A Role for Sam68 in Cell Cycle Progression Antagonized by a Spliced Variant within the KH Domain*

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Sam68 is the main tyrosine-phosphorylated and Src-associated protein in mitotic cells. Sam68 exhibits a conserved functional KH (hnRNPK homology) RNA binding domain and binds single strand nucleic acids. Tyrosine phosphorylation mediates the interaction of Sam68 with many SH3- and SH2-containing proteins and negatively regulates its nucleic acid binding properties. But the function and the impact of Sam68 on cell signaling and cell proliferation remains elusive. We report here the identification of a natural isoform of Sam68 with a deletion within the KH domain. This isoform, called Sam68ΔKH, is specifically expressed at growth arrest upon confluency in normal cells. In cells that do not enter quiescence at confluency such as Src-transformed cells, no recruitment of Sam68ΔKH is observed. Transfected Sam68ΔKH inhibits serum-induced DNA synthesis and cyclin D1 expression. Sam68 overcomes these effects, suggesting that isoforms of Sam68 are involved, through KH domain signaling, in cell proliferation, and more precisely in G1/S transition.

Sam68 is the main tyrosine-phosphorylated and Src-associated protein in mitotic cells (1–5). Based on several experimental evidences, Sam68 turned out to be encoded by the hump62 cDNA previously described as the cDNA of the GAP-p62 associated protein (5). Sam68 exhibits a conserved functional KH (hnRNPK Homology) RNA binding domain and RGG boxes, two landmarks of RNA binding proteins (6, 7), and was indeed shown to bind single strand nucleic acids (8, 9). Because Sam68 is tyrosine-phosphorylated in mitotic cells and has five proline-rich domains, Sam68 interacts both through SH2- and SH3-mediated interactions not only with the Src family tyrosine kinases but also with signaling molecules such as Grb2 and phospholipase Cy-1 (10, 11). Sam68 is therefore envisioned as a multifunctional SH3 and SH2 domain binding protein that could link Src family kinases to putative downstream protein and nucleic acid effectors. A physiological function or an impact of Sam68 on signaling events remains to be elucidated.

Materials and Methods

Binding of Sam68, Sam68ΔKH, and Sam68 (203–443) to Poly(U)

Wild type Sam68 (1–443), Sam68ΔKH (1170–208), and Sam68 (203–443) were translated in vitro (Promega). Sam68 (203–443) was obtained by PCR† using specific primers that introduced an in-frame initiation codon. The translated proteins were incubated 30 min at 4 °C in presence of poly(U) beads in binding buffer (Tris-HCl, pH 7.5, 5, 10 mM, MgCl2, 2.5 mM, Triton, 0.5%; NaCl, 100 mM). Beads were washed three times in binding buffer, and bound proteins were eluted in Laemmli buffer for 10 min at 95 °C. Specificity was assessed by competition with an excess of soluble poly(U). The poly(U) binding activity (binding percent) of each polypeptide is expressed as percentage of specific binding relative to the total binding. The results represent the average of four independent experiments.

Tissue Expression of Sam68 and Sam68ΔKH

Analysis of the Expression of Sam68 and Sam68ΔKH mRNAs in Human Tissues by RT-PCR—The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; RT, reverse transcription.

Western Blot Analysis of Sam68 and Sam68ΔKH in Human Tissues—Multiple tissue Western blot (Clontech) was incubated with antibodies according to the manufacturer’s instructions. The anti-Sam68 monoclonal antibody is commercially available (P20120; Transduction Laboratories), and the anti-Sam68ΔKH antibodies were purified from the serum of rabbit injected with a peptide corresponding to the Sam68ΔKH-specific sequence: (H)-Cys-Lys-Gln-Tyr-Pro-Lys-Glu-Glu-(OH). Antibody specificity was checked on recombinant Sam68 and Sam68ΔKH proteins expressed in SF9 using a baculovirus expression system. The expression system allowed to express both Sam68 and Sam68ΔKH cDNAs amplification and corresponds to nucleotides 1101–1125 of Sam68 cDNA. Primers specificities were checked to avoid nonspecific cross-reactions. The Sam68 and Sam68ΔKH cDNAs at concentrations ranging from 100 to 0.1 μg were also amplified by PCR as standards. A 600-base pair fragment was amplified in all samples. The figure depicts 10% of the PCR products separated on a 1.5% agarose gel. As a control, the amounts of cDNA used in each experiment were evaluated by amplifying the cDNA encoding β actin with specific oligonucleotides (12).

