R-esp1, a rat homologue of *Drosophila* Groucho, is differentially expressed after optic nerve crush and mediates NGF-induced survival of PC12 cells

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Abstract The differential display reverse transcription polymerase chain reaction method was used to detect alterations in gene expression in the superior colliculus after optic nerve crush in adult rats. One of the most prominent changes observed was the selective induction of R-esp1, a homologue of the *Drosophila* enhancer of split locus (Groucho). Therefore, we studied the influence of R-esp1 on nerve growth factor (NGF)-induced cell survival of PC12 cells. Overexpression of R-esp1 promotes cell survival even in the absence of NGF and, conversely, it is reduced by antisense-mediated inhibition of R-esp1 expression. In conclusion, we propose a novel model in which R-esp1 protein mediates the NGF-signaling pathway.

Key words: Signaling; Transcription factor; Neurotrophin

1. Introduction

Groucho homologues, bHLH transcriptional co-repressors of the enhancer of split complex, are highly conserved among different species, including mammals [1–10]. They are major constituents of the Notch-signaling pathway. As heterodimers with hairy-like bHLH transcription repressors [11–15], they regulate gene transcription [1,12–14,16]. The Notch-signaling pathway (reviewed in [17,18]) functions in lineage decisions such as T-cell maturation [19,20], myogenesis [21,22] and neurogenesis (reviewed in [18,23,24]). The expression of the enhancer of split complex and Groucho is increased upon binding of specific ligands (Delta or Serrate) to the Notch receptor. Thereby, the transcription of different target genes is suppressed [17,18,25].

R-esp1, a *Drosophila* enhancer of split (Groucho) homologue, is a 24 kDa protein which consists of an amino-terminal domain and a stretch rich in proline/glycine, but lacks WD-40 repeats and other domains typical for Groucho homologues such as the human transducin-like enhancer of split (TLE [9]) or rat enhancer of split-2 (R-esp2 [5]). In PC12 cells, nerve growth factor (NGF) causes changes in the expression pattern of R-esp1 [5]. In addition, the post-translational inhibition of HES-1, a hairy-like repressor of the Notch-signaling pathway, mediates NGF-signaling and inhibits neuronal differentiation of PC12 cells [26].

Here, we studied the role of R-esp1 as a possible modulator of HES-1 DNA-binding activity relevant for differentiation and cell survival.

2. Materials and methods

2.1. Animals and optic nerve crush

Handling and initial surgery of male PVG/OLA-Hsd rats (Harlan-Winckelmann) started at an age of 10 weeks. The optic nerve was crushed as described [27]. Rats were killed 4, 5, 7, 9, 12 or 13 days after crush, brains were removed and both the left and right superior colliculi (SCs) were dissected out.

2.2. RNA isolation and differential display reverse transcription (DDRT) assay

Total RNA was prepared using TRIZOL (Gibco BRL). DDRT reaction was performed by using the Delta RNA Fingerprinting kit (Clontech), Primezyme polymerase (Biometra) and [α-^{35}S]dATP (ICN). Samples were run on a 6% sequencing gel. Differentially expressed cDNAs were eluted from the dried gel, re-amplified, cloned into pCR2.1 vector (Invitrogen) and sequenced.

2.3. Hybridization protocols

PolyA$^+$-RNA prepared 4 days after crush from total RNA using the Oligotex mRNA Midi kit (Qiagen) was labelled by randomly primed reverse transcription using [α-^{32}P]dATP (ICN) and AMV reverse transcriptase (Promega). Serially diluted R-esp1 cDNA was applied onto a Qiabrane nylon membrane (Qiagen) and probed with a [α-^{32}P]dATP-labelled R-esp1 cDNA. Specificity of the probe is shown by hybridizing to a rat multiple tissue Northern blot (Clontech). Blots were analyzed using a BAS3000 phosphoimager (Fuji) and RFLPscan 3.0 software (Scanalytics).

