obtained in an RT–PCR using PTZ/CHX-induced rat hippocampal RNA and primers Snk1 and Snk2 based on the published mouse sequence (Simmons *et al.*, 1992). This fragment was cloned into pCR2.1 to generate pCR2.1-Snk. A 2781 bp rat *Snk* cDNA clone (pSPORT1-Snk; DDBJ/EMBL/GenBank accession No. AF136583) was obtained using this fragment in a screen of a rat hippocampal cDNA library prepared from PTZ/CHX-induced animals (Link *et al.*, 1995). Rat *Cib* cDNA was obtained using primers Cib1 and Cib2 based on several mouse expressed sequence tags. A 822 bp rat *Cib* cDNA clone (pSPORT1-Cib; DDBJ/EMBL/GenBank accession No. AF136585) was obtained in a screen of a rat hippocampal cDNA library. Both strands of the constructs described above were sequenced as double-stranded plasmid with multiple synthetic primers by the dideoxy chain termination method (Sanger *et al.*, 1977).

Northern blot analysis

Northern analysis was performed as described (Qian *et al.*, 1993). A 2 µg aliquot of poly(A)⁺ RNA (*Fnk* and *Cib*) or 5 µg of total RNA (*Snk*) was loaded per lane. Probes specific for *Fnk*, *Snk* and *Cib* were labeled with [α-³²P]dCTP (rediprime™ kit, Amersham). As a loading control, blots were hybridized to a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985). For quantification, Northern blots were analyzed on a bioimaging analyzer (Bas 2000; Fujix). Phosphorescence-stimulated luminescence signals of *Fnk*, GAPDH reference and blank areas were determined using Image Analyze software (Fuji). After background subtraction, *Fnk* signals were normalized relative to cognate GAPDH reference signals as described (Kuhl *et al.*, 1987).

In situ hybridization

In situ hybridization was conducted as described (Link *et al.*, 1995). Frozen brains were cryostat sectioned at 16 µm on the coronal plane. Control and experimental tissue sections were thaw-mounted onto the same slide to ensure identical hybridization conditions. Uridine 5'- (α-[³⁵S]thio)triphosphate-labeled sense and antisense *Fnk* and *Snk* RNAs were transcribed from the T7 promoter of *Bam*HI-linearized pCR2.1 plasmid containing 1846 and 1767 bp cDNA inserts of *Fnk* and *Snk*, respectively. Sense strand controls were generated from *Bam*HI-linearized pCR2.1 plasmid containing identical kinase inserts in reverse orientation.

Expression and purification of recombinant proteins

The coding region of *Fnk*, except 31 amino acids of the N-terminus, and cDNA encoding 262 amino acids of the N-terminus of *Snk* were amplified using primers FnkEx3 and FnkEx4, and pCR2.1-Fnk as template, and primers SnkEx3 and SnkEx8, and pSPORT1-Snk as template, respectively. The amplification products were cloned into the *Eco*RI–*Xho*I sites of pET-28a(+)(Novagen). The integrity of the reading frames was verified by transcription and translation in the presence of [³⁵S]methionine in a reticulocyte system (TNT Coupled Reticulocyte Lysate System, Promega). Recombinant *Fnk* and *Snk* were expressed as bacterial fusion proteins according to the protocol of the manufacturer (Novagen). The fusion proteins were purified over Ni²⁺-nitriloacetic acid–agarose (Qiagen) and used for the generation of *Fnk*- and *Snk*-specific antibodies. In addition, GST fusion proteins were generated in bacteria to (i) purify polyclonal anti-Fnk and anti-Snk sera by affinity chromatography and (ii) verify the protein interactions between Cib and C-terminal deletion constructs of Fnk and Snk *in vitro*. Primers CibEx3 and CibEx8, and pSPORT1-Cib as template, were used in a PCR to amplify cDNA encoding Cib followed by a nine amino acid HA tag (GST–Cib). The amplification product was cloned into the *Eco*RI–*Xho*I sites of the prokaryotic expression vector pGEX-JDK to generate pGEX-Cib-HA. Primers SnkEx3 and SnkEx10, and pSPORT1-Snk as template, were used to amplify cDNA encoding the N-terminal 352 amino acids of Snk (amino acids 1–352) followed by an 11 amino acid Myc tag (GST–SnkNT). The amplification product was cloned as an *Eco*RI–*Xho*I fragment into pGEX-JDK to generate pGEX-SnkNT-Myc. This plasmid was cleaved with *Eco*RI and *Not*I to remove *Snk* sequences and religated using primers Lig3 and Lig4. The resulting plasmid, pGEX-JDK-Myc, encodes the Myc-tagged GST moiety. Primers FnkEx10 and FnkEx9,