Wild type Sam68 and Sam68ΔKH in NIH3T3 Cells—Ten NIH3T3 cells and 10 μg of infected SF9 lysates were used as control of the specificities of the antibodies. Revelations were performed with Enhanced Chemiluminescence (Amersham Corp.). Detection of Sam68ΔKH in NIH3T3 Cells—Ten NIH3T3 cells transformed by c-Src (Y567F) were synchronized by 24 h of serum starvation and then stimulated by 10% of fetal calf serum up to 48 h.

Cells were washed twice in PBS, and total cell lysates were prepared by scraping in Laemmli buffer with 6 μg of protein. For each sample, 10 μg of proteins were analyzed on 10% SDS-PAGE. Antibodies against Sam68 and Sam68ΔKH were as described above.

Transfection of Sam68 and of Sam68ΔKH and Measure of BrdUrd Incorporation—One NIH3T3 cells transfected with myc-tagged

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RESULTS AND DISCUSSION

While using RT-PCR to clone Sam68 cDNA from human placental RNA, we identified a cDNA fragment identical to Sam68 sequence except for a deletion within the KH domain. This in-frame deletion spanned the region encoding amino acids residues 170-to 208 of Sam68 (Fig. 1 g of cell extracts). Three positive clones were isolated after screening of 10^6 recombinant phages (4–20% gradient SDS-PAGE). Totally sates were obtained as described previously. Proteins were detected with the 9E10 anti-Sam68 monoclonal antibody (P20120; Transduction Laboratories) or a cyclin D1 monoclonal antibody (HD11, Santa Cruz). 

Sam68 Implication in Cell Cycle Progression

FIG. 1. Structural differences between Sam68 and Sam68ΔKH. a, schematic representation of Sam68 and Sam68ΔKH structures. Sam68 is composed of RGG boxes, five consensus proline-rich motifs P1, P2, P3, P4, and P5 (black boxes), a tyrosine-rich domain (dotted box), and a KH homology domain (boxed box) almost completely deleted in Sam68ΔKH. Sam68 KH consensus residues are indicated in bold. Square brackets indicate the deletion limits (39 amino acids). The locations of the primers used for RT-PCR experiments are indicated by arrows. b, binding of in vitro translated Sam68, Sam68ΔKH, and Sam68 (205–443) to poly(U). The poly(U) binding activity (Binding %) of each polypeptide is expressed as the percentage of specific binding relative to the total binding. The results represent the averages of four independent experiments.

Sam68 (1 or 5 μg) or myc-tagged Sam68ΔKH (1 or 5 μg) SV40 expression vectors. Transfection experiments were performed using Lipofectamine (Life Technologies, Inc.) and peptide H1 under conditions that ensured the transfection of 60% of the cells, as controlled by β-galactosidase staining. In a first set of experiments, cells were rinsed twice in PBS 24 h after transfection and synchronized by serum starvation during 24 additional hours. Growth was then stimulated by 10% fetal calf serum during 12 h. BrdUrd incorporation was then analyzed both by immunoblotting (5-bromo-2-deoxy-uridine labeling and detection kit III, Boehringer Mannheim) or FACS analysis. In this case, BrdUrd (30 μM) was added 30 min before collection. Cells were stained for DNA content with propidium iodide and for DNA synthesis with a fluorescein-conjugated anti-BrdUrd antibody. The results represent the average of five independent experiments for immunoblot detection, and three for FACS analysis. The 100% value was arbitrary assigned to the BrdUrd incorporation observed after serum starvation of quiescent cells. In a second set of experiments, transfected cells were asynchronously grown, and S phase entry was analyzed 24 h after transfection as described above. In these experiments, protein expression was evaluated by direct Western blot analysis of 10 μg of cell extracts (4–20% gradient SDS-PAGE). Total lysates were obtained as described previously. Proteins were detected with the 9E10 anti-myc monoclonal antibody or a cyclin D1 monoclonal antibody (HD11, Santa Cruz).

