NRAGE associates with the anti-apoptotic factor Che-1 and regulates its degradation to induce cell death

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Summary

Neurotrophin receptor-interacting MAGE homolog (NRAGE) has been recently identified as a cell-death inducer, involved in molecular events driving cells through apoptotic networks during neuronal development. Recently, we have focused on the functional role of Che-1, also known as apoptosis-antagonizing transcription factor (AATF), a protein involved in cell cycle control and gene transcription. Increasing evidence suggests that Che-1 is involved in apoptotic signalling in neural tissues. In cortical neurons Che-1 exhibits an anti-apoptotic activity, protecting cells from neuronal damage induced by amyloid β-peptide.

Here, we report that Che-1 interacts with NRAGE and that an EGFP-NRAGE fusion protein inhibits nuclear localization of Che-1, by sequestering it within the cytoplasmic compartment. Furthermore, NRAGE overexpression downregulates endogenous Che-1 by targeting it for proteasome-dependent degradation. Finally, we propose that Che-1 is a functional antagonist of NRAGE, because its overexpression completely reverts NRAGE-induced cell-death.

Key words: MAGE, NRAGE, Che-1, AATF, Apoptosis, Neural cell-death, Ubiquitin

Introduction

The interplay between apoptosis and cellular growth requires a dynamic process that selectively drives molecules into functional signalling complexes. Consistent with this concept, studies showing new protein-protein interactions may provide important advancements in the knowledge of the molecular events governing cell fate during development.

NRAGE (neurotrophin receptor-interacting MAGE homolog) has been originally identified as a protein that interacts with the juxtamembrane domain of the p75 neurotrophin receptor ($p75^{NTR}$) and favours nerve growth factor (NGF)-dependent apoptosis in sympathetic neuron precursors cells, probably through a Jun N-terminal kinase (JNK)-dependent mitochondrial pathway (Salehi et al., 2000; Salehi et al., 2002). Structurally, NRAGE is characterised by a C-terminal sequence of 200 amino acids, termed MHD [melanoma-associated antigen (MAGE) homology domain] (Salehi et al., 2000). The MHD sequence corresponds to the highly conserved homology region shared by the protein products of each member of the MAGE gene family, an intriguing cluster of genes that are coming under increasing attention because of their involvement in cell cycle control and neuronal development (Chomez et al., 2001; Barker and Salehi, 2002).

NRAGE, also known as MAGE-D1 (Pold et al., 1999) or Dlxin-1 (Masuda et al., 2001), has been reported to physically and/or functionally associate with important regulatory proteins of apoptosis and cell cycle, including cell surface receptors, transcription factors and protein modifiers (Kendall et al., 2002; Sasaki et al., 2005). Interestingly, it interacts with X-linked inhibitor of apoptosis protein (XIAP) and inhibitor of T-cell apoptosis (ITA), members of the apoptosis inhibitor protein (IAP) family that play a crucial role in apoptosis by interfering with the process of caspase activation. The binding of NRAGE to the C-terminal RING domain of IAPs induces cleavage of XIAP and increases caspase-mediated cell death (Jordan et al., 2001).

Although no evidence concerning the direct physical interaction between the p53 tumour suppressor protein and NRAGE has been reported, recent findings showed that NRAGE-mediated cell cycle arrest is p53-dependent, resulting in post-transcriptional modification and upregulation of p53 activity (Wen et al., 2004). Furthermore, NRAGE has also been described as being a transcriptional regulator. It directly contacts Dlx/Msx homeodomain family proteins and its molecular integrity is necessary for Dlx5-dependent transcriptional function, because NRAGE ubiquitylation strongly represses Dlx-5 activity (Masuda et al., 2001; Sasaki et al., 2002). Although the effects of NRAGE on Msx function remain to be elucidated, it has been shown that necdin, another MAGE family member strongly involved in cell growth suppression (Taniura et al., 1998; Barker and Salehi, 2002),

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binds to and regulates Msx2 via NRAGE to favour muscle differentiation (Kuwajima et al., 2004). In addition, Kendall et al. showed that NRAGE is a key molecule involved in cortical neurogenesis because it mediates the cross talk between bone morphogenetic protein (BMP) signalling and the p38 pathway, driving cells towards apoptosis (Kendall et al., 2005). Taken together these findings are in good agreement with the idea that NRAGE might preferentially act as apoptotic inducer by promoting suitable assembly of signalling complexes during the switch from cell cycle progression to terminal differentiation.

Recently, we have focused on the functional role of Che-1 [also known as apoptosis-antagonizing transcription factor (AATF)], a protein highly conserved during evolution, and proposed to be a key molecule in fundamental networks governing cell cycle progression and gene transcription (Fanciulli et al., 2000; Thomas et al., 2000; Lindfors et al., 2000; Page et al., 1999; Monaco et al., 2003; Bruno et al., 2002). Originally identified as novel binding protein of the RNA polymerase II (Fanciulli et al., 2000), Che-1 exhibits a dual role in cell cycle control because it is able to regulate cell proliferation as well as growth arrest. Indeed, it interacts with and affects the growth suppressing activity of retinoblastoma protein (Rb) by interfering with Rb-mediated recruitment of histone deacetylase I (HDAC1) to E2F1-responsive promoters (Fanciulli et al., 2000; Bruno et al., 2002). In addition, reduced expression of Che-1 frequently correlates with cellular transformation, and Che-1 involvement in CDK inhibitor $p21^{WAF}$-induced cell growth arrest has been reported (Di Padova et al., 2003). Recently, we have observed that DNA damage by different genotoxic agents induces post-translational modifications in the Che-1 protein, extending its half-life and functionally linking it to the transcriptional activation of target genes at the G2-M checkpoint (Bruno et al., 2006; Halazonetis and Bartek, 2006). Interestingly, new binding partners of rat Che-1 (Page et al., 1999; Guo and Xie, 2004; Xie and Guo, 2004), suggest that Che-1 also acts as anti-apoptotic factor interfering with neurodegenerative processes. Consistent with these findings, we recently reported a physical interaction between Che-1 and the microtubule-associated protein tau (MAPT), which is involved in the assembly and stabilization of the neuronal cytoskeleton, leading to modulation during rat cerebellar granule neurons (CGNs) apoptosis (Barbato et al., 2003).