2.4. Quantitative PCR

1 μg of total RNA was reverse-transcribed and 1/20 cDNA mixture was used for quantitative PCR by means of the Lightcycler LC24 (Idaho Technology). A 10 μl reaction mixture contained 1×reaction buffer with BSA (Idaho Technology), 2 mM $MgCl_2$, 200 μmol dNTP, 0.4 U InViTaq polymerase (InViTec), 0.2 μl of a 1:1000 dilution of SYBR Green I (Molecular Probes) and 0.5 μmol of the specific primers, either an α-tubulin primer set (Clontech) or R-esp1/5 (5′-CAGCTCGGTCCCTACCCCAAG) and R-esp1/6 (5′-CCCCACTG-CAGTCCCGAGCC). Experiments were performed in triplicate and α-tubulin was used to normalize the sample cDNA content. Resulting data were statistically analyzed using a one-way ANOVA test.

2.5. PC12 cell survival assay

PC12 (DSM) cells were grown in IMDM (Gibco BRL) supplemented with 10% horse serum (Gibco BRL) and 5% fetal calf serum (Gibco BRL). Cells were seeded into 96 well plates at a density of 20 000 cells per well and exposed over a period of 48 h to 5 μM of a R-esp1 antisense phosphothioate oligonucleotide (5′-TCAGCCTGT-

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TTGTGCATCTCGAT) or a nonsense phosphothioate oligonucleotide (A-mix: 5′-CAGTGTCACTGACACGATTGCGAT). After washing twice in IMDM, cells were cultured another 24 h in the presence (control) or absence of serum supplemented with 10 ng/ml β-NGF (R and D Systems). The vital cell number was measured by means of the XTT Cell Proliferation kit II (Boehringer Mannheim). For transient R-esp1 overexpression, about 200 000 cells in 0.5 ml OptiMEM medium (Gibco BRL) were seeded into a well of a 12 well plate and transfected with either pCRESP1 or the pCR3.1 control vector (Invitrogen) using DAC-30 (Eurogentec; 2 μg DNA/4 μg DAC-30). 48 h after transfection, the medium was replaced by standard PC12 medium and cell survival determined as described above.

3. Results

3.1. R-esp1 is differentially expressed after optic nerve crush

To identify genes relevant for functional recovery after neurotrauma, we used an established model of optic nerve crush [27–32]. The affected right and the corresponding left SC were examined by DDRT-PCR using primers P1 and T4 of the Delta Fingerprinting kit (Clontech) at different cDNA concentrations. A 433 bp cDNA fragment, differentially expressed (Fig. 1a), was identified as part of R-esp1 mRNA, a rat homologue of the *Drosophila* enhancer of split (Groucho) locus first described by Schmidt and Sladek [5]. The clone spans from nucleotide position 701 to 1133 of the published sequence (EMBL number L14462) and represents the 3′-noncoding sequence only.

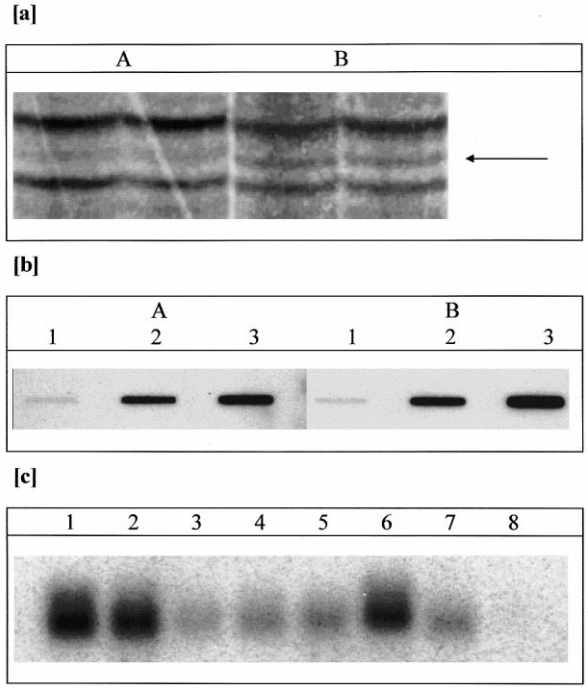

Fig. 1. Differential expression of R-esp1 in the deafferented and contralateral SC after mild optic nerve crush in adult rats. (a) DDRT analysis comparing the unaffected (A) and affected (B) SC 4 days after optic nerve crush. The arrow indicates the R-esp1 cDNA fragment. (b) Reverse Northern slot-blot. Labelled cDNA probes derived from the unaffected (A) or deafferented (B) SC 4 days after crush were hybridized to R-esp1 cDNA (1 = 0.1 ng, 2 = 1 ng, 3 = 10 ng). (c) The rat multiple tissue Northern blot (Clontech, Heidelberg, Germany) was probed with an [α-^{32}P]dATP-labelled R-esp1 cDNA fragment. (1 = heart, 2 = brain, 3 = spleen, 4 = lung, 5 = liver, 6 = skeletal muscle, 7 = kidney, 8 = testis).