RESULTS AND DISCUSSION

While using RT-PCR to clone Sam68 cDNA from human placental RNA, we identified a cDNA fragment identical to Sam68 sequence except for a deletion within the KH domain. This in-frame deletion spanned the region encoding amino acids residues 170-to 208 of Sam68 (Fig. 1). Three positive clones were isolated after screening of 10^6 recombinant phages with a 24-mer oligonucleotide specific for the deletion. Two clones encoun-

passed an entire open reading frame that was identical to that of Sam68 with the exception of the deletion of the KH RNA binding domain (Fig. 1a). We called this open reading frame Sam68ΔKH for deleted form of Sam68. These isoforms probably arise from alternative splicing of a single pre-mRNA species, because the analysis of their remaining common sequences appeared identical, even in the 5′ and 3′ noncoding regions. Both isoforms as well as the carboxyl-terminal of Sam68 which does not contain any known RNA binding motif (8), were in vitro translated. The same amount of each protein was incubated with poly(U)-agarose beads (9, 13), and specifically bound proteins were quantified. Sam68ΔKH bound poly(U) one-fifth as much as did Sam68 (Fig. 1b). However, when compared with the carboxyl-terminal part of Sam68, which was used as a negative control, it appeared that Sam68ΔKH retained some impaired RNA binding properties. This could be due to the presence of RGG boxes in the NH₂-terminal domain of Sam68 that are still present in the deleted isoform and indicates that the deletion of the KH domain does not alter the overall structure of the protein.

Because the predicted sizes of both mRNAs differ by only 117 bases and because Northern blot analysis of several human adult tissue samples revealed only one mRNA species of 2.9 kilobases, we performed RT-PCR to evaluate the expression of Sam68 and Sam68ΔKH mRNAs. Sam68ΔKH mRNA was detected in the different adult tissues (Fig. 2a), and to be able to distinguish between the expression of the two isoforms in human samples, we generated antipeptide antibodies that would only recognize Sam68ΔKH. These antibodies recognized a single band in the different human samples, intensified in skeletal

FIG. 2. Tissue expression of Sam68ΔKH. a, analysis of the expression of Sam68 and Sam68ΔKH mRNAs in human tissues by RT-PCR. 5 μg of total RNA (Clontech) were used for each sample. The figure depicts 10% of the PCR products separated on a 1.5% agarose gel. As a control, the amounts of cDNA used in each experiment were evaluated by amplifying the cDNA encoding β actin with specific oligonucleotides (12). b, Western blot analysis of Sam68 and Sam68ΔKH in human tissues. Multiple tissue Western blot (Clontech) was incubated with anti-Sam68 monoclonal antibody (P20120; Transduction Laboratories) and with anti-Sam68ΔKH antibodies. Antibody (Ab) specificities were checked on recombinant Sam68 and Sam68ΔKH proteins expressed in SF9 using a baculovirus expression system. 10 μg of infected SF9 lysates were used as control.

2 I. Barlat, personal communication.
fluency after stimulation by 10% of fetal calf serum (synchronized by 24 h of serum starvation) and then grown to confluence after stimulation by 10% of fetal calf serum (FCS) up to 48 h. Cells were washed twice in PBS, and total cell lysates were prepared by scraping in Laemmli buffer with 6 M urea. For each sample, 10 μg of proteins were analyzed on 10% SDS-PAGE, and the figure represents the Western blot analysis using either the anti-Sam68 monoclonal antibody (P20120; Transduction Laboratories) or the antibodies against Sam68ΔKH.

Using an anti-Sam68 monoclonal antibody directed against the KH domain, which failed to recognize Sam68ΔKH (Fig. 2b), Sam68 was significantly detected in every sample, although it appeared less represented in brain, skeletal muscle, and liver. These data indicated that Sam68ΔKH is a natural isoform of Sam68 and that the ratio between these two isoforms varies among different adult tissues. These variations could reflect the involvement of Sam68ΔKH in differentiation and/or proliferative processes, which could depend on functional differences between the two isoforms.

Sam68ΔKH has a crippled KH domain and therefore impaired RNA binding properties. It can be envisioned that the two isoforms could have distinct and possibly antagonistic functions. Although no biological role has been assigned yet to Sam68, its specific phosphorylation and Src association are restricted to mitosis and support the hypothesis that Sam68 could be involved in the regulation of cell proliferation.

