A nucleolar targeting sequence in the Werner syndrome protein resides within residues 949-1092

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Summary
Werner syndrome is a premature aging disorder caused by the lack of an active Werner syndrome protein (WRN). The patients suffer from many of the ailments seen at a much later stage in the life of normal individuals. WRN is a nuclear protein and contains a nuclear localization signal (NLS) in its C-terminal region. Inside the nucleus, WRN is mainly located in the nucleoli and in nuclear foci. To begin to understand the role of WRN in the nucleolus, we determined the specific regions of the protein that are responsible for this localization. We have cloned different WRN gene domains fused to enhanced green fluorescent protein (EGFP), and analyzed their intracellular distribution in living cells using confocal microscopy. The region encompassing amino acids 949-1092 of the human WRN, together with the NLS containing amino acids 1358-1432, provides the targeting to the nucleoli. This targeting is observed in three human and one mouse cell line. The NLS-containing region alone is unable to direct EGFP to the nucleoli. The results demonstrate that the human WRN contains a conserved nucleolar targeting sequence residing in a 144 amino acid region (aa 949-1092) and this provides new tools and insight into the biological function of WRN.

Key words: Werner syndrome, Nucleolar targeting sequence, In vivo localization

Introduction
Mutations in the WRN gene are the cause of the rare autosomal recessive premature aging disease Werner syndrome (WS). The amino acid sequence of WRN indicates that the protein (WRN) belongs to the RecQ DNA helicase family (Yu et al., 1996). Among the family members, three helicases, WRN, Bloom (BLM) and Rothmund Thomson’s (RTS) are associated with heritable human diseases. WRN possesses three known catalytic activities: 3′-5′ helicase, exonuclease and ATPase (Gray et al., 1997; Suzuki et al., 1997; Huang et al., 1998). WRN is a nuclear protein and its nuclear targeting is due to the presence of a nuclear localization signal (NLS) in the C-terminal region of WRN (aa 1370-1375) (Matsumoto et al., 1997) (see scheme in Fig. 1B). All mutations identified in WS patients result in a C-terminal truncated WRN protein, with loss of the NLS (Matsumoto et al., 1997). Thus, the inability of WRN to be transported into the nucleus seems to be crucial for the pathogenesis of WS. Once in the nucleus, WRN localizes to the nucleoli and to nuclear foci (Yankiwski et al., 2000; Gray et al., 1998; Marciniak et al., 1998; Sakamoto et al., 2001). While there has been great interest in the intranuclear localization and trafficking of WRN, factors directing this traffic have not yet been identified.

The nuclear import and export of macromolecules depends on energy and receptor-associated processes (Izaurralde et al., 1997). Molecules below the exclusion limit size (~50 kDa) can cross the nuclear pore complexes by simple diffusion. However, in the case of large macromolecules, consensus sequence elements are required for traffic in and out of the nucleus (nuclear export signal and NLS, respectively) (for a review, see Gorlich and Kutay, 1999). The nucleolus has no known physical barrier separating it from the nucleoplasm, and in principle, any soluble protein should be able to diffuse in and out of the nucleoli. However, it has become clear that many proteins contain sequences that are required for the entry into the nucleolar region, but the biological functions of these regions are not yet understood. Perhaps the nucleolar targeting of proteins is related to direct or indirect interaction with the ribosomal DNA (rDNA) or rRNA (Carmo-Fonseca et al., 2000).

To investigate the regulation of the nucleolar localization of WRN we have used a battery of different WRN regions as EGFP fusion proteins and analyzed their intracellular distribution in living cells by confocal microscopy. Here, we demonstrate the existence of a nuclear targeting sequence (NTS) in the WRN region containing amino acids 949-1092. As expected for a conserved intranuclear targeting sequence, we can demonstrate that this NTS is functional in the different human cell lines that we have tested as well as in a mouse cell line. We also show that the nucleolar localization of this domain requires the WRN NLS, although the presence of the NLS alone does not provide the nucleolar targeting of WRN.

Materials and Methods
Cloning of the EGFP-WRN constructs
To generate EGFP-WRN (aa 1-1432) full length construct, the PCR-amplified WRN from the human WRN gene, was subcloned into the XhoI-XmaI sites of pEGFP-C3 vector (Clontech). The WRN fragment containing the NLS (aa 1358-1432) was generated by PCR using the

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Transfections, localization in living cells and immunofluorescence assays

The U-2 OS and B16F10 cells were from ATCC, and the AG11395 cells were from Coriell. Cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies) and 10% FBS. For in vivo localization assays, the cells were grown in glass-bottom microwell dishes (MatTek) and transfected with the corresponding vectors using the Calphos mammalian transfection kit (Clontech). After 15 hours, dishes (80-90% confluent) were collected. The pellets were resuspended in 200 μl/pellet of SDS sample buffer, boiled and analyzed (~8% of the sample) by SDS-PAGE followed by western blot with anti-GFP monoclonal antibody (Clontech).