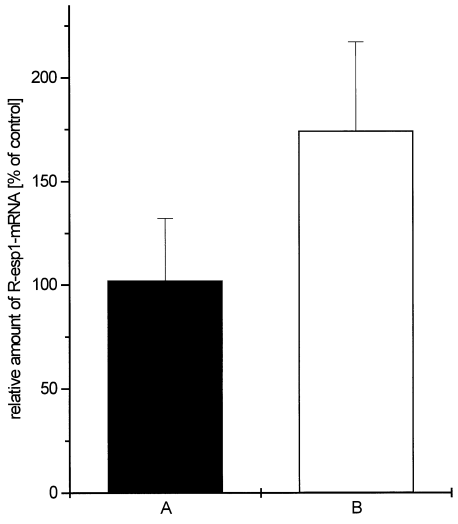

Fig. 2. Quantitative determination of R-esp1 mRNA contents in SC after optic nerve crush. The relative R-esp1 mRNA contents of six animals (4, 5, 7, 9, 12 and 13 days after crush) were analyzed by quantitative PCR.

Differential expression of R-esp1 RNA was confirmed by reverse-Northern slot-blot hybridization, showing a selective increase in R-esp1 mRNA in the deafferented SC (Fig. 1b). This induction developed independently of the left or right optic nerve being crushed. Identical results were obtained if an amplified fragment of the coding region (position 81–681) was applied as R-esp1 cDNA probe instead (not shown).

The crush-dependent induction of R-esp1 mRNA in the deafferented SC was quantified by PCR. In average, there was an increase to 174% of R-esp1 mRNA in the affected SC of crushed rats in the early phase of functional recovery ($n = 6$, $P < 0.05$; Fig. 2). Identical results were obtained if R-esp1 mRNA levels were normalized to α-tubulin or β-actin mRNA (not shown).

R-esp1 shows extensive amino acid sequence homology (87%) to the N-terminal domains of its full-length counterpart, the Groucho homologue R-esp2 [5]. The non-coding region of R-esp1 cDNA differs from the R-esp2 cDNA sequence. Thus, a 3′-fragment of R-esp1 cDNA was used in Northern analysis. A single 1.35 kb mRNA species was detected (Fig. 1c), which is in accordance to the size of the published full-length R-esp1 mRNA [5]. Heart, brain and skeletal muscle showed a 2–3-fold higher R-esp1 expression than kidney, lung and liver. Weak R-esp1 expression was found in spleen and none at all in testis (Fig. 1c). These findings are partially at variance with the results of Schmidt and Sladek [5], who used, however, another rat strain and total RNA instead of PolyA$^+$-RNA.

3.2. R-esp1 mediates NGF-induced survival of PC12 cells

After 24 h of culture in serum-free medium, the number of metabolically active PC12 cells measured by a modified PC12 survival assay [33] decreased to 60% ($n = 30$, $P < 0.05$) compared to cells grown in complete medium (Fig. 3A). In serum-free medium, the addition of 10 ng/ml NGF resulted in a 3.5-fold increase in the cell number ($n = 30$, $P < 0.05$), exceeding even that obtained in the presence of serum (Fig. 3A). This

