The p75 neurotrophin receptor interacts with multiple MAGE proteins*

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Running Title: Multiple MAGE Interactors for p75
Summary

The p75 neurotrophin receptor has been implicated in diverse aspects of neurotrophin signaling, but the mechanisms by which its effects are mediated are not well understood. Here we identify two MAGE proteins, necdin and MAGE-H1, as interactors for the intracellular domain of p75, and show that the interaction is enhanced by ligand stimulation. PC12 cells transfected with necdin or MAGE-H1 exhibit accelerated differentiation in response to NGF. Expression of these two MAGE proteins is predominantly cytoplasmic in PC12 cells, and necdin was found to be capable of homo-dimerization, suggesting that it may act as a cytoplasmic adaptor to recruit a signaling complex to p75. These findings indicate that diverse MAGE family members can interact with the p75 receptor; and highlight type II MAGE proteins as a potential family of interactors for signaling proteins containing type II death domains.

Keywords: MAGE / necdin / neurotrophin / neuronal differentiation / p75
**Introduction**

Neuronal responses to activation of the p75 neurotrophin receptor range from enhanced outgrowth to increased cell death, and p75 null mice exhibit a plethora of defects in both neuronal and non-neuronal systems (1,2). However, the primary interactors and signaling mechanisms activated by p75 are not well understood (3). Although p75 belongs to the TNF receptor superfamily, its intracellular type II death domain does not self-aggregate (4) and does not interact with the major binding partners of type I death domains. Identification of specific signaling partners for p75 has been frustrating, as this particular receptor is a difficult bait in conventional yeast two-hybrid. Nonetheless seven candidate interactors have been reported so far for p75 (3,5). Most of these candidate interactors lack known catalytic domains, suggesting that they must recruit additional binding partners to form a functional signaling complex. There are no general sequence or functional homologies between the described p75 interactors, and the physiological significance of most of them remains to be established.

In order to identify biologically relevant interactors for p75, we used the Ras Rescue System (RRS) for protein interaction trapping in yeast (6). RRS selects for protein interactions in the membrane proximal region of the cytoplasm, and there is no requirement for nuclear or other translocations of the binding partners. The system thus provides an appropriate screening method for the membrane-proximal binding of a receptor intracellular domain with its primary interactors. Our RRS screens identified two MAGE family proteins as novel p75 interactors; and highlight type II MAGE proteins as a potential interactor family for type II death domain proteins.
Materials and Methods

RRS screens, cDNA cloning and sequence analysis- Plasmids, yeast strains and screening protocols for RRS were as described (6). Linker (K274RWN to SLPL342) and death domain (T343KRE to ESLC416) regions of p75-ICD were generated by PCR from rat p75. Synthetic oligonucleotides were designed to encode the nine residue (SESTATSPV) tail domain of p75-ICD. A pADH death domain bait was used to screen a mouse embryonic head cDNA library in pMyr. Alignments of MAGE sequences and phylogenetic trees were constructed using ClustalX.

Expression analysis- Rat tissues for Western blot analysis were flash frozen in liquid nitrogen, macerated to a fine powder, and solubilized in 10mM Tris pH8.0, 150mM NaCl, 10% glycerol, 1mM PMSF, 1mM orthovanadate and proteinase inhibitors (Merck) containing 1% NP-40. Extracts were separated on SDS-PAGE, blotted, and probed with anti-necdin antibodies.

Cell culture, transfections and generation of cell lines- PC12 cells were maintained in DMEM containing 6% fetal calf serum and 6% horse serum. For induction of neuronal differentiation, the cells were seeded at a density of 10-15 × 10^5 cells per cm^2 and differentiated for 2-4 days with NGF (5 ng/ml- 50 ng/ml). Transient transfection of COS cells was with DEAE-dextran.

Transient and stable transfections of PC12 cells lines were carried out by electroporation with 80 µg of DNA per 10^7 cells in 0.4-cm cuvettes on an ECM-300 (BTX) set to 320 V, 6 msec pulse length. Stably transfected PC12 were selected under 0.5 mg/ml Geneticin for approximately 4 weeks until resistant colonies appeared.

Immunoprecipitation (I.p.), Westerns, and Immunofluorescence- Cells were lysed for i.p. in 10mM Tris pH8.0, 150mM NaCl, 10% glycerol, 1mM PMSF, 1mM orthovanadate and proteinase inhibitors (Merck) containing either 0.7% CHAPS or 1% NP-40. Lysates were spun at 10,000 × g for 5 min, and then precleared with 30 µl of a 50% suspension of Protein-G sepharose beads (Pharmacia) before incubation with primary antibody. Immunoprecipitates were eluted
with 80 µl of 1M Guanidin-HCl in 50 mM Tris pH 8.0, before running on SDS-PAGE. Primary antibodies for precipitation: anti-p75 MC192 (Alomone Labs), anti-p75 REX (7), monoclonal anti-HA (Roche). Antibodies and dilutions for Westerns: anti-necdin polyclonal NC243 (8) at 1:5000; anti-necdin polyclonal MNF (9) at 1:1000; anti-HA polyclonal (Santa Cruz) at 1:200; anti-p75 polyclonal 9651 at 1:100; anti-phospho-cdc2 polyclonal (R&D Systems) at 5 µg/ml, and anti-TrkA RTA (10) at 1:1000. Cells were fixed for immunofluorescence microscopy for 20 min with ice cold 3% paraformaldehyde in PBS at room temperature, quenched in 0.1M glycine PBS for 15 min, and permeabilized for 10 min in PBS, 5% donkey serum, 1mg/ml BSA, 0.2% saponin. Permeabilized cells were incubated with anti-HA (1:4000) for 2 hr at room temperature followed by donkey anti-rabbit rhodamine-redX (RRX) conjugated secondary antibody (1:500, Jackson) for 1 hr at room temperature.

**Results**

A mouse embryonic head RRS library was screened against three baits comprising the death domain, the 69 residue juxtamembrane 'linker', and the nine residue C-terminal 'tail' of p75. These screens yielded interacting candidates only for the death domain bait. One of the clones encoded the C-terminal 81 amino acid residues of necdin (11), a MAGE family member thought to act as a cell cycle regulator. Further yeast co-transfections of necdin with the complete panel of p75-ICD subdomains revealed that necdin interacts with both the linker and death domain segments of p75 (Figure 1A). The sequence homologies between necdin and NRAGE, which was previously identified by Barker and colleagues as a p75 interactor (12), prompted us to examine additional family members. Unfortunately, a number of MAGE constructs (e.g.- NRAGE, MAGEL2) were found to associate with yeast plasma membrane, thus precluding their use in the RRS system. MAGE-H1/APR-1 (13) was found to interact robustly with the p75 death domain by RRS (data not shown). Thus, at least three type II MAGE proteins (Fig. 1B) are capable of interacting with p75. Tissue RNA arrays (data not shown) and in silico profiling reveal extensive overlaps in the expression profiles of these genes. Adult rats express
robust levels of necdin protein in the hypothalamus and in dorsal root ganglia (DRG) (Fig. 1C), whereas necdin levels during development are much lower.

Co-i.p. experiments were then carried out in both COS and PC12 cells to confirm the p75-necdin and p75-MAGE-H1 interactions. In accordance with the RRS results, p75 co-precipitated with necdin or MAGE-H1 from transfected COS cells (Figure 2A) or PC12 cells (Fig. 2B). In PC12 cell lines stably transfected with necdin, application of either NGF or BDNF clearly enhanced the interaction of necdin with p75 (Figure 2C). Necdin protein levels did not change during the course of this experiment (data not shown). Finally, a p75-necdin interaction at endogenous levels of expression was demonstrated by co-i.p. from lysates of freshly dissected adult rat DRG (Fig. 2D).

NRAGE was previously found to affect p75-trk association (12), and we therefore examined whether necdin had similar effects in transiently transfected COS cells stimulated with NGF. TrkA co-precipitated together with p75 in the absence of necdin, and this association was lost in necdin-expressing cells (Fig. 2E). Thus, by interacting with p75, necdin can modulate association of p75 with other neurotrophin receptors.

Necdin, like other MAGE family proteins, does not have any known intrinsic catalytic activity, and is likely to signal by recruiting other molecules to a p75 signaling complex. We therefore conducted additional RRS screens using necdin as bait, and identified a number of novel ESTs of unknown function as necdin interactors (data not shown). Interestingly, one known gene obtained in this screen was necdin itself (Fig. 2F, left). Homo-oligomerization of necdin was confirmed by co-i.p. of differentially tagged necdin proteins from transfected COS cells (Fig. 2F, right).

