Nucleocytoplasmic Shuttling of Receptor-interacting Protein 3 (RIP3)

IDENTIFICATION OF NOVEL NUCLEAR EXPORT AND IMPORT SIGNALS IN RIP3*

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Receptor-interacting protein 3 (RIP3), a member of the RIP Ser/Thr kinase family, has been characterized as a pro-apoptotic protein involved in the tumor necrosis factor receptor-1 signaling pathway. In this study, we have mapped a minimal region of RIP3 sufficient for apoptosis induction to a fragment of 31 amino acids in length. This minimal region also functions as an unconventional nuclear localization signal sufficient to confer the import of full-length RIP3 to the nucleus to trigger apoptosis, suggesting that RIP3 is able to play an apoptosis-inducing role in the nucleus. In addition, we have characterized two novel leucine-rich nuclear export signals (NESs) that are responsible for the nuclear export of RIP3 to the cytoplasm via a chromosome region maintenance 1 (CRM1)-dependent pathway and an extra leucine-rich NES in the N terminus of RIP3 that contributes to the cytoplasmic distribution in a CRM1-independent manner. Thus, we provide the first evidence that RIP3 acts a nucleocytoplasmic shuttling protein, which presents a possible link between death receptor signaling and nuclear apoptosis.

The receptor-interacting protein (RIP)† Ser/Thr kinase family consists of four members, including RIP, RIP2 (RICK/CARDIAK), RIP3, and RIP4 (DIK/PKK) (1–4). RIP3 is an important component of the tumor necrosis factor receptor-1 (TNFR1) signaling complex. Sequence analysis reveals that RIP3 contains an N-terminal RIP-like kinase domain (amino acids 21–287) that shares extensive homology with the corresponding N-terminal kinase domain in other RIP family members. Interestingly, in sharp contrast to RIP, which has a C-terminal death domain (DD), RIP2, which has a C-terminal caspase-recruiting domain, and RIP4, which has 11 C-terminal ankyrin repeats, RIP3 possesses a unique C-terminal domain (amino acids 288–518) that shares no significant homology to any known proteins.

It has been shown that the N terminus of RIP3 is required for its kinase activity and autophosphorylation, whereas the C terminus of RIP3 is responsible for caspase activation and apoptosis induction (1–3, 5). Furthermore, RIP3 is recruited to the TNFR1 signaling complex through interaction with RIP via its C-terminal segment, and, once recruited to the complex, RIP3 could exert a pro-apoptotic activity that may be partially accomplished by activating caspases and/or by inhibiting RIP- and TNFR1-induced NF-κB activation (2, 6). Although RIP3 is involved in TNF-α-mediated apoptosis, the mechanism by which RIP3 induces cell death remains largely unclear (7). Because the C terminus of RIP3 is unique and involved in apoptosis induction, we are mainly focused on this unique C-terminal segment to investigate its functional significance in RIP3 signaling.

Previous studies have demonstrated that both TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), two essential DD-containing adapter molecules, are recruited to the TNFR1 complex during TNF-α-induced death signaling, and their function and localization are widely assumed to be cytoplasmic. However, recent studies have shown that TRADD and FADD contain both a nuclear export signal (NES) and a nuclear localization signal (NLS), capable of nuclear export and import and causing proteins to transport between the cytoplasm and the nucleus (8–10). Moreover, TRADD and FADD also appear to have apoptotic activity in the nucleus. The detailed mechanisms of nucleocytoplasmic transport and possible roles of these proteins in nuclear apoptotic signaling remain unclear.

In this study, we have delineated a minimal domain of RIP3 sufficient for apoptosis induction to amino acids (aa) 442–472, a short region of 31 residues in length. This fragment (aa 442–472) also functions as an unconventional NLS and is found to be located predominantly in the nucleus. Furthermore, we present the evidence that RIP3 is a nucleocytoplasmic shuttling protein. This characterized single NLS is capable of conferring the import of full-length RIP3 protein to the nucleus. In addition, we identified two novel leucine-rich NESs that are responsible for the nuclear export of RIP3 via a CRM1-mediated mechanism and an additional leucine-rich NES in the N-terminal region of RIP3, which appears to regulate the cytoplasmic distribution of RIP3 in a CRM1-independent manner.

