Karyopherin-α2 Protein Interacts with Chk2 and Contributes to Its Nuclear Import*

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Chk2 is a nuclear protein kinase involved in the DNA damage-induced ataxia telangiectasia mutated-dependent checkpoint arrest at multiple cell cycle phases. Searching for Chk2-binding proteins by a yeast two-hybrid system, we identified a strong interaction with karyopherin-α2 (KPNA-2), a gene product involved in active nuclear import of proteins bearing a nuclear localization signal (NLS). This finding was confirmed by glutathione S-transferase pull-down and co-immunoprecipitation assays. Of the three predicted Chk2 NLSs, located at amino acids 179–182 (NLS-1), 240–256 (NLS-2), and 515–522 (NLS-3), only the latter mediated the interaction with KPNA-2 in the yeast two-hybrid system, and in particular with its C terminus. Unlike mutations in NLS-1 or NLS-2, which left the nuclear localization of Chk2 unaffected, mutations in NLS-3 caused a cytoplasmic relocation, indicating that the NLS-3 motif acts indeed as NLS for Chk2 in vivo. Finally, co-transfection experiments with green fluorescent protein (GFP)-Chk2 and wild type or mutant KPNA-2 confirmed the role of KPNA-2 in nuclear import of Chk2.

To prevent genomic instability and cancer, DNA lesions arising from external agents or internal metabolism activate multiple checkpoint pathways that coordinate DNA repair and cell cycle progression. The checkpoint protein Chk2, the mammalian homologue of Saccharomyces cerevisiae Rad53 and Schizosaccharomyces pombe Cds1 (1), is a nuclear serine kinase bearing a serine/threonine-(SQ/TQ)-rich region (aa 119–69) with d-galactopyranoside.

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EXPERIMENTAL PROCEDURES

Cells and Transfections—The lymphoblastoid cell line LCL-N from a normal donor was cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 15% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), whereas the U2OS osteosarcoma cell line was maintained in Dulbecco’s modified Eagle’s medium with 10% serum. Cells were transfectioned by the calcium phosphate method. Irradiations were performed in an IBL437CO instrument equipped with a 137Ce source emitting a dose of 8 grays/min.

Yeast Two-Hybrid Screening—The screening in yeast was performed as described (26). Briefly, Chk2 cDNA deleted of aa 76–213 (obtained by digestion of full-length Chk2 cDNA with AocI) was cloned in the EcoRI-SalI sites of pLexA vector in-frame with the Lex A DNA binding domain and used as a bait to transform the EGY48 yeast strain. A selected clone was then transformed with a HeLa c-DNA library cloned in the pG4–5 vector in-frame with the B42 transactivation domain. About 2 × 10⁶ clones were obtained and tested for their capacity to grow in a medium without leucine and by the X-gal filter method. Plasmid DNA was then extracted from positive clones, and the c-DNA inserts were amplified by PCR and analyzed by sequencing. To map the regions of interaction between Chk2 and KPNA-2, deletion mutants for the two proteins were prepared by PCR and cloned in the pLexA and pG4–5 vector. The constructs were then used to transform the EGY48 strain.
and tested for the interaction using a medium containing X-gal or without leucine.

GST Pull-down Assays—The GST-Chk2 recombinant protein was purified from bacteria transformed with the full-length Chk2 cDNA cloned in the EcoRI-NolI sites of pGEX-4T-1 vector. LCL-N cells were lysed in ice-cold ELB buffer (150 mM NaCl, 50 mM Heps, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40), and 1 mg of cell extracts were incubated for 3 h at 4 °C with 10 μg of GST-Chk2 or GST together with glutathione-Sepharose. The beads were extensively washed in lysis buffer, size-fractionated by SDS-PAGE, and immunoblotted for KPNA-2 using a specific monoclonal antibody (Clone 2, Transduction Laboratories).