PC12 cells are a well-established model for NGF-induced differentiation and do not express endogenous necdin (14). We therefore examined the influence of transient transfection of necdin or MAGE-H1 on NGF effects in PC12 cells. Both necdin and MAGE-H1 transfected
cells responded to NGF by a dose-dependent increase in neurite extension, which was greater than observed in vector-transfected cells (Figure 3A). This accelerated neurite extension was quantified in stable necdin-PC12 lines (Figure 3B). Necdin expression levels did not change over the time course of these experiments (data not shown). No effect was observed on the number of differentiated cells. Enhanced neurite extension was not observed when BDNF was applied instead of NGF, or upon application of NGF or BDNF to transfected nnr5 cells (data not shown).

In order to establish whether the enhanced neurite outgrowth might be a consequence of accelerated differentiation, we quantified the phosphorylation levels of cdc2, a cell cycle marker (15). Phosphorylation of cdc2 was decreased approximately five-fold more in necdin-PC12 cells as compared to vector-PC12 cells 24 hr after NGF application (Figure 3C), indicating that the effect of the NGF-p75-necdin signal in these cells is indeed to accelerate differentiation. Finally, we examined the subcellular distribution of necdin expression in both cycling and differentiated PC12-necdin cells. As shown in Fig. 3D necdin protein was primarily cytoplasmic in cycling PC12, and this did not change upon differentiation. An appreciable amount of necdin was found in processes and growth cones of differentiated PC12 (Fig. 3D, lower). Microscopy after harsher permeabilization or Western blot of nuclear extracts revealed lower amounts of necdin in the nucleus (data not shown). The low levels of nuclear necdin did not change after NGF stimulation, or during differentiation (data not shown).

Discussion

These results establish two members of the type II MAGE gene family, necdin and MAGE-H1, as p75 interactors. Necdin and MAGE-H1 are related to NRAGE, which was previously identified as a p75 interactor (12). All three interactors contain a conserved sequence called the MAGE homology domain (MHD), a defining feature of the MAGE gene family (5). Since both necdin and MAGE-H1 are essentially comprised of only the MHD, this structural domain determines the interaction of MAGE proteins with p75. Since most type II MAGE proteins are at least as closely related in sequence to MAGE-H1 or necdin or NRAGE as the latter three are to each other (Figure 1B), additional members of this subfamily may also
associate with p75. The death domain of p75 belongs to the structurally distinct type II fold (4), and the possibility that type II MAGE proteins form a family of binding partners for type II death domain proteins is an intriguing avenue for future study. The multiplicity of potential interactions between these two families may create a compensatory network that is physiologically robust, but at the same time refractory to reductionist analysis. For example, the three MAGE interactors for p75 have overlapping expression profiles, which may complicate the analyses of null alleles for these genes, at least in the context of p75 signaling.

We examined the effects of NGF-induced p75 signaling via the two MAGE interactors in PC12 cells, and found that they cause an acceleration of neuronal differentiation (Figure 3). The p75-necdin signal must synergise with a TrkA signaling pathway, since the effects of necdin on differentiation are not seen in nnr5 cells, which lack TrkA, or upon application of BDNF to PC12 cells. Importantly, the effects of necdin on PC12 differentiation were seen only in ligand-stimulated cells, and are therefore consequent to NGF signaling. Very recently published data from antisense experiments on embryonic DRG neurons also support a differentiation or survival-promoting role for necdin in NGF-responsive neurons (9). This could be due to a change in trk-p75 association (Fig. 2E), thus éfreeingí TrkA from some inhibitory constraint imposed by p75; or could be due to an independent signal emanating from p75 and transduced by necdin. The propensity of necdin for homo-oligomerization (Fig. 2F), and its cytoplasmic localization in PC12 (Fig. 3D), both support the likelihood of necdin acting as a cytoplasmic adaptor for a p75-induced signaling complex. It should however be noted that this does not rule out a nuclear localization or role for necdin in other cell types (16).

Although these in vitro analyses suggest a role for a p75-necdin signal in neuronal differentiation, what might be the in vivo significance of such signaling? The most prominent sites of necdin expression in rodents are the DRG and hypothalamus (Fig. 1C), both of which are known to also express p75 and trk receptors. A significant (and unexplained) loss of DRG neurons has been described in p75 null mice (17), and specific hypothalamic defects have been described in one line of necdin mutant mice (18). Comparative analyses of these mutant mice
may shed light on this interesting question.