Western blot analysis for protein expression levels of the different GFP-WRN fragments

Approximately 15 hours after transfection, cells growing on 10 cm dishes (80-90% confluent) were collected. The pellets were resuspended in 200 μl/pellet of SDS sample buffer, boiled and analyzed (~8% of the sample) by SDS-PAGE followed by western blot with anti-GFP monoclonal antibody (Clontech).

Results

A nucleolar targeting sequence (NTS) of WRN in HeLa cells

To characterize the intranuclear localization of the WRN, we cloned several WRN domains fused to a GFP tag. These are shown in Fig. 1A (EGFP I-IX). The EGFP system allowed us to follow the intracellular localization of the specifically labeled proteins in living cells. It is known that some fixation protocols may alter the localization of proteins, thus leading to misleading conclusions in the analysis of the intracellular distribution of a specific protein. To avoid this problem, we decided to use the confocal microscopy technique with EGFP-transfected living cells.

After transfection in HeLa cells, EGFP-full length WRN (EGFP-WRN) (Fig. 1A, EGFP I) exhibited two main localization patterns: nucleolar-nuclear diffuse (60-80% of the analyzed cells) (Fig. 2Aa-b) and nuclear foci-nuclear diffuse staining (40-20%) (Fig. 2Ac-d). When the exonuclease and helicase domains together (aa 54-946 +NLS) (Fig. 1A, EGFP VII) were targeted to the nucleus, this construct showed (100% of the cells) a nuclear diffuse staining and interestingly, total nucleolar exclusion (Fig. 2Ae-h). This pattern was also seen when the exonuclease domain alone was targeted to the nucleus with the NLS sequence (data not shown). The C-terminal region of WRN (aa 949-1432) (Fig. 2Ai-l, Fig. 1A, EGFP II), exhibited nuclear diffuse staining and clear nucleolar staining. This result demonstrates that the C-terminal region of WRN contains a NTS. To further map the NTS region within the C-terminal domain, we constructed various deletion mutants in this region. Fig. 2B shows the localization pattern of three representative mutants. An EGFP protein fused to a region of WRN containing the NLS (aa 1358-1432) (Fig. 1A, EGFP VI), consistently showed a clear nuclear diffuse staining (Fig. 2Ba-
A nucleolar targeting sequence of WRN

The same nuclear diffuse pattern was seen when the cells were transfected with a longer C-terminal region of WRN that also included the NLS (aa 1072-1432) (Fig. 2Be-h; Fig. 1A, EGFP III). However, a WRN region encompassing amino acids 949-1092, when fused to the NLS (Fig. 1A, EGFP IV), showed clear nucleolar staining (100% of the analyzed cells) (Fig. 2Bi-l). This nucleolar signal was demonstrated by colocalization analysis, using a specific antibody recognizing a nucleolar protein (B23) in the EGFP-WRN (949-1092+NLS)-transfected cells (Fig. 2C). We found 100% colocalization of the EGFP-WRN construct with B23, demonstrating that this WRN construct localized specifically to the nucleoli.

To confirm that the EGFP-WRN fragments used for the mapping of WRN NTS were expressed and were of the expected size, we performed western blot analysis (Fig. 2D). The expression levels of all the fusion proteins were almost identical, except for EGFP-WRN (54-946+NLS), which was expressed at a lower level (Fig. 2D, lane 8).

The NTS-containing WRN region, when not targeted to the nucleus (without the NLS) (EGFP-WRN 949-1092; Fig. 1A, EGFP V) showed cytoplasmic and nuclear diffuse staining, resembling the localization of EGFP alone (Fig. 3Aa-d). This construct (EGFP-WRN 949-1092) is small enough (<50 kDa) to cross the nuclear pore complex by simple diffusion. However, once inside the nucleus, this WRN fragment was not targeted to the nucleoli, indicating that this sequence (aa 949-1092) does not contain nucleolar accumulation properties. Thus, the nucleolar staining of EGFP-WRN (949-1092+NLS)
is an active process, coupled to the nuclear import machinery (WRN-NLS-dependent process). The exonuclease and helicase domains together (aa 54-946) as an EGFP fusion protein without the NLS (Fig. 1A, EGFP VIII), localized completely to the cytoplasm (Fig. 3e-f), which was also expected based on its size (>50 kDa).