Co-immunoprecipitations—LCL-N, unirradiated or irradiated with 10 grays of IR, and U2OS cells transiently transfected with pCDNA3-HA-Chk2 and pCDNA3-FLAG-KPNA-2 were lysed in ELB buffer. After preclearing with protein A/G-coupled Sepharose beads, lysates were immunoprecipitated with an anti-FLAG polyclonal antibody (Sigma) and extensively washed. Immunoprecipitates were analyzed by Western blot with monoclonal antibodies anti-KPNA-2 (Clone 2, Transduction Laboratories) and anti-Chk2 (Clone 44D4-21, generated in our laboratory) and anti-Chk2 (Clone 2, Transduction Laboratories) and anti-Chk2 (Clone 2, Transduction Laboratories). As Chk2 protein could not be detected in KPNA-2 immunoprecipitates, we transiently co-transfected U2OS cell lines with FLAG-KPNA-2 and HA-Chk2 constructs and analyzed by Western blot with monoclonal anti-KPNA-2 antibody sits in the region binding to HA-Chk2 protein associated with KPNA-2 (Fig. 2c).

Identification of the Sites of Interaction between Chk2 and KPNA-2—To map the region of interaction between Chk2 and KPNA-2, three cell lines were transfected with pCDNA3-FLAG-KPNA-2 or pCDNA3-HA-Chk2 with FLAG-KPNA-2 constructs and analyzed for the interaction between Chk2 and KPNA-2. In cells treated with 10 grays of IR and harvested 3 h later, Chk2 co-precipitated KPNA-2 to the same extent as in untreated cells (Fig. 2b, lane 7). As Chk2 protein could not be detected in KPNA-2 immunoprecipitates (possibly because the epitope recognized by the monoclonal anti-KPNA-2 antibody sits in the region binding to Chk2, see below), we transiently co-transfected U2OS cell lines with FLAG-KPNA-2 and HA-Chk2 constructs and analyzed Chk2 in KPNA-2 immunoprecipitates obtained with anti-FLAG antibody. Under these conditions, we were able to find HA-Chk2 protein associated with KPNA-2 (Fig. 2c, lane 4). Altogether, these results demonstrate an interaction between Chk2 and KPNA-2 in human cells.

RESULTS

Yeast Two-hybrid Screenings for Chk2 Interactors—The yeast two-hybrid system was employed to search for Chk2 interactors. The bait consisted of Chk2 deleted of aa 76–213 rather than full-length Chk2 (Fig. 1, a and b), because in preliminary tests, the latter partially inhibited the growth and consequently the transformation efficiency of yeast (data not shown). Approximately two million colonies were screened by the X-gal filter method and for their capacity to grow without leucine. Of 45 sequenced positive clones, 33 encoded the full length or fragments of the karyopherin-α2 (KPNA-2) protein, the shortest of which represent aa 285–529. KPNA-2 (21, 22) is a nuclear transporter of proteins bearing an NLS, structurally characterized by an N terminus importin-β binding (IBB) domain necessary for nuclear translocation, eight armadillo repeats involved in the binding and recognition of NLSs, and a C-terminal acidic domain (Fig. 1c).

Chk2 Interacts with KPNA-2 Both in Vitro and in Vivo—The physical interaction between Chk2 and KPNA-2 was initially examined in pull-down assays in which extracts from normal lymphoblastoid cells (LCL-N) were incubated with Sepharose-bound recombinant GST-Chk2. Under these conditions, the endogenous KPNA-2 bound GST-Chk2 but not GST (Fig. 2a), thus confirming the interaction seen in yeast. To verify this interaction in vivo, we determined whether Chk2 immunoprecipitates with KPNA-2. The results (Fig. 2b, lane 3) showed that Chk2 from LCL-N cells co-precipitated with KPNA-2, and the specificity of this finding was demonstrated by the absence of a KPNA-2 signal when immunoprecipitating with a control antibody (Fig. 2b, lane 2). As Chk2 is involved in the response to DNA damage (1, 2), we tested the effect of IR on the interaction between Chk2 and KPNA-2. In cells treated with 10 grays of IR and harvested 3 h later, Chk2 co-precipitated KPNA-2 to the same extent as in untreated cells (Fig. 2b, lane 7). As Chk2 protein could not be detected in KPNA-2 immunoprecipitates (possibly because the epitope recognized by the monoclonal anti-KPNA-2 antibody sits in the region binding to Chk2, see below), we transiently co-transfected U2OS cell lines with FLAG-KPNA-2 and HA-Chk2 constructs and analyzed Chk2 in KPNA-2 immunoprecipitates obtained with anti-FLAG antibody. Under these conditions, we were able to find HA-Chk2 protein associated with KPNA-2 (Fig. 2c, lane 4). Altogether, these results demonstrate an interaction between Chk2 and KPNA-2 in human cells.

