Nerve growth factor and epidermal growth factor stimulate clusterin gene expression in PC12 cells

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Clusterin (apolipoprotein J) is an extracellular glycoprotein that might exert functions in development, cell death and lipid transport. Clusterin gene expression is elevated at sites of tissue remodelling, such as differentiation and apoptosis; however, the signals responsible for this regulation have not been identified. We use here the clusterin gene as a model system to examine expression in PC12 cells under the control of differentiation and proliferation signals produced by nerve growth factor (NGF) and by epidermal growth factor (EGF) respectively. NGF induced clusterin mRNA, which preceded neurite outgrowth typical of neuronal differentiation. EGF also activated the clusterin mRNA, demonstrating that both proliferation and differentiation signals regulate the gene. To localize NGF- and EGF-responsive elements we isolated the clusterin promoter and tested it in PC12 cell transfections. A 2.5 kb promoter frag-

ment and two 1.5 and 0.3 kb deletion mutants were inducible by NGF and EGF. The contribution to this response of a conserved activator protein 1 (AP-1) motif located in the 0.3 kb fragment was analysed by mutagenesis. The mutant promoter was not inducible by NGF or EGF, which identifies the AP-1 motif as an element responding to both factors. Binding studies with PC12 nuclear extracts showed that AP-1 binds to this sequence in the clusterin promoter. These findings suggest that NGF and EGF, which give differential gene regulation in PC12 cells, resulting in neuronal differentiation and proliferation respectively, use the common Ras/extracellular signal-regulated kinase/AP-1 signalling pathway to activate clusterin expression.

Key words: activator protein 1, differentiation, neuronal cells.

INTRODUCTION

Clusterin (apolipoprotein J) is an extracellular heterodimeric glycoprotein that has been characterized from a number of species and is expressed in various tissues (reviewed in [1,2]). Human clusterin was detected in all body fluids that have been investigated, including serum, where the protein is found as part of the high-density lipoprotein fraction [1–5]. Clusterin is synthesized at an elevated level during a number of physiological processes such as development and differentiation, and in various pathological disorders [3,6,7]. Clusterin expression was shown to be correlated with apoptosis (programmed cell death) in a number of systems, where it is expressed not in the dying cells but in the surviving neighbour cells [2,8,9]. Furthermore, clusterin has been identified as an inhibitor of complement lysis [10]. These findings suggested that, rather than being involved in the cell death programme, the protein might protect the vital neighbouring cells from cell remnants and help with the deposition of cell debris [2].

A recent study demonstrates an active function of clusterin in the reverse transport of cholesterol by stimulating its export from macrophage cells [5], a process that could have a role in counteracting the fatal role of foam-cell macrophages in the formation of atherosclerotic plaques. Reverse cholesterol transport from peripheral cells could also be a key function of the protein in apoptotic tissues, where the surviving cells that phagocytose the dead cell remnants accumulate cholesterol. Clusterin expression studies in the brain (reviewed in [11]) have revealed another important aspect of its function: the protein is overexpressed in the brain of Alzheimer patients, where it binds

to amyloid deposits of senile plaques; this complexing with Alzheimer plaques is due to specific binding to amyloid β ($A\beta$) protein [11,12]. Moreover, clusterin was recently shown to provide a specific transport system for $A\beta$ protein across blood–brain and blood–cerebrospinal fluid barriers [13], which involves glycoprotein 330/megalin, the recently identified receptor for clusterin [14]. Clusterin therefore functions as a specific carrier for $A\beta$, and possibly for other proteins; its overexpression in Alzheimer's disease could have a role in preventing amyloid plaque formation. The up-regulation of clusterin expression in various diseases and tissue rearrangement processes such as differentiation have been documented; however, the signals involved in the induction process have not yet been analysed.