MATERIALS AND METHODS

Reagents—Hoechst 33342 and general caspase inhibitor Z-VAD-Fmk were purchased from Sigma. MitoTracker Red CMXRos was obtained...
from Molecular Probes. NES inhibitor leptomycin B (LMB) was a generous gift from Dr. Minoru Yoshida (RIKEN Institute, Japan).

**Plasmid Construction**—The cDNA encoding full-length RIP3 was isolated by PCR from human brain cDNA library (Clontech). The amplified fragment was digested with BglII and XhoI and then cloned into BglII-XhoI sites of pEGFP-C1 (Clontech) to generate pEGFP-C1/RIP3. The EcoRI- and XhoI-digested segment coding for the truncated mutant RIP3\(^{N223}\) was inserted into EcoRI-SalI-digested pEGFP-C1 to yield pEGFP-C1/RIP3\(^{N223}\). The fragment coding for RIP3 was digested with SalI and NotI and subcloned into the compatible sites of pCI-neo/Myc to create pCI-neo/Myc-RIP3. Various N-terminally GFP-tagged RIP3 mutants used for mapping the minimal region and attesting both NESs and NLSs were created by inserting EcoRI-XhoI-digested fragments into EcoRI-SalI sites of pEGFP-C1. The fragments coding for Bcl-rambo\(^{H9004N204}\) or amino acid residues 1–56 from Smac/DIABLO were digested and inserted into EcoRI-SalI sites of pEGFP-C1 or BglII-XhoI sites of pEGFP-N1 (Clontech), respectively. The BamHI-NotI-digested fragment encoding GFP-RIP3\(^{H9004N223}\) or GFP-RIP3 was subcloned into the same sites of pHis-TAT-PTD-GFP (11) to create pHis-TAT-PTD-GFP-RIP3\(^{H9004N223}\) or pHis-TAT-PTD-GFP-RIP3. All the primers used in this study and construction details are available on request.

**Cell Culture and Transfection**—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO\(_2\). Transfections of cells were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's specifications.

**Apoptosis Assays**—Apoptosis assays were performed mainly as described previously (12) with a slight modification. HeLa cells in 24-well plates were cultured to subconfluence (5 × 10^5 cells) and transiently transfected with the indicated plasmids. At 24 h post-transfection, both floating and adherent cells were fixed and stained with 2 μg/ml Hoechst 33342. At least eight different fields were selected randomly, and the percentages of apoptotic cells among the total number of cells were counted based on the dead cells having DNA fragmentation and/or chromatin condensation under a fluorescence microscope and morphological changes under phase contrast. The data are the means of at least three independent experiments.

**Western Blotting**—Western blot was carried out as described by Yang et al. (11). Briefly, cells were harvested, washed, and then resuspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Science) on ice for 30 min. The lysates were pulled.
centrifuged, and both supernatants and pellets were mixed with an equal volume of 2× Laemmli buffer, respectively. The samples were separated by 12% SDS-polyacrylamide gel, and the resolved proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). The blot was blocked with 5% milk in Tris-buffered saline separated by 12% SDS-polyacrylamide gel, and the resolved proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). The blot was blocked with 5% milk in Tris-buffered saline. The resolved proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). The blot was blocked with 5% milk in Tris-buffered saline.

