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Disruption of ShcA signaling halts cell proliferation — characterization of ShcC residues that influence signaling pathways using yeast

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Abstract

Shc adapter proteins are thought to regulate cellular proliferation, differentiation and apoptosis by activating the SOS-Grb2-RAS-MAPK signaling cascade. Using the small hairpin RNA (shRNA) technique, we found that decreasing ShcA mRNA reduced the proliferative ability of HEK293 mammalian culture cells. We then recapitulated phosphorylation-dependent Shc-Grb2 complex formation in *Saccharomyces cerevisiae*. Immunoprecipitation followed by Western analysis demonstrated that activated TrkB, composed of the intracellular domain of TrkB fused to glutathione S-transferase (GST-$TrkB_{ICD}$), promoted the association of ShcC and Grb2 in yeast. The Ras-recruitment system (RRS), in which a myristoylated (Myr)-bait and son of sevenless (hSOS)-prey are brought together to complement the defective Ras-cAMP pathway in a thermosensitive cdc25H mutant yeast strain, was used to validate a phenotypic assay. Yeast cells transformed with both Myr-ShcC and hSOS-Grb2 (referred to as scheme 1) or Myr-Grb2 and hSOS-ShcC (scheme 2) did not grow at non-permissive temperature; the additional transformation of GST-$TrkB_{ICD}$ enabled growth. GST-$TrkB_{ICD}$ also enabled growth with hSOS-Grb2 and either Myr-ShcA or Myr-SHP2. Mutational analysis of TrkB showed that its kinase activity was essential for complementation, while its docking site for Shc proteins was not. Mutational analysis of ShcC showed that the PTB and SH2 domains were not essential for complementation but phosphorylation at Y304 in the CH1 domain was. Phosphorylation at Y304 could not be substituted by an acidic amino acid. The RRS provides a genetic system to probe Shc proteins and potentially identify member specific protein partners and pharmacological reagents.

Keywords: Trk; Shc; Grb2; shRNA; Knockdown; Ras; MAPK; Yeast

1. Introduction

The Shc adapter protein links agonist-bound receptor protein tyrosine kinases (RPTKs) to cytoplasmic signal transduction pathways, of which the best characterized is the Grb2-SOS-Ras-MAPK pathway. Evidence indicates that Shc regulates the duration of the MAPK pathway activation and thereby determines whether a cell undergoes proliferation, differentiation, apoptosis or senescence [1]. Despite much research, the exact mechanisms by which Shc acts are unclear. Progress is hampered because all model mammalian systems express multiple Shc protein members that lead to the activation of multiple signal transduction pathways. Yeast cells, which lack Shc and have been successfully used for the functional expression of recombinant mammalian proteins, may provide a means to delineate the mechanisms by which Shc influences cellular signaling pathways [2].

Shc may enable diverse biological responses by at least three possible mechanisms. (1) Shc is a family of proteins. Seven isoforms (ShcA, p46, p52, p66; ShcB p47, p52; and ShcC, p55, p69) are generated from three genes by alternative initiation usage and splicing patterns. (2) The Shc proteins are differentially expressed. ShcA is expressed in peripheral tissue, in the developing brain, and only marginally in the adult brain; ShcB is expressed peripherally

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and in the adult brain; and ShcC is expressed exclusively in the adult brain [1]. An abrupt transition from ShcA to ShcC expression is reported to drive the survival and differentiation of stem progenitor cells to neuronal cells during neurogenesis, a process considered to occur throughout adulthood [3]. (3) The Shc proteins show considerable diversity. Shc, characterized by a (CH2)-PTB-CH1-SH2 domain structure, differs from other docking proteins by two properties: the placement of a PTB (phosphotyrosine-binding) domain before a SH2 (Src-homology) domain is found only in Shc proteins [4] and CH (collagen-homology) domains have been identified only in Shc proteins. The CH1 domains, which are the least conserved among each other (40%) as compared to the PTB and SH2 domains (70%), contain the Grb2-binding motifs. Interestingly, the Y+3 position in a Grb2-binding motif, which is considered essential for Ras-MAPK activation, differs in ShcA (Y^{317}VNV) as compared to ShcB and ShcC (Y^{304}VNT). A CH2 domain is found at the N-terminal of some Shc isoforms. Such differences, whether alone or combined, may contribute to differences in affinities and kinetics of association with the same effectors, such as RPTK or Grb2, and result in differential activation of the same signal transduction pathway [1]. For example, transient or persistent activation of the MAPK pathway has been correlated with cellular proliferation or differentiation, respectively [1,5,6].