Here, we report that Che-1 is a NRAGE-interacting protein and characterise the protein domains directly involved in this interaction. Surprisingly, we show that ectopic co-expression of NRAGE and Che-1 affects the sub-cellular distribution of Che-1 and prevents its nuclear localization by sequestering it within the cytoplasm. Furthermore, we report that NRAGE downregulates Che-1 via an ubiquitin-dependent degradation mechanism. Finally, we provide evidence that Che-1 acts as a functional NRAGE antagonist responsible for reverting NRAGE-induced cell-death.

Results

NRAGE interacts with Che-1

We used the yeast two-hybrid system to search for proteins capable of physically interacting with the C-terminal region of Che-1. Since most of the C-terminal region of Che-1 has been conserved in evolution (Lindfors et al., 2000) a cDNA fragment encoding the C-terminal 88 amino acid residues (Che-1-C)

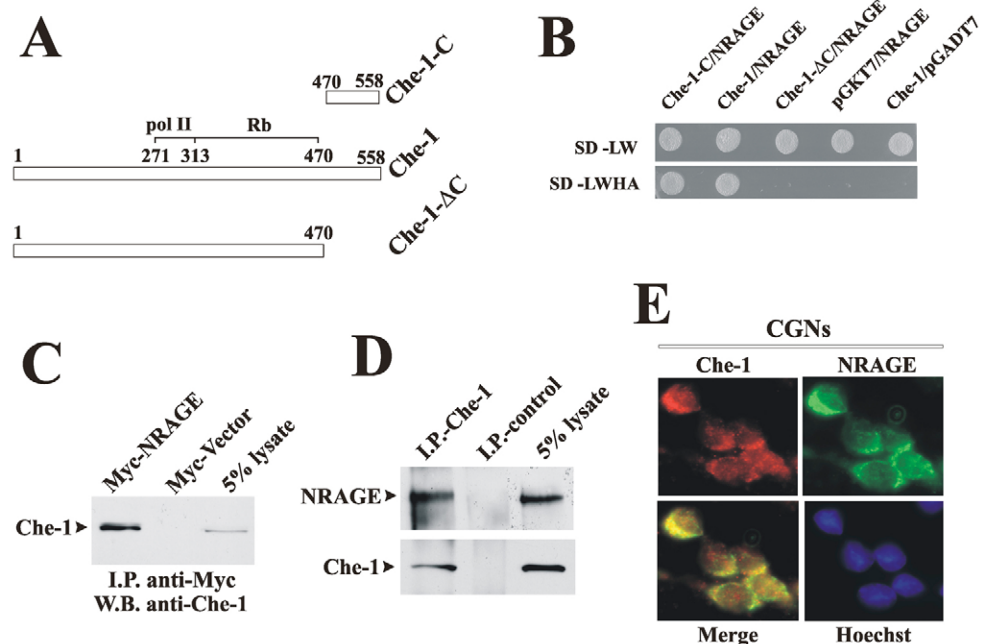

Fig. 1. NRAGE interacts with Che-1. (A) Schematic representation of Che-1 C-terminal deletion mutants and full-length proteins. Construct names are indicated on the left. (B) Yeast two-hybrid assay. AH109 yeast cells were co-transformed with the indicated constructs and plated onto SD media lacking leucine and tryptophan (–LW) to verify the expression of both bait (W^+) and prey (L^+) plasmids, or onto media lacking leucine, tryptophan, histidine and adenine (–LWHA) to examine the interaction between bait and prey proteins. (C) Western blot analysis of lysates from NIH3T3 cells, transfected with expression constructs for Myc-NRAGE or vector expressing Myc-tag only, immunoprecipitated (IP) with anti-Myc monoclonal antibody. Anti-Che-1 polyclonal antibody was used to co-immunoprecipitate endogenous Che-1 protein in Myc-NRAGE expressing cells. (D) CGNs obtained from day 8 postnatal (P8) rats cultured in vitro for 6 days were processed for co-immunoprecipitation assay. Immunoblotting with anti-NRAGE polyclonal antibody showed endogenous NRAGE protein in samples immunoprecipitated with anti-Che-1 rabbit serum (I.P.-Che-1). No immunoreactivity from samples immunoprecipitated with normal rabbit serum (I.P.-control) was detected. (E) Dual-label fluorescence microscopy was performed in fixed CGNs to obtain immunolocalization of endogenous NRAGE (green) and Che-1 (red), as described in Material and Methods. Extensive colocalization (yellow) between NRAGE and Che-1 is visualised by the merged-colour image.