**Indirect Immunofluorescence**—HeLa cells were grown on glass coverslips and then transiently transfected with the indicated plasmids. At 24 h after transfection, cells were washed once with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 in PBS, and then blocked with 3% bovine serum albumin in PBS for 30 min. The cells were incubated with mouse anti-Myc monoclonal antibody (1:500, Clontech) for 1 h at room temperature, followed by rhodamine-conjugated goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch Laboratories) for another 1 h. After rinsing three times with PBS for 5 min, the cells were stained with 1 μg/ml Hoechst 33342 for 1 min, mounting medium containing an anti-fading agent (Vector Laboratories) was added, and the cells were visualized under a fluorescence microscope using standard filter for red fluorescence. Images were captured with an Olympus IX70 microscope controlled by Viewfinder Lite (version 1.0) armed with a ×40 objective lens.

**Mapping the Minimal Region of RIP3 Sufficient for Apoptosis Induction**—Previous studies have shown that expression of RIP3 induces apoptosis, but the minimal domain of RIP3 that promotes apoptosis remains uncharacterized. To pinpoint the minimal region of RIP3 that is sufficient to induce cell death, we prepared a series of constructs by linking GFP to the N-terminus of RIP3 deletion mutants (Fig. 1A). The expression of the GFP fusion constructs was verified by Western blot (Fig. 1B). As shown in Fig. 1 (A and C), unlike RIP3ΔN223, which was shown to promote cell death (2), RIP3-224–460 lacking the C-terminal 58 residues failed to induce apoptosis, indicating that aa 461–518 were required for its pro-apoptotic activity. However, RIP3ΔN460 was found unable to mediate apoptosis, suggesting that the region spanning residue 460 appeared to be crucial for its apoptotic function. As shown in Fig. 1 (A and C), besides RIP3ΔN350 and RIP3ΔN428, a smaller fragment RIP3-(429–483) with only 55 residues in length was also able to induce apoptosis spontaneously.

To more precisely map the minimal region, we started with amino acid 460 as a central residue and generated six additional RIP3 deletion mutants by gradually deleting about 10 residues each time from either the N or the C termini. As...
shown in Fig. 1 (A and C), although RIP3-(429–472) promoted apoptosis, RIP3-(429–460) failed to induce cell death, implying that the region from aa 461 to 472 must be included for its apoptosis induction. In addition, although RIP3-(442–483) was shown to induce apoptosis, RIP3-(451–483) failed to do so, suggesting aa 442–450 was indispensable. Finally, RIP3-(442–472) containing both aa 461–472 and aa 442–450 was found to induce significant apoptosis, whereas RIP3-(451–472) lacking aa 442–450 failed to elicit apoptosis. Based on these results, we have delineated the minimal region of RIP3 sufficient for apoptosis induction to aa 442–472, a fragment of 31 residues in length, that is sufficient to trigger apoptosis upon ectopic expression alone.

**RIP3 Is Localized to the Cytoplasm, but Not to Mitochondria**—Cellular compartmentation is essential for proper function of a protein. RIP3 is recruited to the TNFR1 complex and is thus assumed to be primarily cytoplasmic. However, the precise subcellular localization of RIP3 from limited published data remains controversial (5, 6). To ascertain the intracellular localization of RIP3 in living cells, GFP, GFP-tagged RIP3, or GFP-tagged RIP3ΔN223 was transfected into HeLa cells, respectively (Fig. 2A). The expression of constructs was confirmed by Western blotting (Fig. 2B). As shown in Fig. 2C, GFP alone was distributed evenly throughout the cells (panel a). In contrast, the full-length RIP3 fused to the C terminus of GFP was located specifically in the cytoplasm in a diffused pattern (panel e). Similarly, fusion of RIP3ΔN223 with GFP also displayed a cytoplasmic localization, but predominantly in a punctuated pattern within the cytoplasm (panel i). To examine whether these two different cytoplasmic localization patterns resulted from both full-length RIP3 and the N-terminal-truncated RIP3ΔN223 could be attributed to the kinase activity in the N terminus of RIP3, we generated a point mutant RIP3 (K50A), in which Lys50 was replaced by alanine to abrogate the kinase activity of RIP3 (1, 2). As shown in Fig. 2C, the K50A substitution significantly increased the number of cells with punctate structures within the cytoplasm (panel m), indicating that the loss of kinase activity may associate with the accumulation of punctate structures within the cytoplasm. It is worthwhile to point out that our conclusion regarding cytoplasmic localization of RIP3 differed from a previous report in which they claimed that RIP3 was solely localized to the mitochondrion (5). To have a sustainable support for our conclusion, we further utilized two bona fide mitochondrial proteins, namely Bcl-rambo and Smac/DIABLO (15, 16), as references for mitochondrial location. As shown in Fig. 2 (A and C), GFP fusion protein, with truncated Bcl-rambo (Bcl-ramboΔN204) containing its C-terminal membrane anchor, was found exclusively in the mitochondrion (Fig. 2C, panel q). Likewise, when a segment consisting of the first 56 residues (mitochondrial targeting sequence (MTS)) from Smac/DIABLO was fused to the N terminus of GFP (Fig. 2A), this GFP fusion protein was specifically distributed in mitochondria (Fig. 2C, panel u). As expected, the merged images for both GFP-Bcl-ramboΔN204 and sMTS/56-.
RIP3 is an LMB-sensitive Nucleocytoplasmic Shuttling Protein—Recently, it has been shown that TRADD can be transported to the nucleus from the cytoplasm, which is regulated by inhibiting the CRM1-mediated nuclear export with leptomycin B (LMB) (8). Similar to TRADD, RIP3 is also recruited to the TNFR1 signaling complex and is primarily localized in the cytoplasm as described above; it is reasonable for us to inquire whether RIP3 undergoes a similar nucleocytoplasmic shuttling. In HeLa cells, GFP-RIP3 was localized mainly in the cytoplasm in the absence of LMB. However, LMB treatment at 37 °C markedly changed the subcellular distribution of GFP-RIP3, which was relocated to the nucleus and distributed in both the nucleus and cytoplasm with a slightly more nuclear accumulation (Fig. 3A, left panel), indicating that the effective export of RIP3 from the nucleus to the cytoplasm is CRM1-dependent. This nuclear export is hindered upon LMB treatment, and as a result, import RIP3 synthesized in the cytoplasm starts to accumulate in the nucleus. Furthermore, we also tested the response of GFP-RIP3 to LMB treatment at 4 °C. In this case, not only CRM1-mediated nuclear export was blocked due to the presence of LMB, but also active nuclear import was stagnant because of low temperature. As expected, GFP-RIP3 remained in a cytoplasmic localization pattern (Fig. 3A, middle panel). As a negative control, we examined the effect of the cellular distribution of YFP3, a triple yellow fluorescent protein, to LMB treatment. YFP3 was primarily localized within the cytoplasm. In distinct contrast to GFP-RIP3, the cellular localization of YFP3 remained unchanged upon treatment with LMB at 37 °C (Fig. 3A, right panel). Collectively, our results demonstrated that, on one hand, export from the nucleus is in a CRM1-dependent fashion and, on the other hand, transport of RIP3 into the nucleus is a temperature-sensitive process.

To rule out the possibility that GFP could have caused an interference with RIP3 shuttling, we fused RIP3 with Myc epitope to generate Myc-RIP3, and its subcellular distribution was further assessed by indirect immunofluorescence. As shown in Fig. 3B, Myc-RIP3 was excluded from the nucleus and localized exclusively to the cytoplasm in the absence of LMB (upper panel). Myc-RIP3 was evident in both nucleus and cytoplasm when Myc-RIP3-expressing cells were treated with LMB at 37 °C (Fig. 3B, lower panel), confirming that RIP3 itself was able to travel between the cytoplasm and the nucleus independent of its fusion partner.