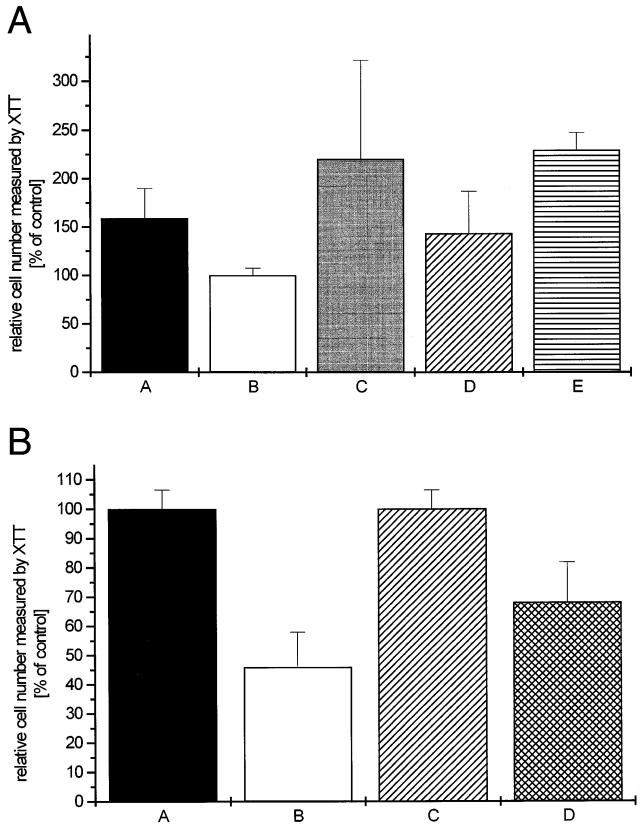

Fig. 3. (A) Influence of R-esp1 antisense oligonucleotides on PC12 cell survival in complete medium (A), serum-free medium (B), serum-free medium supplemented with NGF (C) and serum-free medium supplemented with NGF plus R-esp1 antisense oligonucleotides (D) or nonsense A-mix antisense oligonucleotides (E). (B) Increased survival of PC12 cells by overexpression of R-esp1 (control plasmid pCR3.1: columns A and B, expression plasmid pCRESP1/20: columns C and D). A and C: relative cell number with serum, B and D: without serum.

finding is in full accordance with data reported by others [33]. Inhibition of R-esp1 gene expression by a specific phosphothioate antisense oligonucleotide (position 322–300, 5 μM) specifically blocked these trophic effects of NGF (Fig. 3A), as suggested by the observed reduction of the PC12 cell number to 60% ($n = 30$, $P < 0.05$). On the contrary, the application of a nonsense phosphothioate antisense oligonucleotide (A-mix) had no effect on the NGF-induced cell survival ($n = 36$, Fig. 3A).

Overexpression of R-esp1 by transient transfection with pCRESP1/20 protected PC12 cells from cell death due to growth factor depletion (Fig. 3B). Cells transfected with the control plasmid pCR3.1 (Invitrogen) responded to serum deprivation with a 50% reduction in cell number ($n = 20$, $P < 0.05$; Fig. 3B). Compared to cells transfected with pCR3.1, transfection with pCRESP1 increased the cell number to 140% ($n = 20$, $P < 0.05$).

4. Discussion

We studied expression changes in a well-established neurotrauma animal model [27,29–32] by DDRT-PCR analyses. In this model, the optic nerve of rats is unilaterally crushed, leading to a 90% deafferentation in the SC and a temporary impairment of vision. 3 Weeks following mild injury, there is a nearly complete behavioral recovery [28–31].

Among the genes differently expressed in the affected SC, we identified the R-esp1 gene [5]. This suggests a role of R-esp1 in the post-injury response of the adult mammalian brain. Studying the role of R-esp1 in vitro, we show profound effects of a modulation of R-esp1 expression on the NGF-dependent PC12 cell survival. In the absence of NGF, R-esp1 overexpression significantly increased cell survival and, thus, mimicked effects resulting from NGF receptor triggering. Correspondingly, the inhibition of R-esp1 expression completely abolished this survival-promoting effect of NGF on PC12 cells but increased cell death instead. This suggests a direct linkage of R-esp1 to the NGF receptor-signaling pathway in PC12 cells.

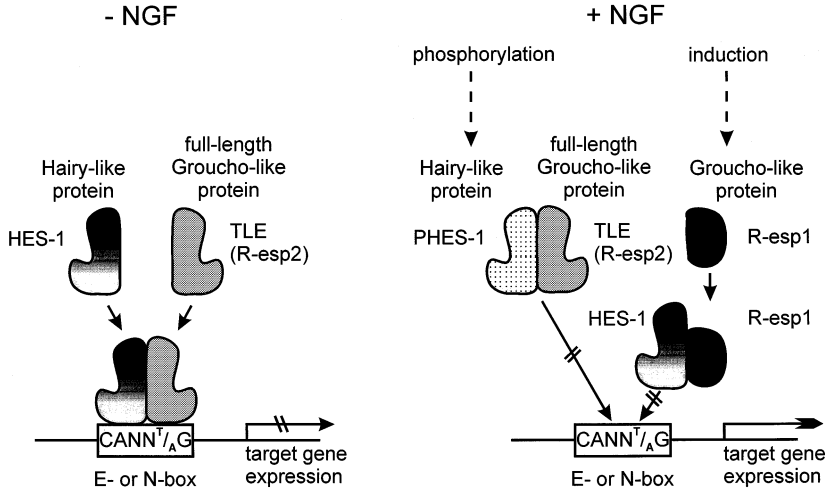

Fig. 4. Model of linkage of NGF- to Notch-signaling pathways by R-esp1 protein. Left panel: effect of HES-1 repressor during NGF deprivation. Right panel: mode of action of induced R-esp1 gene expression following NGF-signaling.