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(Fig. 1A) was fused in-frame with the yeast Gal4 DNA-binding domain in the vector pGBKT7 (Clontech) and used to screen a two-hybrid cDNA library prepared from human adult brain. Twelve positive cDNAs with different degrees of interaction with Che-1 were isolated and one, encoding for NRAGE, was further characterised. To confirm this novel interaction and to define which domains of Che-1 are required for NRAGE binding, Che-1-C and PGBKT7 constructs containing full-length Che-1 or Che-1 lacking the C-terminal region (Che-1-ΔC) (Fig. 1A) were used in the yeast two-hybrid assay. Both full-length Che-1 and also Che-1-C bound NRAGE (Fig. 1B). By contrast, Che-1-ΔC did not interact with NRAGE (Fig. 1B).

To provide further evidence that Che-1 binds NRAGE in mammalian cells, an expression vector encoding Myc-tagged NRAGE (Myc-NRAGE) was transfected into NIH3T3 fibroblasts. Immunoprecipitation of Myc-NRAGE, followed by western blot analysis revealed Che-1, indicating its interaction with NRAGE (Fig. 1C). To determine whether endogenous Che-1 and NRAGE can be co-immunoprecipitated without overexpressing proteins, extracts of rat cerebellar granule neurons (CGNs) were immunoprecipitated using an anti-Che-1 antibody and then assayed by western blot for the presence of NRAGE (Fig. 1D). In order to visualise the extent of colocalization of NRAGE and Che-1 in CGNs, dual-label fluorescence microscopy was performed (Fig. 1E). Fluorescence from NRAGE antibodies revealed a typical cytoplasmic distribution with a perinuclear orientation of the protein. Merged images clearly show an extensive colocalization of NRAGE and Che-1 proteins. Taken together, both experimental strategies demonstrate that cellular Che-1 is indeed complexed with endogenous NRAGE protein indicating a physiological interaction between Che-1 and NRAGE. Therefore, we conclude that Che-1 binds NRAGE.

To determine regions of NRAGE involved in this interaction, constructs encoding Myc-tagged Che-1 (Myc-Che-1) and a series of eight Flag-tagged polypeptides covering the whole NRAGE protein (Fig. 2A) were made and co-transfected into NIH3T3 cells, and co-immunoprecipitation assays performed. In addition to the full-length Flag-NRAGE, five peptide fragments within the MDH domain bound the Myc-Che-1 fusion protein (Fig. 2B), indicating the presence of two consecutive, distinct regions within the MDH domain involved in contacting Che-1.

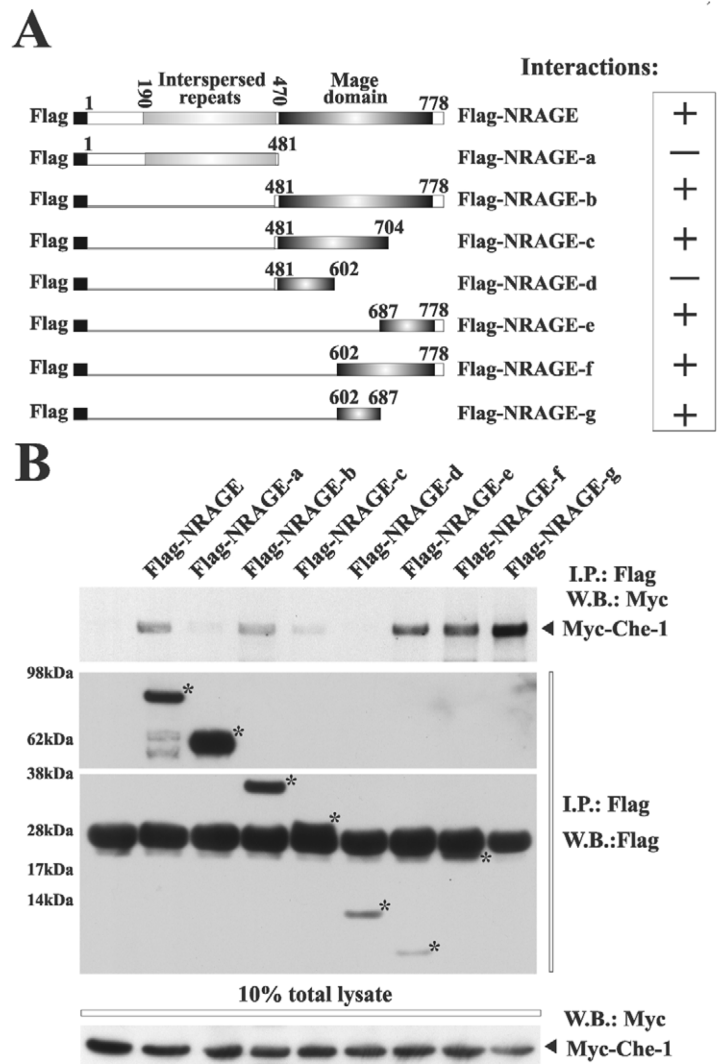

Fig. 2. The NRAGE MHD domain is required for Che-1 binding. (A) Schematic representations of both Flag-tagged NRAGE full-length and its derived deletion mutants. Construct names and binding activities are indicated on the left. (B) Cell lysates from NIH3T3 cells co-transfected with expression vectors encoding the indicated Flag-NRAGE proteins and Myc-Che-1 were immunoprecipitated with anti-Flag antibody. Immunoprecipitated samples were immunoblotted with anti-Myc monoclonal antibody to identify NRAGE domains involved in Che-1 interaction (top) and with anti-Flag antibody for the presence of Flag-NRAGE deletion mutants. Note that, the Flag-NRAGE-g mutant molecule was not revealed in the blot shown here, but under different – more appropriate – gel condition (data not shown).