FIG. 1. Domain structure of human Chk2 and KPNA-2. In a, the diagram depicts the TQ/SQ-rich region and the FHA and kinase domains of Chk2 protein. b, the Chk2 bait deleted of aa 76–213 used in the yeast two-hybrid screening. c, schematic representation of the IBB domain, the armadillo repeats, and the acidic domain of KPNA-2 protein.
were tested in yeast against full-length Chk2. As neither KPNA-2\textsubscript{1–460} nor KPNA-2\textsubscript{1–369} interacted with Chk2 (Fig. 3b), it can be concluded that aa 461–529 of KPNA-2 are required for binding to Chk2. The expression of KPNA-2\textsubscript{1–460} and KPNA-2\textsubscript{1–369} proteins in yeast was demonstrated by Western blot with an anti-LexA antibody. Of note, these mutant proteins were, in contrast to full-length KPNA-2, unreactive with the anti-KPNA-2 antibody (Fig. 3c), suggesting that aa 461–529 contain the anti-KPNA-2 binding epitope.

Identification of Chk2 NLS—As KPNA-2 mediates the nuclear import of proteins carrying the NLS sequence (22), we determined the possible involvement of such an NLS of Chk2 in the interaction with KPNA-2. A PSORT II computer program analysis of Chk2 sequence identified three putative NLSs, a monopartite NLS-1 (KRRP, aa 179–182), a bipartite NLS-2 (RKTCKKVAIKIISKKRF, aa 240–256), and a monopartite NLS-3 (PSTSRKRPR, aa 515–522) (Fig. 4a). To study the function of these putative NLSs, the localization of various Chk2 NLS mutants expressed in transiently transfected U2OS cells was assessed by immunofluorescence. Like full-length FLAG-Chk2, FLAG-Chk2 deleted of aa 76–213 (and therefore of NLS-1) was exclusively expressed in the nucleus, thus suggesting that NLS-1 is dispensable for Chk2 nuclear localization. By contrast, a cytoplasmic localization became evident (Fig. 4b) when the lysine 520 and arginine 521 of NLS-3 were substituted with alanine (Table I). These changes are thought to destroy the putative consensus sequence. Mutations in NLS-2 motif had no effect on Chk2 nuclear localization (Table I and Fig. 4b). In accordance with these findings, KPNA-2 failed to interact in yeast with Chk2 mutated in NLS-3 (Fig. 4c). These results underscore the role of NLS-3 in the interaction with KPNA-2 and nuclear localization of Chk2.

Function of the Chk2/KPNA-2 Interaction in Vivo—To determine the \textit{in vivo} function of Chk2/KPNA-2 association, we examined the effect of ectopically expressed KPNA-2 on the nuclear translocation of Chk2. U2OS cells were transiently co-transfected with plasmids encoding GFP-Chk2 and wild type or mutant KPNA-2 and examined by fluorescence microscopy. Of the fluorescent cells detected in co-transfections with GFP-Chk2 and an empty vector, 51\% showed a nuclear labeling, whereas the remaining, in addition, showed a cytoplasmic labeling whose intensity was in some cases much weaker than the nuclear fluorescence. After co-transfection with wild type KPNA-2, the number of cells with nuclear GFP-Chk2 (Fig. 5) rose to 66\%, whereas after co-transfection with KPNA-2 deleted of either the Chk2-interacting region (KPNA-2\textsubscript{1–460}) or the IBB domain (KPNA-2\textsubscript{1–529}, unable to translocate to the nucleus), the number dropped to 41\%, indicating a role for KPNA-2 in the nuclear translocation of Chk2 kinase.

Since certain kinases modulate their nuclear import by direct phosphorylation of KPNA-2 (28), we assessed whether KPNA-2 is a target substrate for Chk2 kinase. For this purpose, \textit{in vitro} kinase assays were performed using catalytically active recombinant GST-Chk2 and full-length GST-KPNA-2 as a substrate. However, whereas GST-Chk2 phosphorylated, as expected, Cdc25C, it failed to phosphorylate KPNA-2 (Fig. 6a),
thus excluding a phosphorylative event in the interaction of these proteins. This finding is concordant with the lack of a Chk2 consensus substrate motif within KPNA-2 (as determined by Scansite analysis) and with the normal nuclear localization of ectopically expressed Chk2 kinase-dead (Fig. 6b).