To study neuronal differentiation the cell line PC12 serves as a widely accepted model system (reviewed in [15,16]). PC12 cells can be induced to differentiate into a neuronal phenotype by nerve growth factor (NGF) [17] or to proliferate by epidermal growth factor (EGF) [18]. NGF and EGF signal transduction have several pathways in common [15,19–21] and it has been a critical question for the understanding of neuronal differentiation to identify the pathway(s) distinguishing between proliferation and differentiation. The Ras/extracellular signal-regulated kinase (ERK; mitogen-activated protein kinase) signalling pathway has a key role in the specific response of NGF signalling: NGF causes a longer activation of the Ras/ERK cascade than EGF [22]; furthermore, the Ras/ERK cascade seems to be both necessary and sufficient for PC12 cell differentiation [23]. On the basis of these findings it has been proposed that the length of stimulation of this cascade resulting in protein kinase ERK translocation into the nucleus is the signal required for the

Abbreviations used: $A\beta$, amyloid β ; AP-1, activator protein 1; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated kinase; NGF, nerve growth factor; SV40, simian virus 40; TEF1, transcriptional enhancer factor 1; TGF- β , transforming growth factor β .

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neuronal differentiation of PC12 cells [15]. Because stimulation of the Ras/ERK pathway could lead to the activation of at least two transcription factors, activator protein 1 (AP-1) [24] and cAMP-response-element-binding protein [25], NGF-induced neuronal differentiation could require the induction of a set of differentiation-specific genes [15]. To understand the mechanisms of neuronal differentiation it will therefore be essential to compare NGF gene and EGF gene regulation in neuronal systems.

To address these questions we have analysed gene expression in response to growth factor signalling by using the clusterin gene as a model system. We show here that in PC12 cells two growth factors, NGF and EGF, induce clusterin mRNA. An AP-1-binding site in the clusterin promoter was identified as the regulatory element required for the induction of NGF and EGF. This indicates that the Ras/ERK signalling cascade leading to AP-1 activation is required for the control of the clusterin gene by both NGF and EGF, suggesting that additional pathways might be required for differentiation-specific gene expression.

EXPERIMENTAL

Cell culture, RNA isolation and Northern blotting

PC12 cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, 10 i.u./ml penicillin, 10 µg/ml streptomycin and 4 mM glutamine; incubation was performed at 37 °C in humidified air/CO₂ (9:1). The cells were split by treatment with trypsin when it was necessary to separate the cells by extensive suspension, to avoid the formation of lumps, and were seeded in cell culture flasks that had been coated with collagen (0.1 mg/ml) overnight before use. NGF and EGF, both from mouse submaxillary gland, were obtained from Boehringer Mannheim and added to the culture medium as indicated. Total RNA isolation and Northern blotting was done as described [26]. Each sample of total RNA (10 µg) was run on 1.2% (w/v) agarose/0.66 M formaldehyde gels and blotted to nylon membranes (Boehringer, Mannheim). Prehybridization and hybridization were performed in RapidHyb buffer (Amersham) at 65 °C. Hybridization solution contained 25 ng of [³²P]dCTP-labelled random-primed DNA probes. The probes used for hybridization were the 1.6 kb SGP-2 cDNA, obtained from FRT cells by PCR, and a 2 kb human actin cDNA (purchased from Clontech, Palo Alto, CA, U.S.A.). Membranes were washed with 2 × SSC/0.1% SDS for 30 min and 0.1 × SSC/0.1% SDS for 30 min at 65 °C. The blots were then exposed to Hyperfilm (Amersham) at -80 °C with an intensifying screen.

Cloning and sequencing

A 3 kb *Pst*I genomic fragment containing the clusterin promoter was isolated from a mouse spleen genomic library in phage λ (Stratagene, Heidelberg, Germany), which was initially screened with the canine clusterin ('gp80') probe [27], resulting in a 21.4 kb clone. From this clone, which contained a 5' upstream sequence plus exons 1–6, a *Pst*I fragment was subcloned that was identified by using a mouse cDNA probe. Automated DNA sequencing was performed by the dideoxy method on Applied Biosystems devices. Sequence alignments and searches for transcription-factor-binding sites were performed with the HUSAR program (German Cancer Research Center, Heidelberg, Germany).

Construction of plasmid chimaera and mutagenesis

pGL2-2500nt (-2464 to -21) was obtained by restriction digestion with *Kpn*I of the *Pst*I genomic subclone containing the

5' region of the clusterin gene (see above). pGL2-1500nt (-1558 to -21) was obtained by digestion with *Bgl*II. The promoter fragments were ligated into the promoterless luciferase expression plasmid pGL2-Basic (Promega, Heidelberg, Germany). pGL2-300nt (-338 to -21) was created by PCR and ligation into pGL2-Basic. pGL2-500nt (-2298 to -1806) was also constructed by PCR and ligated into pGL2-promoter, which contained a fragment from the simian virus 40 (SV40) promoter (Promega). The mutant promoter plasmid MutAP-1-300nt (-338 to -21) was obtained from pGL2-300nt by site-directed mutagenesis with the use of PCR, as described [28]. Four base pairs were changed in the putative AP-1 consensus sequence by using the mutagenic oligonucleotide 5'-CTGGGCGTGA-TATCCGCAGGTTTGCAGCCAGC-3', plus two flanking primers for amplification of the promoter. The resulting mutated promoter fragment was ligated into pGL2-Basic.

DNA transfection and luciferase assay

For transient transfections, PC12 cells were plated on 6-well plates 24 h before transfection. The cells were transfected with 4.5 µg of clusterin-promoter/luciferase constructs, 3 µg of cytomegalovirus-β-galactosidase plasmid and 45 µl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate (DOTAP; Boehringer Mannheim), then incubated at 37 °C for 24 h. After transfection the cells were incubated with 50 ng/ml EGF or 100 ng/ml NGF for 48 h. Cells were harvested and luciferase activity was assayed. Transfection efficiency was monitored by assaying the β-galactosidase activity. The results presented resulted from three independent experiments with triplicate transfections.

Preparation of nuclear extracts and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared from untreated PC12 cells essentially as described [29]. The protein concentration of each extract was determined with a bicinchoninic acid protein assay from Pierce (Rockford, IL, U.S.A.) with BSA as a standard. Mobility-shift assays were performed by using the radiolabelled double-stranded oligonucleotides described in the legend to Figure 9. The competitor fragments were (only one strand shown): 5'-GATCTTCTAGTGCATGAGTCAGACTTGATA-3' (AP-1 binding site of the human collagenase gene), 5'-TCGATCTCGAGACTGAATCACTATGTACATTGTGTGTCATG-3' (AP-1 site of HPV-16 [30]) and binding motifs from viral enhancers 5'-TCGATCTCGAGTAAACTGCACATGGGTGTGTGCAAACCG-3' (CACCC motif 'M' from HPV-16 [31]), 5'-TCGACAACATAATGTACCCCTAG-3' (CACCC-box of HPV-16 [31]), 5'-CTAGATTACAGCATATTTGGCATAAGGTTAT-3' (NF-1 site of HPV-16 [31]) and 5'-GATCCCAAGTGAATGTGTGGAATGTGTCTG-3' [the transcriptional enhancer factor 1 (TEF1) site of SV40]. The double-stranded oligonucleotides were labelled by filling in 5' protruding ends with [³²P]dATP by T7 DNA polymerase (Stratagene); 15 µg of nuclear cell extract was used in each assay. The proteins were incubated for 10 min at room temperature in binding buffer [20 mM Hepes (pH 7.5)/50 mM KCl/5 mM MgCl₂/0.2 mM EDTA/1 mM 1,4-dithiothreitol/20% (v/v) glycerol/12 mM spermidine] in the presence of 1.5 µg of poly(dI-dC) as a non-specific competitor (Pharmacia), and approximately 40 fmol of ³²P-labelled probe; the binding reaction was loaded on a 5% (w/v) polyacrylamide gel (acrylamide/bisacrylamide 29:1, w/w) and run in 0.5 × Tris/borate/EDTA buffer at 4 °C for 4 h at 180 V. The gels were dried and analysed by autoradiography.