In addition, a construct coding for a chimeric fusion protein containing an exogenous NLS from HIV-1 TAT protein transduction domain (PTD) (11) at the N terminus of GFP-RIP3 was transiently transfected into HeLa cells (Fig. 3C). Unexpectedly, TAT-PTD-GFP-RIP3 was found to localize mainly in the cytoplasm instead of being targeted into the nucleus by TAT-PTD, indicating that the nuclear export activity of RIP3 may have overcome the nuclear targeting ability of TAT-PTD, resulting in a net export of TAT-PTD-GFP-RIP3 from the nucleus.
vestigate whether the cytoplasmic location of RIP3ΔN223 is necessary for its apoptotic activity, we once linked a TAT-PTD (NLS) to RIP3ΔN223 to change its subcellular location from the cytoplasm to the nucleus. To our surprise, TAT-PTD (NLS), when fused to the N terminus of GFP-RIP3ΔN223, failed to dislocate the TAT-PTD-GFP-RIP3ΔN223 into the nucleus (Fig. 4A), implying that a cytoplasmic retention sequence or NES may exist in this region. To address this point, we scrutinized the amino acid sequence in this region and found two distinctive leucine-rich NES-like sequences, one is 255LEGLKELMQL (NES-1) near the C terminus of the kinase domain, and the other is 344MVSEWLNKLNL (NES-2) present at the unique C terminus of RIP3 (Fig. 4B). These sequences resembled those previously identified leucine-rich NESs characterized by the presence of four closely spaced hydrophobic residues, most often leucine (17). Sequence alignments revealed that two NES-like motifs of RIP3 were closely similar to the NES consensus (Fig. 4C).

To investigate whether these putative leucine-rich sequences can truly function as NESs, we examined the subcellular localizations of a series of GFP-tagged RIP3 deletion mutants by fluorescence microscopy. As seen in Fig. 4D, different GFP-RIP3 fusion proteins containing either NES-1 or NES-2 or both displayed predominantly cytoplasmic localizations, despite the fact that RIP3-(224–287) containing only NES-1 exhibited a relatively modest export activity compared with RIP3-(288–358) containing only NES-2. To further characterize a shorter NES-2-containing sequence, we generated a RIP3-(333–358) mutant that contains only 26 residues. This fusion protein was

| Name | Sequence |
|------|----------|
| NES-3 | LEGLKELMQL |
| I | 255LEGLKELMQL |
| II | 344MVSEWLNKLNL |
| NES-4 | LHKLADFGL |
| NES-5 | LGYAPFLF |

* Highly conserved hydrophobic amino acids are in bold.
still found in the cytoplasm (Fig. 4D), indicating that aa 333–358 were sufficient to export GFP protein to the cytoplasm. Thus, we have identified two novel leucine-rich NESs in RIP3, which were capable of directing GFP proteins to the cytoplasm.

It has been shown that the leucine residues within the leucine-rich NES are critical for its nuclear export activity (18). To elucidate the functional significance of these hydrophobic leucine-rich NES are critical for its nuclear export activity (18). Thus, we have identified two novel leucine-rich NESs in RIP3, which were capable of directing GFP proteins to the cytoplasm. As shown in Fig. 4E, both RIP3-(224–287) and RIP3-(288–358) were sensitive to LMB treatment and resulted in nuclear accumulation by passive diffusion because of their small size, confirming that both NES-1 and NES-2 are capable of directing proteins from the nucleus to the cytoplasm in an LMB-sensitive, CRM1-dependent manner.

In addition, the time course of LMB treatment on the nuclear export activity of NES-2 was also examined. Cells expressing RIP3-(288–358) were incubated with LMB at 37 °C for the indicated periods of time and further examined by fluorescence microscopy. As seen in Fig. 4F, RIP3-(288–358) rapidly entered the nucleus as early as 5 min after LMB treatment, and an increasing nuclear accumulation was observed as incubation time proceeded.

