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Requirement of an additional Sam68 domain for inhibition of human immunodeficiency virus type 1 replication by Sam68 dominant negative mutants lacking the nuclear localization signal

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Abstract

Human immunodeficiency virus type 1 (HIV-1) replication requires active nuclear export of unspliced and incompletely spliced HIV-1 RNA transcripts. This process is evolutionally made possible by expression of HIV-1 Rev, one of the three HIV-1 proteins encoded by completely spliced HIV-1 RNAs. Evidence has accumulated to suggest that Sam68 plays an important role in HIV-1 replication through HIV-1 Rev protein. In the present study, we further examined the structure–function relationship of Sam68 protein in relation to HIV-1 replication. We identified a Sam68 domain located between aa269 and aa321 to be involved in the HIV-inhibitory effects of Sam68 dominant negative mutants lacking the nuclear localization signal (NLS). Deletion of this domain abrogated inhibition of HIV-1 replication by these mutants. HIV-1 Rev protein appeared to mediate the HIV-inhibitory effects of these mutants and by this domain, as assessed by Rev-dependent chloramphenicol acetyltransferase reporter gene assay, in trans rev-defective HIV-1 complementation assay, and RNase protection assay. The HIV-inhibitory mutants containing this domain were further found to have diminished binding affinity to the wild-type Sam68 and to be associated with cytoplasmic retention of exclusively nuclear localized wild type Sam68. Taken together, these results further ascertain the important role of Sam68 in HIV-1 Rev function and viral replication, and suggest that the HIV-inhibitory effects of Sam68 dominant negative mutants directly result from their binding to endogenous Sam68 and their interference with nuclear localization of endogenous Sam68.

1. Introduction

Sam68 was initially isolated as a p120-RasGTPase-activating protein (Ras-GTP)-binding protein, or p62 (Ellis et al., 1990). The protein was later identified to be a Src-associated protein in mitosis with a molecular mass of 68 kilodaltons (kDa), and thus renamed as Sam68 (Fumagalli et al., 1994; Taylor and Shalloway, 1994). Structurally, Sam68 contains the heteronuclear ribonucleoprotein particle K homology (KH) domain, proline-rich domains (P_0 to P_5), a tyrosine-rich domain, and a nuclear localization signal (NLS) (Fig. 1A). Several biological functions have so far been attributed to Sam68. These include cell cycle regulation, intracellular signal transduction,

Abbreviations: Sam68, Src-associated protein in mitosis; HIV-1, human immunodeficiency virus type 1; NLS, nuclear localization signal; CRM1, chromosome region maintenance 1; GFP, green fluorescence protein; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldyhyde-3-phosphate dehydrogenase; DAPI, 4′, 6′-diamidino-2-phenylindole; Rev, to regulate expression of the virion; RRE, Rev-responsive element; β-Gal, β-galactosidase.

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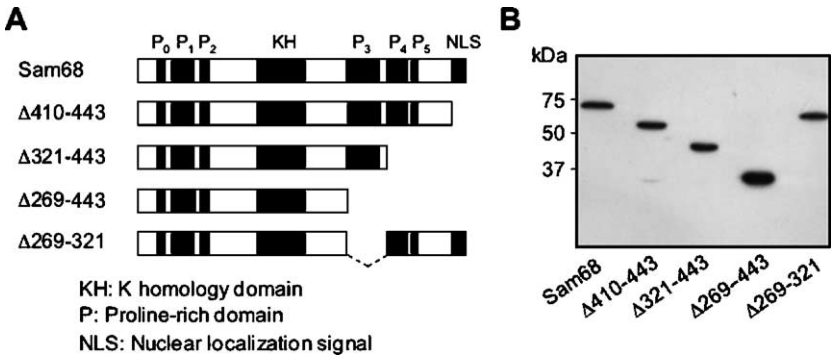

Fig. 1. Sam68 and its mutants. (A) Diagram of wild-type Sam68 and its mutants. HA was tagged at the N terminus of wild-type Sam68 and all mutants were constructed in the context of the wild-type Sam68 plasmid. Functional domains were marked as shown. (B) Expression of Sam68 and its mutants. Plasmid DNAs were transfected in 293T cells and protein expression was determined by Western blot analysis using an anti-HA monoclonal antibody. There was no non-specific reactivity of the anti-HA antibody.

tumorigenesis, and RNA metabolism [for a review, see Ref. (Lukong and Richard, 2003)].