NRAGE affects Che-1 nuclear localization

In primary CGNs, where we observed a particular high level of NRAGE protein, Che-1 appears to accumulate in the cytoplasmic compartment and colocalise with NRAGE (Fig. 1E). To investigate whether NRAGE is able to affect Che-1 sub-cellular localization, we performed experiments in which NIH3T3 cells were co-transfected with Myc-Che-1 fusion constructs and constructs encoding enhanced green fluorescent protein (EGFP) fused to NRAGE (EGFP-NRAGE). As shown in Fig. 3, cells overexpressing Myc-Che-1 alone exhibited typical nuclear Che-1 localization. By contrast, concomitant expression of EGFP-NRAGE and Che-1 caused Che-1 relocalization to the cytoplasm. The involvement of NRAGE in this phenomenon was confirmed by co-transfecting EGFP-NRAGE with Myc-Che-1-C or Myc-Che-1-ΔC (Fig. 1A). Myc-Che-1-C colocalises with EGFP-NRAGE to the cytoplasm, whereas Myc-Che-1-ΔC did not. Taken together, these findings strongly support the hypothesis that NRAGE is directly involved in the cytoplasmic recruitment of Che-1.

NRAGE induces ubiquitin-dependent Che-1 degradation

The data presented above suggest that the binding of NRAGE

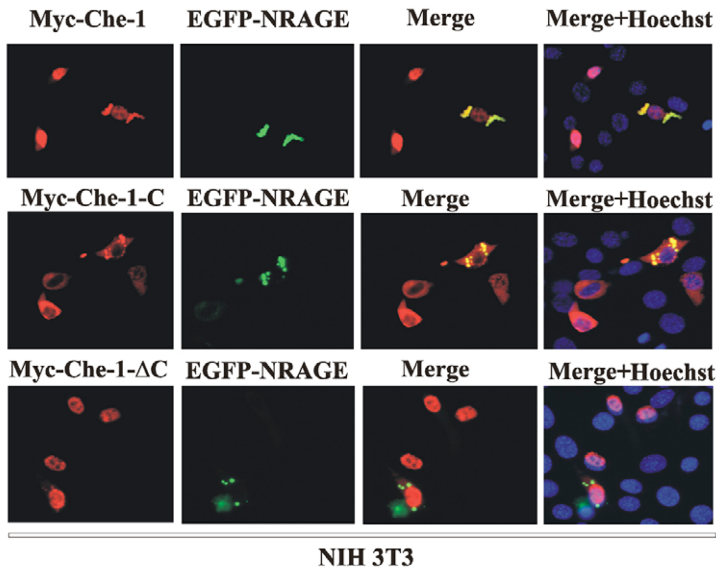

Fig. 3. NRAGE affects Che-1 cellular localization. For co-expression experiments, NIH3T3 cells were doubly transfected with EGFP-NRAGE and Myc-Che-1 or one of the two Myc-Che-1 deletion mutants (Myc-Che-1-C, Myc-Che-1-ΔC). After 48 hours, cells were fixed and immunostained with antibody against Myc-epitopes as described in Materials and Methods. Sub-cellular distribution and colocalization of Myc-tagged proteins (red) with EGFP-NRAGE (green) were analysed using fluorescence microscopy. No overlapping staining was observed in cells co-expressing Myc-Che-1-ΔC and EGFP-NRAGE fusion proteins. Note that, EGFP-NRAGE expression affects the nuclear localization of Myc-Che-1.

to Che-1 has important implications for Che-1 function. In order to evaluate the effects of NRAGE overexpression on endogenous Che-1, SY5Y neuroblastoma cells were transiently transfected with EGFP-NRAGE. As shown in Fig. 4A, in cells overexpressing EGFP-NRAGE, Che-1 was barely detectable, suggesting that NRAGE promotes Che-1 degradation. This was confirmed in HeLa cells transfected with Myc-NRAGE, in which overexpression of NRAGE led to a marked and progressive reduction of Che-1 protein expression (Fig. 4B).

We have recently observed that the proteasome-system regulates the stability of Che-1 (Bruno et al., 2006); we therefore evaluated NRAGE effects on Che-1 ubiquitylation. For this purpose, HeLa cells were co-transfected with Myc-NRAGE and hemagglutinin (HA)-tagged ubiquitin (HA-ubiquitin) encoding constructs. Immunoprecipitated Che-1 was ubiquitylated in cells transfected with Myc-NRAGE, particularly the proteasome inhibitor MG132 (Fig. 4C) was present, confirming that Che-1 levels are regulated by proteasome-system and suggesting that NRAGE can use this mechanism to negatively regulate Che-1 expression.

Che-1 counteracts NRAGE-induced apoptosis

Since NRAGE is involved in triggering cell-death, whereas Che-1 exhibits a strong anti-apoptotic activity (Page et al., 1999; Bruno et al., 2006), it is reasonable to speculate that NRAGE promotes Che-1 degradation in order to switch cells to an apoptotic phenotype. To test this hypothesis, we determined whether Che-1 overexpression can affect NRAGE-dependent apoptosis. For this purpose, we used