Identification of an LMB-insensitive NES in the N Terminus of RIP3—To further investigate whether there exits additional NESs in the N-terminal region of RIP3 that may regulate the cytoplasmic distribution, we examined carefully the RIP3 primary sequence ranging from aa 1 to 223. Three presumed leucine-rich NES-like sequences were categorized, and interestingly, one of which (NES-3) was shown to contain two overlapping NES-like motifs I and II (Table I). To determine whether these NES-like sequences are functional, each putative NES-like motif was fused to GFP and their subcellular distributions were then examined under a fluorescence microscope. As shown in Fig. 5A, RIP3-(143–173), RIP3-(174–223), or RIP3-(143–223), respectively, containing NES-4, NES-5, or both was found unable to export GFP to the cytosol, demonstrating that neither NES-4 nor NES-5 was functional. In contrast, RIP3-(1–223), RIP3-(1–142), or RIP3-(104–142), each of which contains NES-3, showed a considerable cytosolic distribution, implying that the short leucine-rich sequence NES-3 is functional and sufficient to export GFP protein to the cytoplasm.

To further test whether the nuclear export mediated by NES-3 is CRM1-dependent, we examined the effects of LMB on the subcellular localization of RIP3-(1–223), RIP3-(1–142), or RIP3-(104–142). Much to our surprise, the nuclear export of RIP3-(1–223), RIP3-(1–142), or RIP3-(104–142) was not affected by LMB treatment (Fig. 5B), indicating that, unlike NES-1 and NES-2, NES-3 exhibits nuclear export activity in a CRM1-independent manner. Thus, we identified an additional nuclear export sequence NES-3 residing in the N-terminal region of RIP3, which may contribute to the nuclear export of RIP3 by a CRM1-independent mechanism.

RIP3 Contains a Putative Nuclear Localization Signal—Mutant RIP3ΔN350, which lacks NES-1, NES-3, and part of NES-2, was found to accumulate mainly in the nucleus, which strongly prompted us to hypothesize that a potential NLS might be present in this region. After careful inspection, we deduced three discernible NLS-like sequences in the aa 224–518 region (Fig. 6A), however, these underlined NLS-like mo-
tifis were eventually demonstrated to be nonfunctional (data not shown).

If only judged by the consensus sequence of NLS, there appeared to be no functional classic NLS present in this region, thus we inquired whether there exists an "unconventional" NLS that could lead RIP3/N350 to the nucleus. Based on the results described in Table II, deletion mutants RIP3N428, RIP3N429–483, and RIP3-(442–472) displayed similar nuclear localization. Of all these segments, an 442–472 appeared to be the smallest region that can target GFP protein to the nucleus, because aa 451–472, a region smaller than aa 442–472, has lost the ability of nuclear import ability (Fig. 6B). We thus defined aa 442–472 of RIP3 as an unconventional NLS despite the fact that this sequence does not represent a typical NLS.

To examine whether this NLS is truly responsible for nuclear localization of these RIP3 deletion mutants, HeLa cells were transfected with the indicated constructs shown in Fig. 6C and incubated in the presence of Z-VAD-Fmk dissolved in Me2SO to prevent the massive cell death. Cells expressing RIP3N350, RIP3N428, or RIP3-(429–483), each of which contains this putative NLS, showed remarkable nuclear accumulation, whereas other mutants, including RIP3N460 and RIP3-(429–460), both of which lack an intact NLS, were distributed throughout the cells under the same conditions. Treatment with Me2SO alone showed no effect on this nuclear translocation. In addition, the nuclear localization of RIP3 constructs is not confined to HeLa cells; a similar result was obtained in other cell lines such as MCF-7. All these combined data clearly demonstrated that this unique NLS (aa 442–472) might represent a novel NLS that has not been characterized previously. To further clarify the role of this unique NLS in the context
of full-length RIP3 protein, we first created a point mutant GFP-RIP3-NES-1m-223, in which all conserved leucine residues in NES-1 and NES-2 were replaced by alanine to abolish the nuclear export activity (Fig. 6D). Following transient transfection in HeLa cells, despite the finding that more fluorescence was observed in the nucleus than that of wild-type RIP3 (Figs. 6D and 2C, panel e), suggesting that the intact NES-3 may regulate the nuclear export of RIP3 and counteract the nuclear import by NLS. To confirm this premise, we further constructed two deletion mutants, namely GFP-RIP3-NES-1m-2mΔN142, which lacks the first 142 residues at its N terminus, and GFP-RIP3-NES-1m-2mΔNES-3, which is only missing NES-3 (Fig. 6D). As expected, both RIP3-NES-1m-2mΔN142 and RIP3-NES-1m-2mΔNES-3 were found to be localized in the nucleus with some noticeable dot-like structures in the cells (Fig. 6D), demonstrating that NLS is truly responsible for the nuclear import of full-length RIP3.