**DISCUSSION**

Two-hybrid screenings in yeast have enabled us to identify a new physical and functional interaction between Chk2 kinase and KPNA-2, a family member of proteins involved in the active transport from the cytoplasm to the nucleus of cargo proteins containing an NLS (21, 22). Two types of NLS motifs exist; one, exemplified by the SV40 large T antigen PKKKRKV, contains a short stretch of basic amino acids (monopartite), and the other, first reported in nucleoplasmin (KRPAATKKAGQQKKKLDK), contains two clusters of basic amino acids separated by a spacer region of 10–12 amino acids (bipartite).

To cross the nuclear membrane and deliver its NLS-tagged cargo, KPNA-2 has to bind karyopherin-β (23, 24). In the nucleus, these complex components, including the cargo, are released, allowing the recycling of karyopherins to the cytoplasm (25).

**TABLE I**

**Chk2 mutants in the NLSs**

| Mutants     | Sequences                                    |
|-------------|----------------------------------------------|
| Chk2 wild type | 240RKTCKVKAIIISKRF256--515PSTSRKRP522       |
| NLS-2       | 240AATCKVKAIIISKRF256--515PSTSRKRP522       |
| NLS-3       | 240RKTCKVKAIIISKRF256--515PSTSRPAAP522      |

**FIG. 4.** Immunofluorescence staining of Chk2 mutants in the putative NLSs. a, the positions along the Chk2 c-DNA sequence of the three putative NLSs, a monopartite at aa 179–182, a bipartite at aa 240–256, and another monopartite at aa 515–522, predicted by the PSORT II computer program. b, U2OS cells were transiently transfected with FLAG-Chk2 constructs and immunostained with anti-FLAG antibody. Note the nuclear localization of Chk2 deleted of aa 76–213 or mutated in the NLS-2 and the cytoplasmic localization of Chk2 mutated in the NLS-3. W.T., wild type; DAPI, 4',6 diamidino-2-phenylindole. c, yeast two-hybrid results showing the disruption of the interaction between KPNA-2 and Chk2 mutated in the NLS-3 sequence.

**FIG. 5.** KPNA-2 overexpression and Chk2 subcellular localization. The intracellular distribution of GFP-Chk2 was examined by fluorescence microscopy after transient co-transfection of U2OS cells with wild type or deleted forms of FLAG-KPNA-2. The mean number (± S.D., shown as error bars) of cells with a nuclear fluorescence intensity (N; dark shaded columns), cells with similar nuclear and cytoplasmic fluorescence intensity (N=C; light shaded columns), and cells with much dimer cytoplasmic than nuclear fluorescence (N>C; open columns) are shown. Note that the nuclear localization of GFP-Chk2 increases when overexpressing wild type KPNA-2 and decreases when overexpressing mutant KPNA-2.

We confirmed the interaction seen in yeast between Chk2 and KPNA-2 also in pull-down assays between recombinant GST-Chk2 and endogenous KPNA-2 from total cell lysates. We have additionally demonstrated that Chk2 immunocomplexes from unirradiated or irradiated cells, the latter expressing hyperphosphorylated Chk2 (1, 2), contain KPNA-2. The detection by mass spectrometry of KPNA-2 in FLAG-Chk2 immuno-
FIG. 6. Recombinant Chk2 does not phosphorylate KPNA-2. Enzymatically active recombinant GST-Chk2 was tested for its capacity to phosphorylate KPNA-2 or GST-Cdc25c (the latter used as positive control). No KPNA-2 phosphorylation was detected (a). Normalization with Coomassie Blue staining is shown. b, normal nuclear localization of FLAG-Chk2-KD (kinase-dead) transiently expressed in U2OS cells.

complexes extracted from cells\(^2\) lends further support to these results. Although we were unable to co-precipitate Chk2 with anti-KPNA-2 antibodies, reasonably because the epitope that it recognizes maps within the region of interaction with Chk2 (as we have shown in Western blots of KPNA-2 deletion mutants expressed in yeast) and may thus be unavailable when bound to Chk2, we could nevertheless demonstrate Chk2 in FLAG-KPNA-2 complexes immunoprecipitated with an anti-FLAG antibody from cells ectopically expressing FLAG-KPNA-2 and HA-Chk2.