The domains of Shc have specific functions in bridging the agonist-bound RPTK to Grb2. The N-terminal PTB domain binds at the Shc-binding site on tyrosine phosphorylated RPTK. As a result, RPTK can tyrosine-phosphorylate Shc's CH1 domain on three tyrosines (referred to as Y239, Y240 and Y317 by their position in $p46^{ShcA}$) and facilitate binding of Grb2. A role for the C-terminal SH2 in the Grb2-SOS-RAS-MAPK pathway is unassigned [7,8]. In mammalian cells, SOS, the guanine-nucleotide exchange factor for Ras, is constitutively associated with Grb2 in the cytoplasm. RPTK-bound Shc recruits the Grb2-SOS complex enabling guanine-nucleotide turnover on Ras and activation of the MAPK pathway.

Homologues of Shc proteins are present in all metazoan organisms analyzed to date, from nematodes to humans and have evolved in two directions. First, the family has expanded from one gene in *Drosophila* to three in mammals. Second, the family has evolved to acquired new biological processes, such as the ability to activate Ras-MAPK pathway [4]. For example, *Drosophila* deleted of Shc shows defects in a subset of RTPKs, e.g., EGF-receptor but not sevenless, which activate Ras [9]. Alternatively, in mammalian cells, some RTPKs, such as EGF, can bind Grb2 either directly or through Shc leading to Ras-MAPK activation [5].

The absence of Shc in yeast suggests that yeast may serve as a model heterologous system in which to analyze Shc. An interaction between Grb2 and the protein tyrosine phosphatase SHP2 was detected in the classical yeast-transcription based two-hybrid system [10]. A limitation of this two-hybrid system is that the interaction occurs in the nucleus rather than at the normal milieu of Grb2, the inner leaflet of the plasma membrane. The yeast Ras-recruitment system (RRS) is different in that it complements the loss of the Ras function with a hybrid that is plasma membrane-localized in an interaction-dependent manner. Proper interaction is required for growth under selective conditions; permissive conditions are also possible that allow easier construction and manipulation of strains, as well as experimental controls. We wanted to adapt the RRS system to elucidate the association between Shc and Grb2.

In this report, the role of Shc proteins in cell division was investigated using small hairpin RNA (shRNA) technique to knockdown (KD) Shc expression in HEK293 cultured cells. Our results indicate that ShcA is essential for serum-mediated proliferation. We reconstituted phosphotyrosine-dependent interaction between Grb2 and Shc in yeast cells. To obtain a phenotypic assay, we used the RRS approach with Grb2 and its partners, ShcA, ShcC and SHP2. Our results suggest that yeast cells could be used to characterize residues, domains, and mechanisms by which TrkB, ShcC and Grb2 interact in mammalian cells. Insights from using the RRS approach may provide tools with which to unravel the more complex processes that occur in mammalian cells. Determining the mechanisms by which Shc links RPTK to its downstream effectors to regulate cellular plasticity/morphology may help in understanding the etiologies of various diseases, as well as, for developing pharmacotherapy for medical intervention.

2. Materials and methods

2.1. ShcA shRNA plasmid construction

A 21 nucleotide sequence complementary to human ShcA mRNA was selected for shRNA construction according to the published guidelines [11,12]. They include: h558 (sense GGTCACCAGGGAGGCCATCAG), h660 (sense GGAGGAGTAACCTGAAATTTG), h780 (sense CCACATGCAATCTATCTCATT) and h840 (sense GACCCTGTGAATCAGAGAGCC), and h660* (GGAGGAGTAACCTGAA**G**TTTG), which harbors a mutation indicated in bold. A pair of complementary 51 oligonucleotide sequences containing an inverted pair of 21 nucleotide sequence RNAi intervened by 6 nucleotide sequence spacer were annealed and ligated into pSilencer 2.0 following the manufacturer's recommendations (Ambion). A green fluorescence protein (GFP) expression cassette consisting of CMV/b-actin promoter and EGFP was subcloned downstream of the multiple cloning site to facilitate identification of transfected cells in vitro. Following sequence verification, the plasmid pSilencer-caggsGFP was transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen).