

Analysis of Epitope-Tagged Forms of the Dyskeratosis Congenita Protein (Dyskerin): Identification of a Nuclear Localization Signal

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ABSTRACT: The X-linked form of the bone marrow failure syndrome Dyskeratosis congenita is caused by mutations in dyskerin, a 514 amino acid protein that is presumed to play a role in ribosome biogenesis. Here we report that dyskerin tagged with the human immunoglobulin epitope localizes to nuclei of transfected HeLa and COS-1 cells. A carboxyl-terminal domain consisting of amino acids 467-475 and encoding KKEKKKSKK is both necessary and sufficient to mediate nuclear entry. Immunoglobulin-tagged dyskerin did not interact with the Fanconi anemia group A protein, FANCA. These results suggest a nuclear role for dyskerin. Moreover, hematopoietic failure observed in both Dyskeratosis congenita and the most common type of Fanconi anemia is unlikely to have a common mechanism resulting from abnormal physical interactions between the respective gene products of these disorders.

Keywords: Dyskeratosis congenita, hematopoietic failure, Fanconi anemia, subcellular localization

INTRODUCTION

Dyskeratosis congenita (DC) is a genetically heterogeneous disorder characterized by bone marrow failure, abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia (1,2). Progressive pancytopenia develops in over 80% of DC patients by age 30 years and is a major cause of morbidity and mortality (3). There is an increased incidence of malignancies (1), and chromosomal instability is occasionally encountered in fibroblasts and bone marrow cells of DC patients (4). Although some of these features are reminiscent of Fanconi anemia FA; ref. (5)), DC lymphocytes do not appear to be sensitive to the clastogenic effects of mitomycin C and diepoxybutane (6). In addition, the most common subtype of DC is transmitted as an X-linked recessive trait (MIM 305000), while FA is autosomal recessive. The DKC1 gene mutated in the X-linked subtype is localized to the distal end of Xq28 and has recently been isolated by positional cloning (7). The spectrum of mutations identified to date include partial gene deletion, putative splice site mutation, and a variety of missense mutations (7,8). The protein product of DKC1, called dyskerin, is a highly basic 514-

amino-acid polypeptide that has homologues in many other species. By analogy with the functions of the homologues, postulated roles for dyskerin include involvement in centromere function (9,10), nucleocytoplasmic trafficking (11,12), rRNA transcription (13), stability of RNA particles (14), ribosome biosynthesis (15), and RNA pseudouridylation (14,16). To begin to address the function of dyskerin, we prepared an epitope-tagged form of dyskerin and studied its localization in transfected mammalian cells.

MATERIALS AND METHODS

Cloning of DKC1-Immunoglobulin

The pBS-DKC construct consisting of the full-length human DKC1 cDNA was a generous gift of Dr. Nina Heiss and Dr. Annemarie Poustka (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (ref. (7)). Using this vector as a template, we used polymerase chain reaction (PCR) amplification to generate epitope-tagged expression constructs in pcDNA3 (Invitrogen, Carlsbad, CA). The full-length DKC1 cDNA was modified to encode a Kozak consensus sequence

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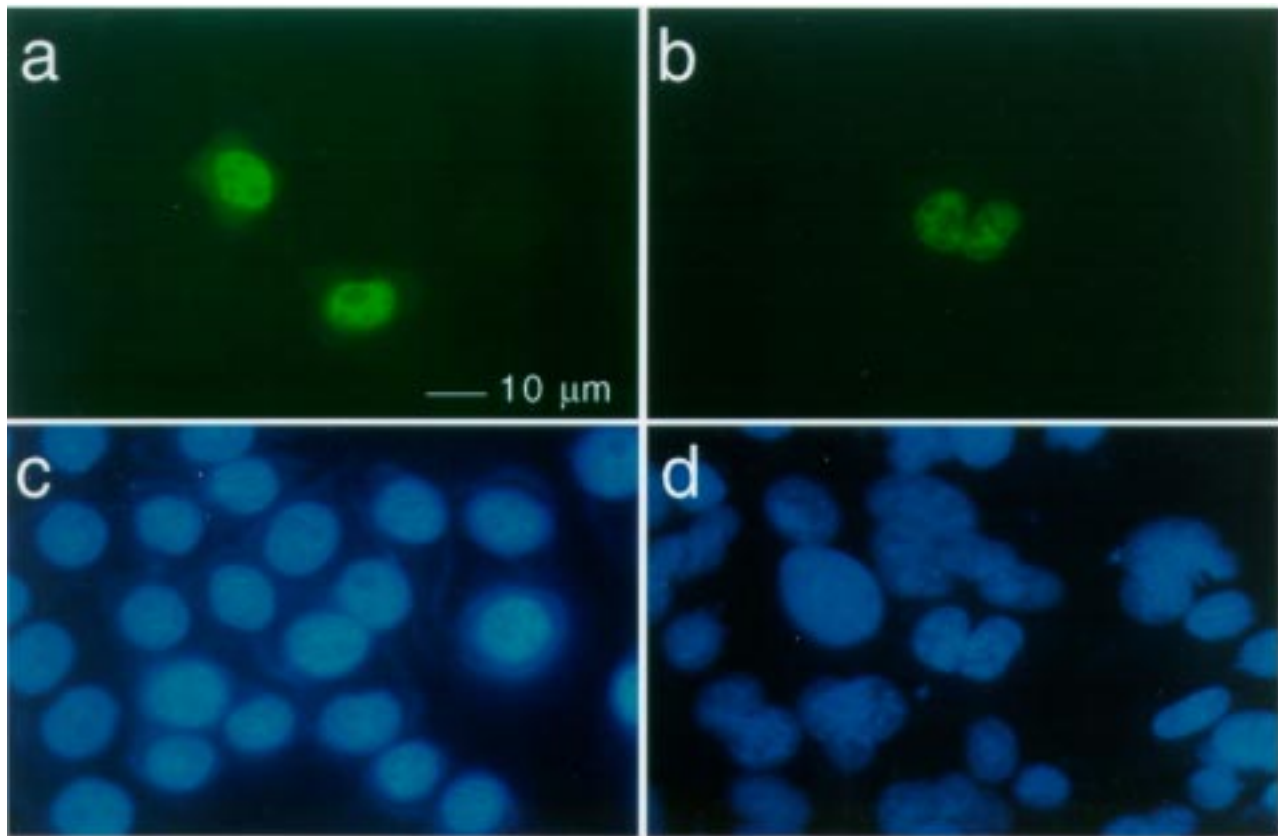


Figure 1. Nuclear localization of DKC-Ig. Transfected HeLa (a,c) and COS-1 cells (d,b) grown on coverslips were visualized with FITC-conjugated anti-human IgG (a,c), and nuclei were stained with bisbenzimidide (c,d).

at the 5' end and a mutated stop codon at the 3' end, which facilitated subcloning to the downstream human immunoglobulin IgG1 epitope to yield DKC-Ig. A unique BglIII site was introduced at the stop codon to encode an Asp residue, which was ligated to a BamHI site at the 5' end of IgG1, as described previously (17). Deletion mutants were also derived by PCR. All PCR-derived products were sequenced to exclude the introduction of spurious mutations. The FANCC-Ig fusion protein (17) and the pcDNA3-FANCA constructs (18) have been described previously.

Cell Culture and Transfection

HeLa and COS-1 cells were grown in Dulbecco's Minimal Essential Medium (DMEM; GIBCO-BRL, Grand Island, NY) with 10% FCS.

COS-1 cells were transfected using the DEAE-dextran, and HeLa cells were transfected using Superfect (QIAGEN, Valencia, CA), as described (18).

Immunofluorescence Microscopy

Transfected cells were plated on glass coverslips after 24 h and processed for immunofluorescence microscopy after 48 h, as before (18). Fluorescein-conjugated goat-anti-human IgG (Cappel Laboratories, West Chester, PA) was used for detection of the fusion protein. Nuclei were visualized by staining with bisbenzimidide (Sigma Chemical Co., St. Louis, MO). Experiments were performed in triplicate. Fifty transfected cells from each coverslip were scored as nuclear, cytoplasmic, nuclear and

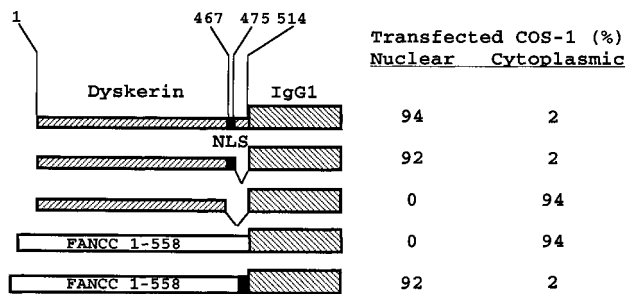


Figure 2. Schematic representation of dyskerin constructs and localization. Deletion mutants of dyskerin fused to human IgG1 and their localization after transfection in COS-1 cells are shown. The percentage of transfected cells having exclusively nuclear and exclusively cytoplasmic patterns are shown. The remainder of the transfected cells were either indeterminate or showed localization in both the nucleus and cytoplasm. The positions of dyskerin amino acid residues are shown on top. Constructs (from top to bottom) are: DKC-Ig; DKC 1-475-Ig; DKC 1-466-Ig; FANCC-Ig; FANCC 1-558/DKC 467-475-Ig.

cytoplasmic or indeterminate and the percentage of the mean recorded.