Table I. PCR primers

<i>Snk1</i>	5'-GCAACGATGGAGTCTCTGCGGACTA-3'
<i>Snk2</i>	5'-CGGGAGATCACCACCATCCATGAGG-3'
<i>SnkEx3</i>	5'-TCCGAATTCGCAACGATGGAGTCTCTGCGGACTA-3'
<i>SnkEx5</i>	5'-TCAGGATCCGTTACATCTCTGTAAGAGCATGTTACAG-3'
<i>SnkEx6</i>	5'-TCAGGATCCGTGGAATCTGGAATGTGTGGCA-3'
<i>SnkEx7</i>	5'-TCCGAATTCCACTTGTCAAGCCCAGCCAAGAAT-3'
<i>SnkEx8</i>	5'-GTGCTCGAGGGCCAGATGTCTGATTCACAGCCG-3'
<i>SnkEx10</i>	5'-TCAGTCGAGATTCAGATCCTCTTCTGAGATGAGTTTTGTTCGGCGCCGCGTGGAAATCTGGAAGTGTGTGGCA-3'
<i>SnkEx11</i>	5'-GCCTCATCAGGCGGCCGTTACATCTCTGTAAGAGCATGTTACAG-3'
<i>SnkEx12</i>	5'-CATGCCATGGAGTGGGTCACCAATGGGTCGACTAC-3'
<i>SnkEx13</i>	5'-CATGCCATGGAGCTAGAAAACATGCCTGAAGCTGAC-3'
<i>SnkEx14</i>	5'-TCAGGATCCGCGTTGTTGAAAAGGACTCCAAC-3'
<i>SnkEx15</i>	5'-TCAGGATCCTTGGCCAAGTTCGCAATAATAGT-3'
<i>SnkEx16</i>	5'-TCCCTCGAGGCAACGATGGAGTCTCTGCGGACT-3'
<i>Fnk1</i>	5'-ATGCCTCCCCGCGATCGGAACCTGA-3'
<i>Fnk2</i>	5'-GGGCTTTGGTCCCTGAGCAGGGCA-3'
<i>Fnk3</i>	5'-CCCAGACCTGACACCCCCAACCC-3'
<i>Fnk4</i>	5'-GGTTCTGTCCGCTGGGGCATG-3'
<i>FnkEx3</i>	5'-TCCGAATTCGCCTCCCCGCGATCGGAACCTGA-3'
<i>FnkEx4</i>	5'-GTGCTCGAGGCTTTGGTCCCTGAGCAGGGCA-3'
<i>FnkEx5</i>	5'-TCCGAATTCATGGAGCCCGCCGCGCTTCTTG-3'
<i>FnkEx7</i>	5'-TCACTCGAGAGCAGGGCTTTGGTCCCTGAGCAG-3'
<i>FnkEx8</i>	5'-TCCCGAATTCGAATGGAGCCCGCCGCGCTTCTTG-3'
<i>FnkEx9</i>	5'-GCCTCATCAGGCGCCGAGCAGGGCTTTGGTCCCTGAGCAG-3'
<i>FnkEx10</i>	5'-TCCGAATTCACACCCCCAACCTGCGAGGAGT-3'
<i>FnkEx11</i>	5'-TCCGAATTCGAACACCCCCAACCTGCGAGGAGT-3'
<i>FnkEx12</i>	5'-CATGCCATGGAGTGGGTCAGCAAGTGGGTTGACTAC-3'
<i>FnkEx13</i>	5'-CATGCCATGGAGGCTTATGCCCCAGCTGAACAG-3'
<i>FnkEx14</i>	5'-TCAGGATCCGCGTCAATTGAAGAGCACGGTAC-3'
<i>FnkEx15</i>	5'-TCAGGATCCTTCTGTGCTGGTGGGTTGTAGTG-3'
<i>FnkEx16</i>	5'-TCCCTCGAGATGGAGCCCGCCGCGCTTCTTG-3'
<i>Cib1</i>	5'-GGATCCATGAGGACGGAAGTGAGAA-3'
<i>Cib2</i>	5'-TGCCACATCTCAGCAGTGGCCAGGT-3'
<i>CibEx1</i>	5'-TCAGGATCCTTATGGGAGGTTCCGGCAGTCCGCTG-3'
<i>CibEx2</i>	5'-TCCGAATTCGGGGGAGAGTCTCCCTGCCAGG-3'
<i>CibEx3</i>	5'-TCCGAATTCATGGGAGGTTCCGGCAGTCCGCTG-3'
<i>CibEx8</i>	5'-TCACTCGAGAGCGTAATCTGGAACATCGTATGGGTAGGCGCCGCCAGGACAATCTTAAAGGAGCT-3'
<i>MycEx1</i>	5'-TCAGAATTCATTCAGATCCTTCTGAGATGAG-3'
<i>Lig3</i>	5'-AATTCAATTCTAGACTCCATGGGTCGACTGC-3'
<i>Lig4</i>	5'-GGCCGACGTCGACCCATGGAGTCTAGAATTG-3'