Sam68 was first demonstrated to be capable of substituting for and synergizing with HIV-1 Rev protein in 1999 (Reddy et al., 1999). Using the subtractive cDNA cloning strategy, we have independently identified Sam68 to be responsible for the Rev functional defect in HIV-1 replication-restricted astrocytes (Li et al., 2002b). Since then, more evidence has accumulated to support the important function of Sam68 in HIV-1 Rev-mediated nuclear export of HIV-1 RNA transcripts. However, much remains inconclusive or controversial. One major disagreement is whether Sam68 shares the same nuclear export function as HIV-1 Rev and synergizes with HIV-1 Rev. The early study has shown that Sam68 is able to substitute for and synergizes with HIV-1 Rev in HIV-1 replication (Reddy et al., 1999). However, other studies including ours have failed to show the ability of Sam68 to function in place of HIV-1 Rev in nuclear export of HIV-1 RNAs and viral replication (Soros et al., 2001; Li et al., 2002b). Moreover, we have shown that the synergy between Sam68 and HIV-1 Rev only occurs in cells such as human astrocytes, in which a low level of constitutive Sam68 is expressed (Li et al., 2002a). Accordingly, two competing pathways have been proposed for Sam68 function in nuclear export of HIV-1 viral RNAs: CRM1-independent (Reddy et al., 1999) and CRM1-dependent (Li et al., 2002a). The other major disagreement is how the Sam68 dominant negative mutant lacking the C-terminal domain or the nuclear localization signal inhibits HIV-1 replication. The early study has shown that this mutant impedes Rev nuclear localization (Reddy et al., 1999). But a recent study has shown that this mutant sequesters unspliced and singly spliced RNAs from the translation apparatus in the cytoplasm (Soros et al., 2001). In addition to these disagreements, the molecular mechanisms of Sam68 function in HIV-1 Rev-mediated nuclear export pathway have not fully been elucidated. Thus, the skepticism about the importance of Sam68 function in HIV-1 replication remains. In the present study, we further dissected Sam68 function in HIV-1 replication using a series of Sam68 mutants. These results support the important role of Sam68 in HIV-1 Rev function and viral replication, and suggest that the HIV-inhibitory effects of Sam68 dominant negative mutants are likely a direct result of cytoplasmic retention of endogenous nuclear Sam68 protein.

2. Materials and methods

2.1. Cells, transfection, and plasmids

293T, BOSC23 and Jurkat cells were purchased from the American Tissue Culture Collection (ATCC, Vanassas, VA) and maintained in Dulbecco's modified Eagle's medium (for 293T and BOSC23) or RPMI (for Jurkat) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Cell transfection was performed by the standard calcium phosphate precipitation method. The sources of plasmids used in the studies were: HIV.env$^-$ and HIV.env$^-$.rev$^-$ were from M. Emerman (Lewis et al., 1992), pCMV.Rev and Rev dominant negative mutant M10 from B. Cullen (Malim et al., 1989), pRRE-CAT from T. Hope (Hope et al., 1990), pSV2-CAT from J. Roth (Ristea et al., 2000), and pMX-puro from T. Kitamura (Onishi et al., 1996). pcDNA3 was purchased from Clontech (Chalbard, CA). Sam68, Sam68Δ410–443, Sam68Δ329–443, Sam68Δ269–443, and Sam68.GFP were described elsewhere (Li et al., 2002b). Sam68Δ269–321 deleted the region between aa269 and aa321 was constructed using a PCR-based ExSite™ PCR-Based Site-Directed Mutagenesis Kit, Sam68 as template, and primers 5′-GCC ATC ACC AGA GGT GCC ACT-3′ and 5′-AAA TTG CTC CTG ACA GAT ATC ATC CAT CAT ATC CG-3′. Similarly, wild-type Sam68 and all deletion mutants were constructed in the backbone of pGBT9 expressing the Gal4 DNA binding domain and pGAD424 expressing the Gal4 activation domain, respectively, or in the backbone of pMX-puro using the standard PCR cloning technique. Positive controls pGBT-p53, pGAD424-T, and parental plasmids pGBT9 and pGAD424 were from Clontech (Palo Alto, CA). All recombinant plasmids were verified by double strand DNA sequencing. Cell transfections were performed by the calcium phosphate precipitation method, which usually gives rise to 80% or higher transfection efficiency in 293T and BOSC23 cells. pcDNA3 was used to equalize the amount of DNA transfected among all transfections throughout the studies. pCMVβGal was included to