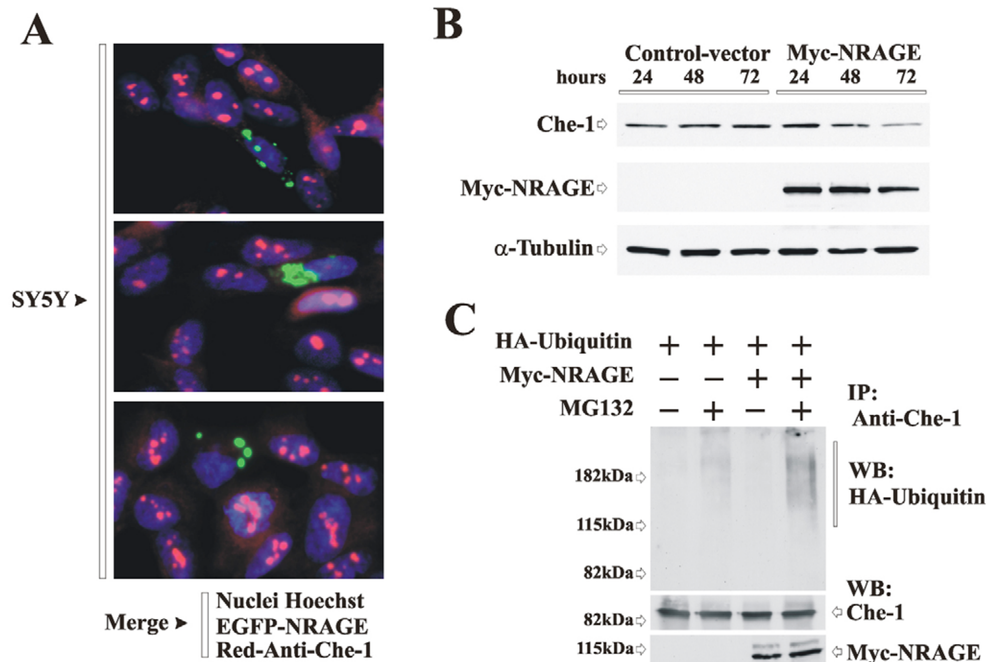

Fig. 4. NRAGE induces ubiquitin-dependent Che-1 degradation. (A) Three representative sections showing endogenous Che-1 in SY5Y cells transfected with expression vector expressing EGFP-NRAGE fusion protein, subjected to indirect immunofluorescence analysis as described in Materials and Methods. In the merged image, no fluorescence signal derived from Che-1 (red) was detectable in cells overexpressing EGFP-NRAGE (green). (B) Western blot analysis of HeLa cells transfected with either Myc-NRAGE or with empty control vector, harvested at the indicated times and analysed on SDS-PAGE. The immunoreactivity of Che-1 and Myc-NRAGE proteins was detected by probing blots with anti-Che-1 and anti-Myc antibodies, respectively. Samples were normalised for protein content by reprobing blots with anti α-tubulin. (C) In vivo ubiquitylation assay was performed with HeLa cells transiently co-transfected with plasmids expressing HA-tagged ubiquitin and Myc-NRAGE. After transfection, cells were treated without or with 20 μM MG132 for 4 hours and subjected to immunoprecipitation (IP) and western blotting (WB) analysis with the indicated antibodies. Che-1-ubiquitin conjugates were detected by using anti-HA monoclonal antibody.

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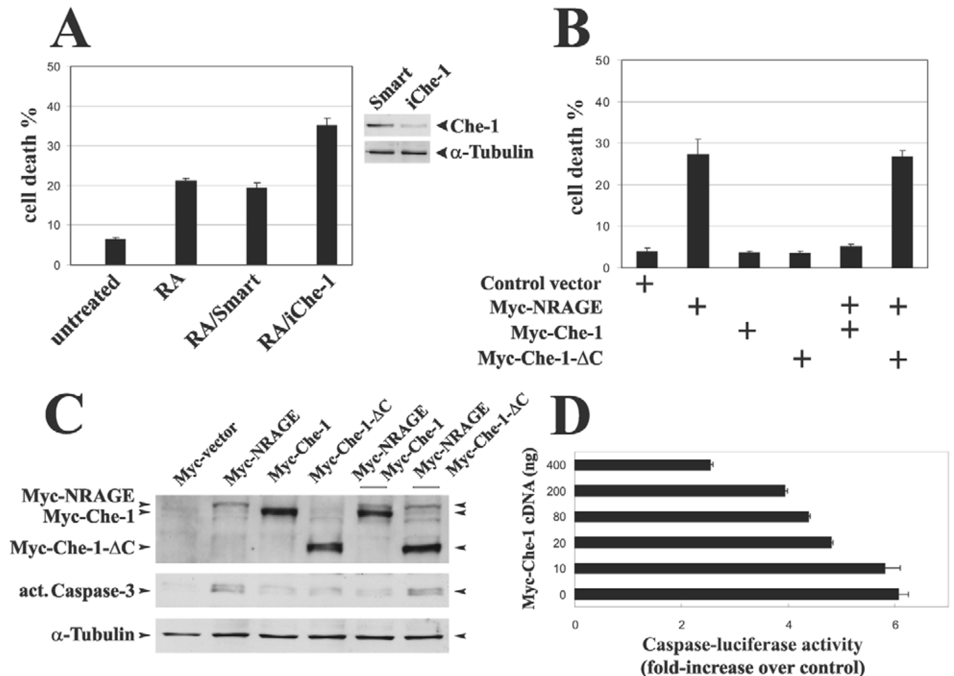