and pSPORT2-Fnk as template, were used to amplify cDNA encoding 313 amino acids of the C-terminus of Fnk (amino acids 335–647). Similarly, cDNA encoding 331 amino acids of the C-terminus of Snk (amino acids 352–682) was amplified using primers SnkEx7 and SnkEx11, and pSPORT1-Snk as template. The amplification products were inserted individually as *EcoRI*–*NotI* fragments into pGEX-JDK-Myc to generate pGEX-FnkCT-Myc and pGEX-SnkCT-Myc, respectively. Following transformation of *Escherichia coli* BL21 cells (Novagen), the expressed GST fusion proteins were purified using glutathione–Sepharose 4B beads (Pharmacia) according to the manufacturer's protocol.

Generation of polyclonal antisera

Rabbits were immunized with recombinant His₆-tagged Fnk and Snk proteins according to a standard immunization protocol (Eurogentec). Antisera were purified over affinity columns containing cyanogen bromide-activated Sepharose 4B (Pharmacia) coupled to the C-terminal domain of Fnk and the N-terminal domain of Snk, respectively. Antibodies were eluted in glycine buffer (pH 2.5). Western blots showed specific binding of the antisera to the corresponding recombinant kinase protein, and no cross-reactivity was observed. Depletion was achieved by overnight incubation of the purified sera with redissolved cyanogen bromide-activated Sepharose 4B coupled to the corresponding recombinant protein.

Immunohistochemistry

Free floating 35 µm vibratome sections were pre-incubated in normal horse serum (10%) and then incubated overnight in purified rabbit polyclonal antisera to Fnk (1:5, 1% normal horse serum as diluent) and to Snk (1:10) as well as in antisera depleted by pre-absorption with the corresponding kinase protein. For detection of Cib protein, a mouse monoclonal antibody (1:50) was used (Naik *et al.*, 1997). Localization

of the kinase proteins and Cib was detected using the Vectastain Elite Avidin-Biotin-Complex system (Vector Laboratories) with nickel-intensified diaminobenzidine (DAB) as chromagen.

Yeast two-hybrid assays

For yeast two-hybrid-based interaction studies, the open reading frame of *Cib* was amplified using primers CibEx1 and CibEx2, and pSPORT1-Cib as template. The amplification product was inserted in-frame into the *BamHI*–*EcoRI* sites of the yeast expression vector pACTII (a gift from Dr S.J.Elledge) to generate pACTII-Cib. The complete coding sequence of *Snk*, the N-terminal 352 (SnkNT) and the C-terminal 331 amino acids (SnkCT) of *Snk* were amplified using the following primer pairs: SnkEx3 and SnkEx5 for Snk, SnkEx3 and SnkEx6 for SnkNT, and SnkEx7 and SnkEx5 for SnkCT. In all three PCRs, pSPORT1-Snk served as a template. The *Snk* amplification products were cloned in-frame into the *EcoRI*–*BamHI* sites of pAS2-1 (Clontech) generating pAS2-1-Snk, pAS2-1-SnkNT and pAS2-1-SnkCT, respectively. The entire coding region of *Fnk* was amplified using primers FnkEx5 and FnkEx6, and pSPORT2-Fnk as template, and cloned in-frame into the *EcoRI*–*SalI* sites of pAS2-1 to yield pAS2-1-Fnk. Sequence analysis of each construct verified correct in-frame cloning to the respective GAL4 domain. Each of the above constructs was transformed into Y187 cells and tested in a filter assay for autonomous β-galactosidase activity using Bluo-GAL (Gibco) as a substrate (Matchmaker Two-Hybrid System 2 manual, Clontech). Yeast cells harboring pAS2-1-Snk and pAS2-1-SnkCT alone exhibited autonomous transactivation of the *LacZ* reporter. Therefore, all kinase fragments were cloned into pACTII and *Cib* was cloned into pAS2-1. The *EcoRI*–*NcoI* fragment of pACTII-Cib was cloned in-frame to the GAL4 DNA-binding domain of pAS2-1 to generate pAS2-1-Cib. The *SfiI*–*BamHI* fragments of pAS2-1-Snk, pAS2-1-SnkNT and pAS2-1-SnkCT were inserted into pACTII to yield pACTII-Snk, pACTII-SnkNT and pACTII-SnkCT. The entire coding region of

Fnk and cDNA encoding the C-terminal 313 amino acids of Fnk were amplified using primers FnkEx8, FnkEx7 and FnkEx11, FnkEx7, and pSPORT2-Fnk as a template. The amplification products were cloned into the *EcoRI-XhoI* sites of pACTII to yield pACTII-Fnk and pACTII-FnkCT. Amplification products encoding the Snk polo-box of 30 amino acids (SnkPolo30) and the polo-box plus an additional 20 amino acids of N-terminal and C-terminal flanking sequences (SnkPolo70) were generated using primer pairs SnkEx12 and SnkEx14, and SnkEx13 and SnkEx15, and pSPORT1-Snk as template. Similarly, corresponding polo constructs of Fnk (FnkPolo30 and FnkPolo70) were amplified using the primer pairs FnkEx12 and FnkEx14 for FnkPolo30, and FnkEx13 and FnkEx15 for FnkPolo70, and pSPORT2-Fnk as template. All polo-box fragments of Snk and Fnk were inserted in-frame into the *NcoI-BamHI* sites of pACTII. No autonomous expression of β -galactosidase was observed in single-transformed Y187 cells with any of these plasmids. Each of the *Fnk*- and *Snk*-containing pACTII plasmids was co-transformed with pAS2-1-Cib into Y187 cells and assayed for β -galactosidase expression as described above. Specificity of the interaction was determined in co-transformation experiments using the full-length kinase constructs, and pAS2-1 encoding only the DNA-binding domain and pLAM5'-1 (Clontech) encoding a DNA-binding domain-lamin C fusion protein as negative controls. As an additional negative control, pGAD10-trypsinogen encoding a GAL4 activation domain-trypsinogen fusion protein was tested for interaction with pAS2-1-Cib. Snf4 and Snf1, two yeast proteins that previously were shown to interact (Fields and Song, 1989), served as a positive control. For quantitative analyses, each co-transformant was assayed for β -galactosidase activity in a liquid culture assay using CPRG (Boehringer Mannheim) as a substrate as described in the Matchmaker Two-Hybrid System 2 (Clontech, 1996). Enzymatic assays represent the average of three independent yeast clones.