As described above, aa 442–472 functions not only as an NLS, but also as an apoptosis-inducing domain. Any the RIP3 deletion version, as long as it contains aa 442–472, was observed in the nucleus than that of wild-type RIP3 (Figs. 6D and 6E). Importantly, RIP3-(442–472) happens to contain the core 16 residues of RIP homotypic interaction motif (aa 451–466) that is necessary, but not sufficient, for interaction with RIP (6). Sun et al. (6) reported that aa 411–474 of RIP3 is the minimal region for its association with RIP, and if this was the case, the defined aa 442–472 would not be able to interact with RIP, implying that the apoptotic function of RIP3 may not have to rely on RIP-RIP3 interaction.

The subcellular localization studies of RIP3 have remained controversial based on the limited evidence from two groups (5, 6). In accordance with the observation from Sun et al. (6), we have shown that RIP3 is primarily localized in the cytoplasm, but not to mitochondria. Prediction of the RIP3-sorting sequence by PSORTII failed to disclose any recognizable mitochondrial targeting or anchor sequence within RIP3, which partially supports our conclusion that RIP3 is unlikely to localize to mitochondria. Although we have shown that the punctate structures accumulated in the cytoplasm appear to be closely related to the loss of RIP3 kinase activity, the possibility that some other cellular proteins prefer to associate with RIP3ΔN223 to form punctate structures in the cytosol can not be excluded completely.

Leucine-rich NESs consist of four or five hydrophobic residues within a region that is ~10 amino acids in length. The widely accepted NES consensus is LX_2,L[LIVFM]X_2,LX[X,L] (17). In this study, we have identified and characterized two novel leucine-rich NESs in RIP3, which are not only structurally similar to consensus, but also functionally active. NES-1 and NES-2 were shown to powerfully export proteins from the nucleus to the cytosol via an LMB-sensitive, CRM1-dependent pathway. Moreover, sequence alignments of human RIP3 with mouse RIP3 (mRIP3) (3) revealed two previously unidentified leucine-rich NES-like motifs in mRIP3; one is 260LEK-LKPEMI (6) and the other is 328SVKMI-DRLHL (34), which, respectively, correspond to NES-1 and NES-2 delineated in this study. These data suggest that leucine-rich NESs might be highly conserved in RIP3 across different species and further imply that they must have played indispensable roles during the evolution of RIP3 in determining cytoplasmic localization.

In addition to NES-1 and NES-2, we have discovered another novel leucine-rich NES-3 at the N terminus of RIP3, which is also involved in regulating nuclear export of RIP3. However, different from NES-1 and NES-2, the nuclear export activity of NES-3 was found to be LMB-insensitive, thus it functions as a CRM1-independent NES. This conclusion was further validated by a complementary observation that two novel splicing variants of RIP3, both retaining NES-3 but lacking NES-1, NES-2, and NLS, were found to be localized predominantly in the cytoplasm and insensitive to LMB inhibition.2 Similar to RIP3, α-catenin was recently reported to contain a weak LMB-insensitive NES-1 and a strong LMB-sensitive NES-2, both involved in the nuclear export of α-catenin via a CRM1-dependent pathway (21).