Fig. 5. Che-1 counteracts NRAGE induced apoptosis. (A) P19 cells were transfected with siRNA targeting Che-1 (iChe-1) or with a non-specific siRNA control pool (Smart). After 24 hours, apoptosis was induced with 1 μM retinoic acid (RA). Cell-death was measured by Trypan Blue exclusion after 48 hours. Transfected cells were subjected to western blot analysis to verify reduced Che-1 expression levels after siRNA treatment (top). Note that cells with low Che-1 levels are more responsive to RA treatment. (B) P19 cells were transfected with Myc-tag expression vectors for NRAGE, Che-1 and Che-1-ΔC proteins. After 48 hours, cell death was measured by Trypan Blue exclusion. Each point represents the mean of three independent experiments performed in duplicate. Note that, in co-expressing cells Che-1 but not its deletion mutant Che-1-ΔC abrogates the ability of NRAGE to induce cell death. (C) Whole-cell extracts from transfected P19 cells were subjected to western blot analysis. Immunoblotting with anti-Myc monoclonal antibody was performed to visualise expression of Myc-tagged proteins, NRAGE (upper band), Che-1 and Che-1-ΔC (middle and lower bands, respectively). The same samples were monitored for endogenous level of activated caspase-3 using antibody against cleaved caspase-3. Samples were normalised for protein content by reprobing blots with a monoclonal antibody to α-tubulin. (D) Caspase-3 activity was measured in P19 cells in which Myc-NRAGE was co-transfected with increasing amounts of Myc-Che-1, as indicated. Caspase activity is shown as the n-fold increase in caspase activation by comparing the levels of luciferase activity of transfected cells with those of untreated control cells. Equal numbers of cells were analysed. Each point represents the mean of three different experiments performed in triplicate.

P19 mouse embryonal carcinoma cells, because recent research showed that NRAGE activity is necessary and sufficient for retinoic acid (RA)-induced apoptosis (Kendall et al., 2005) in these cells. NRAGE knockdown in P19 cells fail to activate caspase-3 and this correlates with enhanced cell survival after RA treatment (Kendall et al., 2005). By contrast, we found that P19 cells whose Che-1 expression was diminished by specific small interfering RNA (siRNA) were more responsive to RA-induced apoptosis (Fig. 5A). This result was expected owing of the anti-apoptotic function of Che-1, and suggests that NRAGE and Che-1 have opposite functions within the same apoptotic pathway.

Then, because NRAGE-overexpressing P19 cells undergo apoptosis even in absence of RA (Kendall et al., 2005), we determined whether Che-1 overexpression can affect NRAGE-induced cell-death. As shown in Fig. 5B, cell death induced by the expression of Myc-NRAGE was completely counteracted by the co-expressing Myc-Che-1. By contrast, Myc-Che-1-ΔC – which does not interact with NRAGE – failed to revert NRAGE-induced cell death, indicating that the antagonizing effect of Che-1 on NRAGE needs the direct interaction of the two proteins. Then, to confirm the correlation between the apoptotic phenotype and NRAGE overexpression, activation of caspase-3 was determined by western blotting. Consistent with data discussed above, Myc-Che-1 protein was able to neutralize the caspase-3 activation induced by Myc-NRAGE in P19 cells. By contrast, no counteracting effect was observed by Myc-Che-1-ΔC co-expression (Fig. 5C). Furthermore, we measured NRAGE-induced caspase-3 activity when Che-1 was co-expressed, using a caspase assay based on luminescence. In P19 cells, a constant amount of Myc-NRAGE was co-transfected with increasing amounts of Myc-Che-1. As shown in Fig. 5D, Myc-Che-1 significantly decreases NRAGE-induced caspase-3 activation in a dose-dependent manner. These data confirm that Che-1 strongly interferes with NRAGE-induced apoptosis and that, under permissive conditions, NRAGE counteracts the anti-apoptotic activity of Che-1 by triggering its degradation.

Discussion

Some members of the MAGE gene family are attractive targets for cancer immunotherapy because their expression is restricted to tumours. However, recent investigations have focused on the physiological role of a subtype of MAGE genes, classified as type II, expressed in normal tissue and involved in maintaining critical steps during development (Barker and Salehi, 2002). Among the type II MAGE genes, NRAGE has been recently described as an inducer of neuronal cell-death, triggering mitotic arrest and apoptosis during neurons genesis. Although much remains to be elucidated, evidence suggests that NRAGE acts as an adaptor protein in the assembly of multi-protein complexes that have a negative impact on cell life (Sasaki et al., 2005). NRAGE-interacting partners demonstrate that NRAGE leads to cell death by being recruited to the apoptosis-associated membrane receptors $p75^{NTR}$ and UNC5H1 (Salehi et al., 2000; Salehi et al., 2002; Williams et

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al., 2003), or by binding the apoptotic inhibitor XIAP (Jordan et al., 2001). This supports the concept that NRAGE may contribute to one or more phases of apoptotic signalling, because it physically interacts with pro-apoptotic and also anti-apoptotic proteins to mediate both upstream and downstream events that follow commitment to apoptosis.

We provide new insights into the biological role of the MAGE gene family, by showing that Che-1 is a new NRAGE interaction partner. NRAGE binds to the C-terminal region of Che-1 through its MHD domain (Figs 1, 2) and targets Che-1 for the ubiquitin-proteasome pathway by strongly affecting Che-1 expression level (Fig. 4). We support these data of sub-cellular localization studies by showing that NRAGE sequesters Che-1 in the cytoplasm, preventing its nuclear localization (Fig. 3). The functional relevance of Che-1 degradation induced by NRAGE was unveiled in vitro by showing that Che-1 interferes with NRAGE-induced cell death (Fig. 5). These results support the concept that Che-1 and NRAGE proteins play opposite functions within the same apoptotic pathway, and that the ratio of Che-1 to NRAGE modulates cell survival and apoptosis. Indeed, an anti-apoptotic activity for Che-1 has already been discussed. In rat, AATF antagonises prostate apoptosis response-4 (Par-4)-induced apoptosis, associated with neuronal degeneration observed in Alzheimer disease. AATF binds Par-4 through its leucine-zipper domain and its expression blocks aberrant production and secretion of neurotoxic amyloid β peptide 1-42 (Guo and Xie, 2004).