In vitro binding experiments

Myc-tagged GST fusion proteins comprising homologous C-terminal domains of Snk (GST-SnkCT) and Fnk (GST-FnkCT) and Myc-tagged GST protein (GST) alone as a control were cloned and bacterially expressed as described above. Each of these proteins was immobilized identically overnight at 4°C on seven Maxi sorb immuno plates (Nunc) in twelve 1:2 dilution steps ranging from 500 ng to 240 pg. After overnight adsorption, each protein dilution was incubated for 1.5 h at room temperature in 200 μ l of blocking solution containing 2% bovine serum albumin (BSA), 0.01% thimerosal, 0.1% Tween-20 in PBS and subsequently washed four times with PBS, 0.1% Tween-20. Adsorbed proteins on plates 1 and 2 were detected with a monoclonal mouse anti-Myc antibody (1:500) (Evan *et al.*, 1985) and quantified in a microplate reader (Model 3550, Bio-Rad) by a peroxidase-labeled anti-mouse antibody conjugate (1:4000; Bio-Rad) and OPD (0.4 mg/ml, 200 μ l/well) (Sigma) as a substrate. The obtained values served for normalization of adsorbed recombinant kinase proteins on plates 3–7. Each well on plates 3–7 was incubated for 1 h at room temperature with 1 μ g of HA-tagged GST-Cib protein solubilized in blocking solution. Following four washing steps, bound GST-Cib was detected using monoclonal mouse anti-HA antibody (1:500) (Boehringer Mannheim), washed four times, and quantified in a peroxidase reaction as described above.

Co-localization assays

The entire coding region of *Fnk* and cDNA encoding the complete open reading frame of *Snk* were amplified using primers FnkEx16 and MycEx1, and pSPORT2-Fnk as template, and SnkEx16 and MycEx1, and pSPORT1-Snk as template, respectively. The amplification products were cloned in-frame into the *XhoI-EcoRI* sites of the GFP expression vector pEGFP-C3 (Clontech). An *EcoRI-XhoI* fragment of pGEX-Cib-HA encoding the open reading frame of *Cib* plus nine amino acids of an HA tag was cloned into pCDNA3 (Invitrogen). COS7 and N2A cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated bovine calf serum and a 1:100 dilution of a penicillin-streptomycin solution. The cells were expanded by trypsin-EDTA treatment and subcultured at a split ratio of 1:5 every 2–3 days. Cells were transfected with Lipofectamine (Gibco) following the manufacturer's recommendations. Briefly, 1 μ g of DNA for single transfections or 1 μ g of each recombinant plasmid DNA for double transfections was incubated with 10 μ l of Lipofectamine in 0.1 ml of DMEM for 60 min at room temperature. One milliliter of DMEM was then added and the mixture was applied to the DMEM-washed cells. After 5 h of incubation at 37°C, the mixture was replaced with complete medium. A mouse monoclonal anti-HA tag antibody (1:500 dilution, BabCo) was used as primary antibody and Cy3 (red) goat anti-

mouse antibody (1:100 dilution, Dianova) as secondary antibody in immunostainings. Cells processed for immunofluorescence were washed with PBS and fixed for 15 min at 4°C with 100% methanol. The samples were then blocked with 10% calf serum in PBS for 15 min at room temperature. Two additional 5 min washes were carried out before the coverslips were mounted in glycerol. For Snk/Cib co-localization experiments in rat brain, free floating 25 μ m sections were incubated with Snk- (1:10) and Cib- (1:50) specific primary antibodies. Localization of Snk protein was detected by anti-rabbit Cy2 (green) (1:1000, Amersham) as secondary antibody. Cib protein was detected using a biotinylated secondary anti-mouse antibody and a streptavidin-coupled Cy3 (red) (1:1000, Amersham). Samples were examined in dual channels using a Leica TCS-NT confocal microscope and images were processed using Leica TCS-NT VI.6.551 (Wetzlar, Germany) and Imaris (Bitplane, Zürich, Switzerland) software.

Acknowledgements

We thank Christine Gall and Paul Skehel for comments on the manuscript, Oliver Sperl for preparing the figures, Lisa Kurunci, Uwe Holtrich and Monika Vöth for experimental help, and Ulhas Naik (University of Delaware) for Cib monoclonal antibodies. S.J. was supported by the Graduiertenkolleg grant GRK255 (D.K.). This research was supported by the Bundesministerium für Bildung und Forschung (D.K.), the Deutsche Forschungsgemeinschaft grants SFB 444, Ri 192/21-1-3 (D.K.), STR 336/5-1 (K.S.), the Deutsche Krebshilfe grant W 102/93/Rü2 (K.S.) and by the Whitehall Foundation (U.S.).

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Received June 9, 1999; revised and accepted August 27, 1999