Although CRM1-mediated export remains the most exten-
sively studied export pathway to date, other export receptors have been identified (17). Recently, calreticulin was shown to mediate export of protein kinase inhibitor in an NES-dependent, LMB-insensitive manner, indicating that CRM1 might not be the only export receptor recognizing the leucine-rich NES (22). Therefore, in the case of RIP3, more experiments are needed to investigate the detailed mechanism of how the nuclear export by NES-3 is achieved in a CRM1-independent fashion.

Entering the nucleus by GFP-tagged RIP3 can not be a form of passive diffusion, because its molecular size (80–90 kDa) is much bigger than the size limit (40–60 kDa) of nuclear pore complex (23) and this nuclear import is completely abolished at 4 °C. A cytoplasmic protein, YFP-α, with a comparable size (>80 kDa) but lacking any endogenous NLS, is incapable of entering the nucleus under similar conditions. These data indisputably demonstrate that the nuclear translocation of RIP3 is an active, temperature-dependent process.

The nuclear import of proteins involves the recognition of NLS by importins α and/or β, translocation through the nuclear pore complex, and release in the nucleus (24). The best characterized “classic” NLS consists of either one (monopartite) or two (bipartite) clusters of basic amino acid residues, predominantly lysine or arginine. However, a variety of other sequences that bears no obvious resemblance to the classic NLS is also found to mediate nuclear import of proteins (25). In the present study, we have defined a unique NLS in RIP3, which does not share the preserved futures of classic NLS yet is able to mediate the nuclear import. Deletion experiments showed that there appears to be only a single NLS in RIP3, which is localized at its C terminus. In terms of its transporting ability, this unconventional NLS seems to be less powerful than NESs characterized in this study, because the net flow of RIP3 is normal from the nucleus to the cytosol, which is evidenced by strong cytoplasmic location of RIP3. To demonstrate that this NLS is sufficient to confer the nuclear import of full-length RIP3, we mutated NES-1 and NES-2 and deleted NES-3, the resultant RIP3 was found in the nucleus with dot-like fluorescence scattering in the cells. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome. However, a variety of other sequences that bears no obvious resemblance to the classic NLS is also found to mediate nuclear import of proteins (25). In the present study, we have defined a unique NLS in RIP3, which does not share the preserved futures of classic NLS yet is able to mediate the nuclear import. Deletion experiments showed that there appears to be only a single NLS in RIP3, which is localized at its C terminus. In terms of its transporting ability, this unconventional NLS seems to be less powerful than NESs characterized in this study, because the net flow of RIP3 is normal from the nucleus to the cytosol, which is evidenced by strong cytoplasmic location of RIP3. To demonstrate that this NLS is sufficient to confer the nuclear import of full-length RIP3, we mutated NES-1 and NES-2 and deleted NES-3, the resultant RIP3 was found in the nucleus with dot-like fluorescence scattering in the cells. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome. However, a variety of other sequences that bears no obvious resemblance to the classic NLS is also found to mediate nuclear import of proteins (25). In the present study, we have defined a unique NLS in RIP3, which does not share the preserved futures of classic NLS yet is able to mediate the nuclear import. Deletion experiments showed that there appears to be only a single NLS in RIP3, which is localized at its C terminus. In terms of its transporting ability, this unconventional NLS seems to be less powerful than NESs characterized in this study, because the net flow of RIP3 is normal from the nucleus to the cytosol, which is evidenced by strong cytoplasmic location of RIP3. To demonstrate that this NLS is sufficient to confer the nuclear import of full-length RIP3, we mutated NES-1 and NES-2 and deleted NES-3, the resultant RIP3 was found in the nucleus with dot-like fluorescence scattering in the cells. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome. Due to drastic changes in their structures, it is not irrational to speculate that some proportion of RIP3 mutants could be located in cytoplasmic aggresomes (dot-like structures) before they are targeted to the proteasome.

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