We have recently demonstrated also that Che-1 activity is regulated through post-transcriptional modifications, and that its degradation via the proteasome system is a key step in induction of apoptosis (Bruno et al., 2006; Halazonetis and Bartek, 2006). Despite their apparent opposite roles in cell cycle control, NRAGE and Che-1 share important features. Both were highly conserved during mammalian evolution and are ubiquitously expressed in adult tissue (Chomez et al., 2001; Sasaki et al., 2005). Both proteins interact with and modulate cell cycle regulatory molecules, involved in survival and apoptosis. Che-1 binds Rb and inhibits its growth-suppression activity by removing HDAC1 from E2F-target promoters (Bruno et al., 2002). NRAGE interacts with and regulates necdin (Kuwajima et al., 2004), a MAGE protein described as a Rb-like growth suppressor, highly expressed in postmitotic neurons and associated with human neurological diseases (Taniura et al., 1998; Sasaki et al., 2005). NRAGE activity is also regulated via the proteasome-system. In fact, Praja1 directly promotes the ubiquitin-proteasome-dependent degradation of NRAGE and this is crucial for Dlx5-dependent transcriptional activity (Sasaki et al., 2002). Moreover, NRAGE expression is subject to strong post-transcriptional regulation during murine embryogenesis and exhibits temporal and spatial tissue-specific regulation, whereas NRAGE mRNA is constitutively expressed in all tissues during development though adulthood (Kendall et al., 2002).

In conclusion, we hypothesise that NRAGE and Che-1 act as opposite programme executors, respectively ensuring the commitment to specific cellular programs that regulate cell death and survival. Since, perfect coordination of cell division and cell death is essential for normal tissue growth during development, functional defects in NRAGE or Che-1 proteins may result in reciprocal perturbation of these signalling pathways, significantly contributing to cellular pathology.

Materials and Methods

Yeast two-hybrid selection

The cDNA region encoding the C-terminal part of human Che-1 (aa 470 to 558) was cloned into the pGBKT7 vector (Clontech) in frame with the *Gal4* DNA-binding domain (pGBKT7-Che-1-C) and used as bait for yeast two-hybrid screening. Yeast strain AH109, bearing UASg-His3, UASg-ADE2 and UASg-LacZ as reporter genes, was co-transformed with the bait pGBKT7-Che-1-C and an adult human brain cDNA library (Clontech) fused to the *GAL4* activation domain, in the vector pGAD10 (Clontech). Transformation was performed using the lithium acetate method (Gietz et al., 1992). Cells were plated directly on minimal synthetic-defined (SD) medium: 2% glucose, 0.67% Bactoyeast nitrogen base (Difco), supplemented with the required bases and amino acids, except tryptophan (Trp), leucine (Leu), adenine (Ade) and histidine (His). Plates were incubated for 7 days at 30°C, at which time $Ade^{+}His^{+}$ transformants were isolated. The $Ade^{+}His^{+}$ colonies were replica-plated on SD -Leu -Trp -His -Ade medium, and $LacZ^{+}$ clones identified by a filter-lifting assay for β-galactosidase activity (Gietz et al., 1992). Plasmid DNA was prepared from candidate clones and electroporated into *E. coli* XL1-blue competent cells (Stratagene). The recovered library-derived plasmids were then analysed as positive candidates. Liquid Y187 yeast cultures were assayed for beta-galactosidase activity to quantify two-hybrid interactions (Schneider et al., 1996). Bait interaction specificity was further analysed cloning in pGBKT7 vector both the complete open reading frame of human Che-1 (pGBKT7-Che-1) and the Che-1 C-terminus deletion mutant from aa 1 to aa 470 (pGBKT7-Che-1-ΔC).

Plasmids

The Myc-tagged pCS2-Che-1 (Myc-Che-1) construct and its deletion mutants Myc-Che-1-C and Myc-Che-1-ΔC were generated by PCR amplification. Full-length human EGFP-NRAGE (vector-pEGFP-N) (Clontech), Myc-NRAGE (vector pCS2MT), Flag-NRAGE (vector-pCMV-Tag2A) (Stratagene) and its seven deletion mutants (designated as Flag-NRAGE-org) were generated by PCR amplification and/or sub-cloning. The HA-ubiquitin construct was a kind gift from Dirk Bohmann (EMBL; Heidelberg). All constructs prepared were controlled by DNA sequence obtained by GeneLab Service (Enea-Casaccia).

Polyclonal antibody production

A 354-aa-long fragment, corresponding to the C-terminal region of human Che-1, was cloned in pQE30 vector, expressed in bacteria and purified according to the manufacturer's protocol (QIAGEN). For mouse antiserum production, mice were immunised twice at an interval of 1 week with 100 μg purified protein in Freund's adjuvant. The antiserum was collected 10 days after the last injection (Harlow and Lane, 1988).

Cell culture and transfection

NIH3T3 mouse fibroblasts and HeLa human cervical adenocarcinoma cell line were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco). Human SY5Y neuroblastoma cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. P19 mouse embryonal carcinoma cells were cultured in α-MEM (Gibco), supplemented with 2.5% heat-inactivated FBS and 7.5% heat-inactivated calf serum (CS; HyClone). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Total amounts of transfected DNA were equalised using empty vectors.