To summarize, we have identified necdin and MAGE-H1 as novel interactors for the intracellular domain of p75, and suggest that type II MAGE proteins will feature prominently in p75 signal transduction.

Acknowledgements
We are grateful to Phil Barker, Moses Chao, and Louis Reichardt for generous gifts of antisera, and to Ríada Massarwa for excellent technical assistance.

References

Footnotes

*This work was supported by grants from the Israel Science Foundation (647/01) and the European Union Fifth Framework Program (QLRT-1999-573) to M.F., and a short-term EMBO fellowship (to M.T.). M.F. is the incumbent of the Daniel Koshland Sr. Career Development Chair at the Weizmann Institute of Science.
Figure Legends

Fig. 1: Necdin, a MAGE family protein, interacts with p75.

(A) Myristylated linker and death domain regions of p75 interact with necdin-Ras to rescue transformed yeast grown at 36°C, whereas no interaction is observed with the tail domain.

(B) Phylogenetic tree showing relationships between human type II MAGE genes, * denotes those shown to interact with p75.

(C) Western blot analysis of necdin expression in rat tissues. 250 µg of tissue protein was loaded in each lane, and the blot was probed with the anti-necdin MNF antibody. Equal loading was verified by stripping and reprobing the blot with anti-tubulin antibody (not shown).

Fig. 2: Association of necdin and MAGE-H1 with p75 in mammalian cells.

(A) Interaction of p75 with MAGE proteins transiently expressed in COS cells. P75 was co-transfected with necdin or MAGE-H1-HA. As a control MAGE proteins were co-transfected with empty vector. Immunoblotting of cell lysates shows equal expression of MAGE proteins with or without p75. P75 was immunoprecipitated with the MC192 antibody and blots were tested for presence of MAGE proteins with anti-necdin and anti-HA antibodies. MAGE proteins were immunoprecipitated only in the presence of p75.

(B) Interaction of p75 with overexpressed MAGE proteins in transiently transfected PC12 cells. PC12 were electroporated with constructs expressing MAGE proteins, and co-immunoprecipitations were carried out with the indicated antibodies. An irrelevant antibody was used as control (- in the figure). Both necdin and MAGE-H1 were
co-precipitated with the p75 antibody, and p75 was co-precipitated with anti-MAGE antibodies.

(C) Interaction of p75 with necdin is enhanced by NGF or BDNF in a stably transfected PC12 line. A stable PC12 cell line expressing HA-necdin was treated with 100ng/ml of NGF or BDNF for 2 hr, followed by immunoprecipitation with the MC192 antibody. Western blotting for necdin reveals increasing amounts of co-precipitated necdin upon ligand stimulation.

(D) Co-immunoprecipitation of necdin with p75 from lysates of freshly dissected adult rat DRG. 40-45 DRG were used for each lane, and lysates were precipitated with the anti-p75 monoclonal MC192, followed by Western with the anti-necdin polyclonal MNF.

(E) Co-immunoprecipitation of TrkA with p75 from transiently transfected COS cells. NGF (100 ng/ml) was added to the cells 44 hr after transfection, and lysates were processed for co-i.p. after 4 hr incubation. P75 pull-down was with the REX antibody, and TrkA detection was with the RTA antibody.

(F) Necdin-necdin interaction exemplified by RRS at 36°C (left panels), and by co-i.p. from transiently transfected COS cells (right panel).
Fig. 3: Necdin and MAGE-H1 accelerate the differentiation of PC12 cells.

(A) PC12 cells were transiently transfected with necdin, MAGE-H1, or vector control, and incubated for three days in 50 ng/ml NGF.

(B) Differentiation of two necdin-expressing stable PC12 lines, and two vector-transfected stable lines, in the presence of 50 ng/ml NGF for two days. Average ± S.E.M. is shown for a representative experiment. 120-180 differentiated cells were measured for each point.

(C) Levels of phospho-cdc2 in necdin versus vector transfected PC12 cells, after 24 hours in 50 ng/ml NGF. Upper panel shows a representative experiment, while the lower panel shows average ± S.E.M. (n = 3).

(D) Immunofluorescence microscopy reveals predominantly cytoplasmic necdin protein in both cycling (upper) and differentiated (lower) PC12-necdin cells.