The fragments EGFP V and VIII were expressed with the expected size, as demonstrated by western blot analysis (Fig. 3B).

The same WRN NTS region localizes to nucleoli in other human cell lines and in mouse cells

We next transfected other cell lines with the WRN constructs to determine whether our observations were specific to HeLa cells or were more general. We tested U-2 OS, a telomerase negative cell line, and an SV40-transformed WS AG11395 cell line, which is telomerase negative and lacks endogenous nuclear WRN. These cell lines were transfected with EGFP-WRN, EGFP-WRN (1072-1432) and EGFP-WRN (949-1092 +NLS) constructs. After transfection we analyzed the intracellular distribution of each construct as before (living cells). As shown in Fig. 4, full length WRN (EGFP-WRN) showed nuclear diffuse and nuclear foci staining (Fig. 4a-d). However, we cannot rule out that some of the nuclear foci are actually within the nucleoli. Importantly, the EGFP-WRN (949-1092 +NLS) construct, as seen in HeLa cells, accumulated in the nucleoli of both cell lines (100% of the cells) (Fig. 4i-l). Similar results were obtained using the SV40-transformed WS AG07066B cell line (data not shown). Thus, the nucleolar targeting of this WRN fragment (aa 949-1092) is independent of the presence of endogenous WRN and of telomerase expression levels.

To investigate the species conservation of the WRN NTS, we examined cells from another species. Mouse B16F10 cells were transfected with either EGFP-WRN, EGFP-WRN (1072-1432) or EGFP-WRN (949-1092 +NLS) constructs. After transfection we analyzed the intracellular distribution of each construct as before (living cells). As shown in Fig. 5, full length WRN (EGFP-WRN) showed nuclear diffuse and nuclear foci staining (Fig. 5a-d). Interestingly, full length WRN also showed a clear nucleolar exclusion pattern. The NLS-containing C-terminal fragment of WRN (aa 1072-1432) was distributed throughout the nucleus (80-90% of the analyzed cells), with some nucleolar exclusion (20-10%) (Fig. 5e-h). In contrast, the NTS-containing region (aa 949-1092 +NLS) was efficiently targeted to the nucleoli in all the analyzed cells (Fig. 5i-l). Thus, this domain of the human WRN protein is as active as an NTS in a mouse cell line.

Nucleolar exclusion pattern of a WRN RQC (RecQ conserved) domain deletion mutant

To further demonstrate that the WRN region comprising amino acids 949-1092 contains a NTS, we generated an EGFP-tagged RQC domain deletion mutant of WRN (EGFP-WRN(D853-1089)) and transfected it into HeLa cells (Fig. 6A,B). After transfection, 80% of the analyzed cells showed nuclear diffuse staining, and more interestingly, total nucleolar exclusion of this construct (100% of the analyzed cells). Thus, the presence of an active NLS in the C-terminal domain of WRN does not alone provide the targeting of the protein to the nucleoli. The nucleolar targeting is dependent on the WRN RQC domain (aa 853-1089), specifically on amino acids 949-1092, as shown before.

Discussion

We show that the WRN region comprising amino acids 949-1092, when fused to the WRN NLS, is targeted to the nucleoli in four mammalian cell lines. This domain contains part of the RQC (RecQ helicase conserved region) domain, a common motif found among the RecQ helicase family members. This region is highly conserved and would be expected to be of biological significance. Its function is not understood, but we have found that this region binds some WRN-interacting proteins including the FEN-1 flap endonuclease (Brosh, Jr et al., 2001). This WRN region (aa 949-1092) also specifically stimulates FEN-1 activity (Brosh, Jr et al., 2001) and thus it
A nucleolar targeting sequence of WRN has biological importance in FEN-1-associated pathways. FEN-1 participates in both DNA replication and DNA base excision repair. We have observed that proteins other than FEN-1 can also bind to this WRN region (C.v.K. and V.A.B.,

Fig. 3. Intracellular localization and expression of EGFP-WRN fragments. (A) HeLa cells were transfected with the vector alone (EGFP) (a,b), EGFP-WRN (949-1092) (c,d) and EGFP-WRN (54-946) (e,f) constructs, and analyzed as described in Fig. 1A,B. One representative picture of each construct is shown. (B) Protein expression of EGFP-WRN constructs shown in panel A. After transfection the cells were analyzed as described in Fig. 2D.