RNA interference was performed as previously described (Bruno et al., 2002). The siRNA-mediated interference experiments targeting Che-1 expression were performed by transfecting cells with Che-1 SMART pool siRNA reagent or, as negative control, with non-targeting siRNA SMART pool (Dharmacon), using Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions.

For induction of apoptosis, P19 cells were cultured in bacteriological-grade culture dishes at a concentration of 10^5 cells per ml, in growth media supplemented with 1 μM all-trans-retinoic acid (RA; Sigma) for 48 hours. Cell viability was determined by Trypan Blue exclusion. An equal volume of 0.4% Trypan Blue solution (Gibco) was added to the cell culture and both total and stained cells were counted using a hemocytometer.

Immunofluorescence

48 hours following transfection, cells were subjected to immunofluorescence assays. Briefly, cells were fixed in 4% formaldehyde for 15 minutes and then permeabilised with 0.2% Nonidet P-40 in phosphate-buffered saline (PBS) for 10 minutes. For detection of epitope-tagged proteins, cells were incubated with a 1:3 dilution, in blocking buffer (1% bovine serum albumin, BSA, in PBS) of anti-Myc hybridoma-conditioned medium (9E10) for 60 minutes, followed by Alexa-Fluor-594-conjugated anti-mouse IgG (Molecular Probes). For detection of endogenous Che-1, cells were incubated for 60 minutes with a 1:200 dilution in blocking buffer of rabbit polyclonal antibodies specific for Che-1 protein (Fanciulli et al., 2000). Alexa-Fluor-594-conjugated anti-rabbit IgG (Molecular Probes) was used as secondary antibody. In dual-staining experiments, colocalization of NRAGE and

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Che-1 proteins was performed incubating fixed cells with the anti-NRAGE and the anti-Che-1, rabbit polyclonal and mouse polyclonal antibodies respectively. As secondary antibodies were used: Alexa-Fluor-488-conjugated anti-rabbit IgG (Melecular Probes) to label anti-NRAGE antibody and Alexa-Fluor-594-conjugated anti-mouse IgG to label anti-Che-1 antibody.

Nuclei were visualised by staining with Hoechst dye 33258 (1 μg/ml) (Sigma). All immunofluorescence steps were performed at room temperature. Stained specimens were examined by conventional epifluorescence microscopy (Olympus BX51; Tokyo, Japan).

Immunoblotting

For the biochemical analysis, we used the following antibodies: anti-Che-1 polyclonal antibody (Fanciulli et al., 2000), anti-NRAGE polyclonal antibody (Upstate), anti-Myc monoclonal antibody (9E10 clone, hybridoma-conditioned medium), monoclonal antibody against α-tubulin (Sigma) and polyclonal antibody cleaved caspase-3 (Cell Signaling Technology). Whole-cell extracts were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA 1% Triton X-100, 1 mM DTT) supplemented with 1 mM PMSF and a proteinase inhibitor cocktail (Complete™, Roche). Total soluble proteins concentrations were determined by Protein Assay (Bio-Rad). 25 μg of cell lysate were electrophoresed through SDS-polyacrylamide gel (SDS-PAGE) under reducing conditions and transferred onto nitrocellulose membranes (Schleicher&Schuell). Blots were probed using appropriate dual immuno-staining with primary antibody and horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology). The immunoreactive bands were visualised by chemiluminescence (ECL plus; Amersham), according to the manufacturer's instructions.

Co-immunoprecipitation

Immunoprecipitation assays were performed using the following antibodies: anti-Myc monoclonal antibody-agarose beads (Clontech), anti-Che-1 polyclonal antibody (Fanciulli et al., 2000) and agarose-covalently attached anti-Flag M2 (Sigma). Briefly, cell lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% NP-40) supplemented with 1 mM PMSF and a mixture of proteinase inhibitors (Complete™, Roche) were immunoprecipitated using standard procedures. Immunoprecipitated proteins eluted from beads by boiling in Laemmli buffer for 10 minutes were electrophoresed by SDS-PAGE. Blots were subjected to immunoblotting as described above.

In vivo ubiquitylation assay

HeLa cells were co-transfected with Myc-tagged NRAGE and HA-tagged ubiquitin expression constructs. After 20 hours, cells were treated with or without 20 μM MG132 (Sigma) for an additional 4 hours to block proteasome activity. Subsequently, cells were harvested and subjected to immunoprecipitation assay. Briefly, whole-cell extracts (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 5 mM EDTA) supplemented with 1 mM PMSF, and a protease inhibitor cocktail (Complete™, Roche), were immunoprecipitated with polyclonal anti-Che-1 antibody and, after protein-A-Sepharose (Pharmacia) adsorption, immunoprecipitated proteins were eluted from beads by boiling in Laemmli buffer for 10 minutes. Proteins were separated by SDS-PAGE and immunoblotted by using monoclonal anti-HA antibody (Santa Cruz).

Caspase activity

Caspase-3 activity was measured using the Caspase-Glo 3/7 assay (Promega), according to the manufacturer's instructions. Briefly, P19 cells were transiently transfected with Myc-NRAGE alone or with increasing amounts of Myc-Che-1. After 48 hours, equal numbers of cells were seeded in 96-wells plates and Caspase-Glo 3/7 reagent was added to each well. Plates were then incubated at room temperature for 1 hour and luminescence was measured using a multi-label counter (Victor [3] Wallac).

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