Protein Interactions

In vitro translated 35S-methionine-labeled proteins were generated using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI). Proteins were allowed to form complexes in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40 supplemented with protease and phosphatase inhibitors. After immunoprecipitation with protein A-agarose (Affi-Gel; Bio-Rad, Hercules, CA), samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

RESULTS AND DISCUSSION

Subcellular Localization

Despite the cloning of the DKC1 gene and the observation that the sequence of dyskerin is highly conserved in evolution, the molecular function of dyskerin and the pathogenesis of DC remain obscure. Here we examined the

subcellular localization of dyskerin in mammalian cells by epitope tagging and immunofluorescence microscopy. In both HeLa and COS-1 cells transiently expressing DKC-Ig, the large majority of cells (over 90%) showed an exclusively nuclear pattern (Figure 1). No clear nucleolar localization was noted; the latter may be an artifact of overexpression. However, we cannot rule out the possibility that nucleolar localization was specifically deficient in these transfected cells or perhaps was obliterated by the overexpression strategy. This is somewhat unlikely as we have previously reported nucleolar localization for other transfected gene products, such as the Werner syndrome (WS) protein (19). Experiments to generate specific antibodies to dyskerin and analyze the endogenous expression of the protein are in progress. As virtually no cytoplasmic localization was observed, we believe that dyskerin has a nuclear function and certain putative cytoplasmic functions (e.g., involvement in nuclear-cytoplasmic transport or ribosome assembly) are less likely.

Carboxy-Terminal Nuclear Localization Signal

Using PROSITE for searches of functional motifs, two predicted nuclear localization signals (NLS) at the amino (KKHKKK) and carboxyl terminal (KRKR) regions of dyskerin were found to be conserved in the rat sequence (7). We generated a panel of deletion mutants to analyze the activity of these putative NLSs (Figure 2). While deletion of residues 476-514 did not disturb nuclear localization, deletion of residues 467-514 completely abolished nuclear localization. We also appended the sequence KKEKKKSKK (residues 467-475) (ref. (7)) to the 3' end of the FA group C protein FANCC) fused to IgG1 (17). This protein has previously been demonstrated to have a predominantly cytoplasmic localization (17). However, transfection of COS-1 cells with the chimeric FANCC-dyskerin (residues 467-475) construct showed a homogeneous nuclear pattern for the

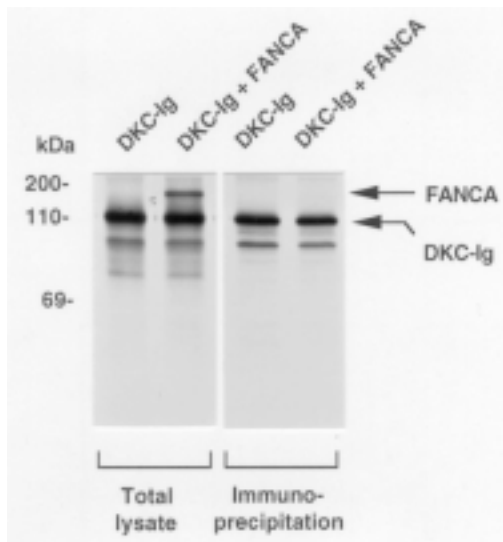


Figure 3. *In vitro* interaction analysis. DKC-Ig and FANCA were translated *in vitro* in the presence of ³⁵S-Methionine, incubated to allow complex formation, precipitated with protein A-agarose, and analyzed by SDS-PAGE and autoradiography. Transfected constructs are shown at the top. For immunoprecipitation experiments, three- to fivefold higher amounts than those depicted in the total lysate lanes were used.

fusion protein in >90% of the transfected cells. These results reveal the presence of a NLS at the carboxy terminal region of dyskerin that is both necessary and sufficient to mediate nuclear entry.

Dyskerin Protein Interaction

The FA complementation A gene product, FANCA, is predominantly nuclear, and mutations in this gene account for the majority of the cases of FA (18). Because both Fa and DC are associated with bone marrow failure, there may be one or more shared pathogenetic defects between these disorders, including faulty protein-protein interactions between their respective gene products. One postulated function for FANCA is that it forms a scaffold for other proteins. Thus, FANCA is capable of interacting with FANCG, the FA group G gene product (20-22), and perhaps with other proteins as well. We evaluated the possibility that DKC-Ig interacts with FANCA. After *in vitro* translation, the radiolabeled products were allowed to form a

complex and pulled down by single-step immunoprecipitation using protein A-agarose. No complexes were detected between DKC-Ig and FANCA (Figure 3). Under the same binding conditions, FANCA readily forms complexes with FANCG (21,22; data not shown). The Ig epitope is also unlikely to interfere with binding, as demonstrated previously (17). These results suggest that failure of protein interactions between dyskerin and FANCA is unlikely to account for the phenotypic similarities between DC and FA.

In summary, to our knowledge this is the first study to establish the subcellular localization of dyskerin in transfected mammalian cells and to identify a functional motif. Carboxy terminal NLSs have been previously noted in other disease-associated gene products, including the WS helicase (22). Such an arrangement would predict that truncated mutants generated by small carboxy-terminal deletions, frameshifts or nonsense mutations proximal to the NLS, will fail to work *in vivo* because of their inability to reach the appropriate subcellular compartment, even if these proteins are otherwise stable or functional *in vitro*. This arrangement may represent a quality control mechanism whereby the nuclear compartment remains free of partially active or potentially toxic protein products.

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