Fig. 4. Intranuclear localization of EGFP-WRN fragments in different human cell lines. U-2 OS (left) and AG11395 cells (right) were transfected with EGFP-WRN (a-d), EGFP-WRN (1072-1432) (e-h) and EGFP-WRN (949-1092 +NLS) (i-l) constructs as indicated on the left. After transfection the localization of each construct was analyzed by confocal microscopy.
X. Author et al., unpublished). These include some proteins that are known to be associated with the nucleolus (C.v.K. and V.A.B., unpublished) and provide biochemical support to the observations reported here.

The human WRN sequence contains a NTS (aa 949-1092) that directs the protein to the nucleoli in a number of human cells and that is recognized by the nucleolar targeting machinery in rodent cells. However, when this sequence is expressed in the context of the full length protein (EGFP-WRN), it is unable to target the protein to the nucleolus in the mouse cell line we studied. This situation resembles the differences in the WRN localization that we observed between the human cell lines. The full length protein (EGFP-WRN) showed predominantly a nucleolar diffuse staining in HeLa cells, whereas in ALT cells (U-2 OS and AG11395 cell lines) it was principally in nuclear foci (compare Fig. 2Aa-d with Fig. 4a-d). In contrast, the EGFP-WRN (949-1092 +NLS) construct was efficiently targeted to the nucleoli in all cell lines tested. In agreement with these data, a WRN RQC deletion mutant (containing an active NLS) showed a nuclear diffuse and nucleolar exclusion pattern (Fig. 6A,B). This result clearly demonstrates that the RQC domain of WRN contains a NTS. The NLS also needs to be present for the protein to reach the nucleolus, but the NLS alone will not target WRN to the nucleolus (Fig. 2B).

Conformational changes (post-translational modifications) may render some regions of WRN more accessible and this may be a determining factor in targeting of WRN to different intranuclear structures or may affect the binding of WRN to interacting proteins. Support for this hypothesis is found in a previous study (Gray et al., 1998), which suggests that tyrosine phosphorylation could modulate the intranuclear trafficking of WRN by either direct (WRN...
phosphorylation) or indirect (phosphorylation of a WRN interacting protein) modification.

Basic components of the intracellular protein trafficking machinery are conserved among different species (i.e. NLS and nuclear export signals) (Dimaano et al., 2001; Schmitt et al., 1999; Jans et al., 2000). Thus, specific intracellular targeting sequences would be expected to function in different species and cell lines, as is the case of the WRN NTS presented here.

Our studies were conducted in living cells. A previous study (Suzuki et al., 2001) was performed with fixed cells and reported that two amino acids (1403-1404) in the C-terminal region of WRN contains the NTS, but no other WRN regions were analyzed. A high concentration of formaldehyde (10%) was used in the fixation protocol in that study (Suzuki et al., 2001). In our hands, the fixation protocol greatly affects the nucleolar localization of the proteins, and we therefore argue that it is of importance to perform this type of analysis in live cells.

The role of WRN in the nucleoli is unclear. WRN may participate in nucleolar processes such as transcription or it may be located there for temporal storage (sequestration), a phenomenon that has been observed for other proteins (Visintin and Amon, 2000). This sequestration could prevent proteins from reaching their targets in other cellular compartments. We have previously shown that WRN is involved in RNA-polymerase-II-directed transcription, but not in RNA polymerase I transcription in vitro (Balajee et al., 1999). While the role of WRN in the nucleoli may not involve rDNA transcription, WRN may function in other rDNA/rRNA metabolic processes.

It has been suggested previously that the nucleolar localization of proteins depends upon the presence of an active NLS (Creancier et al., 1993), thus linking the processes of nuclear import and nucleolar targeting; our results support this notion. It is likely that eukaryotic cells have evolved a mechanism that discriminates between simple nonspecific diffusion and active targeting of proteins to different subnuclear structures.

In summary, using four different cell lines and a battery of different EGFP-WRN fragments, we have demonstrated that amino acids 949-1092 of WRN contain an NTS that is responsible for the targeting and also requires the presence of a NLS. Supporting this data, we also showed that a WRN RQC deletion mutant (lacking amino acids 853-1089) exhibited a complete nucleolar exclusion pattern. The experiments were carried out in living cells. The 949-1092 WRN region not only targets the WRN protein to the nucleolus, but binds (and modulates) some of the WRN-interacting proteins, playing a central role(s) in the regulation and intracellular trafficking of WRN. It is possible that one of the protein binders to this WRN region is specifically involved in the transport to the nucleoli.

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