Having confirmed the interaction between these two molecules, we next determined whether this was mediated by the NLSs. As the PSORT analysis of Chk2 cDNA sequence scored three NLS motifs localized at aa 179–182 (NLS-1; monopartite), aa 240–256 (NLS-2; bipartite), aa 515–522 (NLS-3; monopartite), we examined in the two-hybrid system various misense and/or deletion mutants of Chk2 and KPNA-2 and found that only the C-terminal aa 450–543 of Chk2, which comprises NLS-3, associates with KPNA-2.

To determine the functional role in vivo of the three putative NLSs, the intracellular distribution of FLAG-tagged Chk2 constructs transiently transfected in U2OS cells was analyzed by immunofluorescence. We have shown that mutations in NLS-3, but not in NLS-1 or NLS-2, cause a cytoplasmic redistribution of Chk2. This finding, together with the fact that NLS-3 mutations disrupt the interaction with KPNA-2 in yeast (see above), underscores the importance of NLS-3 for the binding to KPNA-2 and nuclear translocation of Chk2.

KPNA-2 contains an IBB, eight armadillo repeats typically involved in recognition and binding to NLSs, and a C-terminal acidic domain (22). We have established that the C-terminal aa 461–529 of KPNA-2 are indispensable for binding to Chk2. This result is in apparent contrast with the finding that the armadillo repeats mediate the binding to NLS (22), but concordant with others showing that proteins such as RAG-1, BSAP, Stat1, and the export protein CAS interact with the C-terminal region of the karyopherin family members (29–32). It is speculated that the use of C-terminal region for interactions allows karyopherins to bind more than one molecule at the time, giving rise to a scaffold for protein complexes (31).

To establish the role played by KPNA-2 in the nuclear transport of Chk2, the subcellular localization of GFP-Chk2 co-expressed with wild type or mutant versions of KPNA-2 was analyzed by fluorescence microscopy. We have shown that only 51% of GFP-Chk2 cells have a nuclear localization, the remaining exhibiting both nuclear and cytoplasmic fluorescence of various intensities. It is worth noting that this cytoplasmic localization was not observed in cells expressing FLAG-Chk2, suggesting that the conformation of GFP-Chk2 might impair its nuclear transport. Interestingly, when co-expressed with wild type KPNA-2, the fraction of cells with nuclear GFP-Chk2 fluorescence rose to 66%, whereas when co-expressed with KPNA-2 mutated in the Chk2 binding site or in the IBB domain (thus unable to reach the nucleus), this number fell to 41%. Altogether, these results indicate that overexpression of wild type KPNA-2 enhances the nuclear localization of GFP-Chk2, whereas the mutant forms of KPNA-2 do not significantly modify the subcellular distribution of Chk2. These data may not be unexpected since there is no evidence indicating that the KPNA-2 mutants could act as dominant negative. Moreover, given the redundancy of the nuclear import/export pathways, other members of the karyopherin isoforms (33) could possibly complement KPNA-2 defects. This hypothesis is supported by experiments showing that the selective inhibition of KPNA-2 protein expression by small interfering RNA treatment only induces a 2-fold increase in cells with cytoplasmic GFP-Chk2, rather than an abrogation of nuclear GFP-Chk2 import,\(^3\) indicating that other karyopherin family members could partake in Chk2 nuclear import, like certain ribosomal and core histone proteins whose nuclear transport is mediated by at least five different importins (34). It should be noticed, however, that additional two-hybrid screenings in yeast with various Chk2 baits and different cDNA libraries have never revealed interactions with the karyopherin family other than KPNA-2.

As the Itk kinase stimulates its nuclear import through the phosphorylation of KPNA-2 (28), we tried to establish whether Chk2 operates in a similar way. However, we were unable to demonstrate, at least in vitro, any phosphorylation of GST-KPNA-2 by Chk2, indicating that the nuclear transport of Chk2 by KPNA-2 is independent of its enzymatic activity, which actually concurs with the appropriate nuclear localization of the kinase-inactive form of Chk2.

In conclusion, we have shown that Chk2 kinase carries a functional NLS located at aa 515–522 indispensable for KPNA-2 binding and that this interaction contributes to Chk2 nuclear translocation. As proteins of the nuclear import machinery, including KPNA-2, appear to have a role in other physiological processes, e.g. mitotic spindle formation, RNA export, and chromatin structure (35–37), we cannot exclude that Chk2-KPNA-2 association solely regulates Chk2 nuclear import.

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\(^2\) L. Zannini, personal communication.

\(^3\) L. Zannini, unpublished observation.
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