In order to examine whether or not Sam68 and Sam68ΔKH expressions could vary according to cell growth, we performed Western blot experiments on NIH 3T3 cells under different conditions of cell proliferation. Synchronized cells were analyzed as they reached confluency (Fig. 3a). In quiescent cells synchronized by serum starvation for 24 h, only Sam68 expression was observed. Quiescence and cell cycle progression of the cells were checked by FACS analysis, which showed that BrdUrd incorporation was detected 12 h after serum stimulation, indicating the progression of the cells in phase S. 24 h after serum stimulation, both Sam68 and Sam68ΔKH were detected. In cells stimulated by serum for 36 and 48 h, which became confluent, no BrdUrd incorporation was observed, indicating the quiescence of the cells. This correlated with a marked increased in the expression of Sam68ΔKH. These data indicate that Sam68ΔKH is expressed at confluency in NIH3T3 cells, suggesting that this protein could be recruited upon growth arrest. Oncogenic transformation bypass this growth arrest upon confluency in fibroblasts. We performed the similar set of experiments on NIH3T3 cells transformed by c-Src (Y527F) (Fig. 3b). Under the same conditions of culture, the cells continued to incorporate BrdUrd and Sam68ΔKH was not detected. These data indicate that Sam68ΔKH is observed upon confluency only in cells that enter quiescence in these cell culture conditions.

We next addressed whether or not Sam68ΔKH was able per se to inhibit cell proliferation and specifically S phase entry in response to serum stimulation (14). Transfection of plasmid encoding myc-tagged Sam68 did not modify the BrdUrd incorporation of quiescent NIH3T3 cells stimulated by serum and transfected with empty vector (Fig. 4a). In sharp contrast, expression of myc-tagged Sam68ΔKH led to more than 50% inhibition of BrdUrd incorporation. A cotransfection of Sam68 expression vector plasmid was able to rescue the inhibition induced by Sam68ΔKH. The fact that Sam68 had no effect per se in the absence of exogenous Sam68ΔKH suggest that Sam68 is not rate-limiting in NIH3T3 cells. In primary rat embryo fibroblasts, however, exogenous transfected Sam68 increased by 30% the BrdUrd incorporation. The same transfection experiments were realized in exponentially growing NIH3T3 cells and also indicated that Sam68ΔKH inhibited BrdUrd incorporation and that Sam68 rescued this inhibition induced by its isoform (Fig. 4b), the expression of the recombinant Sam68 and Sam68ΔKH being controlled by Western blot analysis using the anti-myc 9E10 antibody (Fig. 4c). Because cyclin D1 is rate-limiting for G1 to S phase entry in fibroblasts (15, 16), we
performed Western blot analysis on the exponentially growing NIH3T3 cells using an anti-cyclin D1 monoclonal antibody in order to further characterize the antagonistic impact of the transfected Sam68 and Sam68ΔKH. Our data indicate that the inhibition induced by Sam68ΔKH and the rescue by Sam68 are respectively correlated with a down-regulation and a recovery of cyclin D1 expression (Fig. 4c).

These results are the first data that document a function for Sam68 and support the hypothesis that Sam68 participates in the control of cell proliferation by promoting the S phase entry. This function of Sam68 appears to involve its KH domain and probably its RNA binding properties. It has been shown that these RNA binding properties are impaired when Sam68 is phosphorylated (9). Because Sam68 is tyrosine-phosphorylated and Src-associated only in mitosis, it is likely that Sam68 impact on G1/S transition does not rely directly on its association with Src. However, because Src is involved both in G1 (17–19) and in G2 (20) and is also activated in mitosis (21, 22), we cannot exclude that Sam68 functions in cell cycle progression depend, directly or indirectly, on Src activity. Our results suggest also that two isoforms of Sam68 compete for common partners to regulate cell growth, although we cannot exclude that they have also specific protein or nucleic targets. Future experiments will try to identify the RNA targets of Sam68 that seem critical for S phase entry and will lead toward the clarification of the role of Sam68 in Src signaling. Sam68ΔKH will prove useful to study Src pathways and cellular differentiation, a paradigm of cell arrest.

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