The bHLH, transcriptional repressor HES-1 mediates the NGF-dependent induction of neurite outgrowth in PC12 cells [26]. Phosphorylation of HES-1 in response to NGF-signaling prevents binding of the HES-1/TLE complex to DNA, leading to neuronal differentiation. Furthermore, overexpression of HES-1 in PC12 cells completely abolished the development of a neuronal phenotype in response to NGF [26]. Our data together with those of Ström et al. [26] identify the bHLH transcriptional repressor HES-1 and its competitor R-esp1 as two important mediators of PC12 cell survival.

Schmidt and Sladek [5] propose a model in which R-esp1 acts as a negative regulator by competing for factors that otherwise form heterodimers with R-esp2. This mode of action and some features in the sequence of R-esp1 [5] make it an attractive hypothesis that R-esp1 acts like an Id protein [34,35]. Accordingly, the Id-like repressor R-esp1 titrates Groucho homologues like R-esp2 away of active HES-1/R-esp2 repressor complexes. Resulting inactive heterodimers lack DNA-binding capability. The nuclear localization signal (NLS) provided by HES-1 may still facilitate translocation of the dimeric complex to the nucleus. This would explain the presence of R-esp1 in the nuclei of both PC12 and GH3 cells despite the absence of a NLS [5]. In support of this view, others [13,14,36] showed homo- and heterodimerization of Groucho-like proteins (GRG or TLE) via a region within the amino-terminal Gln-rich region. Similarly, heterodimerization of TLE/Groucho and HES/hairy and the existence of two repression domains within the TLE protein [11,13,14] have been shown. These repression domains are located within the dimerization and an internal region characterized by an abundance of Ser, Thr and Pro residues [13,14]. R-esp1 protein while lacking this latter domain still contains the amino-terminal glutamine-rich dimerization and glycine/proline-rich region [5]. Thus, hairy-like proteins (e.g. HES) or Groucho-like proteins (e.g. TLE or R-esp2) may heterodimerize with R-esp1 via the Gln domain, forming dimers lacking repressor activity. Based on the Id-like features of R-esp1 and considering both our data and those of Ström et al. [26], we propose the following new model of linkage of NGF-signaling pathways and R-esp1 protein (Fig. 4). Exposure of PC12 cells to NGF leads to a rapid induction of R-esp1. R-esp1 at increased concentrations binds to HES-1, thereby preventing it from forming heterodimers with full-length Groucho homologues (TLE [9] or R-esp2 [5]). As a result, active HES-1/TLE repressor complexes gradually fade out and target gene expression is turned on. According to our hypothesis, neuronal differentiation of PC12 cells [26] and cell survival should be inducible by either an overexpression of R-esp1 or an inhibition of HES-1 expression/activity. In turn, both an inhibition of R-esp1 expression and HES-1 overexpression should abolish NGF-dependent influences on PC12 cells. Surprisingly, Ström et al. [26] did not observe any binding of an Id-like protein to HES-1. Keeping in mind, however, that a subtle balancing of R-esp1 levels is sufficient to determine lineage decisions, an artificial overexpression of HES-1 should by far exceed physiological R-esp1 levels. Induction of HES-1 and Groucho expression occurs in response to Notch receptor-signaling [37–39], whereas R-esp1 expression is induced by NGF (our data).

Presently, we could not explain whatever the function of the R-esp1 gene in vivo may be. The increase in R-esp1 mRNA expression after optic nerve crush in the corresponding SC might protect cells from undergoing cell death as observed in our PC12 experiments.

The high degree of conservation of Groucho genes and their signaling pathways among different species suggest that we are dealing with a fundamental molecular event that is crucial in our understanding of neurotrauma or other pathological conditions.

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