RESULTS

NGF and EGF induce clusterin mRNA in PC12 cells

Clusterin expression is found in neurons of mouse and rat, in both developing and adult brain [11,32], which raises the question of whether neuronal differentiation signals regulate the clusterin gene. Incubation of PC12 cells with NGF (100 ng/ml) led to the cessation of cell proliferation and produced a typical neurite outgrowth after 5 days of NGF treatment (Figure 1). In contrast, the control cells grown in the absence of NGF did not form neurites (Figure 1). To examine clusterin mRNA regulation in PC12 cells under NGF control, total RNA was isolated at various times after the addition of NGF and analysed by Northern blotting with a clusterin cDNA probe; β -actin mRNA expression served as a control. Increased levels of clusterin mRNA were observed after only 1 day of treatment with NGF (Figure 2). After the initial induction the clusterin mRNA level remained elevated until day 5 and was not significantly altered in comparison with the actin control. These findings demonstrate that NGF, which is a neuronal differentiation factor for PC12 cells, produces an induction of the clusterin gene.

In contrast with NGF, EGF does not function as a differentiation factor in PC12 cells but stimulates cell proliferation [15,16]. EGF also failed to cause PC12 neurite formation in the cell system used here (results not shown). We analysed clusterin expression in PC12 cells in the presence of EGF. After the addition of EGF and incubation for 1–5 days, total RNA was isolated from the cells and analysed by Northern blotting. Interestingly, the addition of EGF (50 ng/ml) to PC12 cells produced an induction of clusterin mRNA (Figure 3). The induction was similar to the NGF response described above because it was clearly seen after 1 day and stayed at the induced level for 5 days after the addition of EGF. These results demonstrate that two growth factors that have different effects in PC12 cells, namely NGF, which produces differentiation, and EGF, which stimulates proliferation, promote the induction of clusterin mRNA, possibly by a common pathway.

Figure 2 NGF activates clusterin mRNA in PC12 cells

The results of a Northern blot are depicted in which PC12 cells were incubated with NGF, which induced clusterin mRNA 1 day after NGF addition. The induced level stays unchanged until day 5, in contrast with the actin control. Shown is a typical result, which was seen in three independent experiments, resulting in at least 2-fold increases in the amount of clusterin mRNA. The cells were grown in the presence of 10% (v/v) horse serum and 5% (v/v) fetal bovine serum. At day 0 the medium was changed to serum-containing medium plus 100 ng/ml NGF. At the indicated time points the cells were harvested and RNA was isolated as described [26]. A Northern blot was performed with 10 μ g of total RNA in each lane; the blot was hybridized to a radioactively labelled probe of the rat clusterin gene (SGP-2 [26]); after autoradiography, the clusterin probe was removed and the blot was rehybridized with a probe for the β -actin gene. Abbreviation: d, day(s).

NGF and EGF activate a 300 bp promoter fragment of the clusterin gene

To analyse the regulation of clusterin gene expression in more detail, we isolated the promoter region of the mouse clusterin gene. We constructed a genomic bacteriophage λ library, from which a subfragment was cloned containing an approx. 2800 bp sequence upstream of the promoter start site (EMBL accession number X84792; exon 1 starts at position 2862; see the Experimental section). A search for transcription-factor-binding

Figure 1 NGF induces neurite outgrowth in PC12 cells

PC12 cells were grown in medium as described in the Experimental section, either in the absence (control) or presence of NGF (100 ng/ml). The NGF-treated cells, but not the control cells, underwent neuronal differentiation, as can be seen from the formation of neurites. Treatment with EGF (50 ng/ml) did not produce neurite outgrowth (results not shown). During normal cultivation (in the absence of NGF), the cells tended to form lumps; this was minimized by repeated suspension.

Figure 3 EGF induces the clusterin mRNA

Shown is a Northern blot of PC12 cells, which were grown in the absence or presence of EGF. The induction by EGF of clusterin mRNA was seen in three independent experiments with at least 2-fold higher expression with EGF than in the control. The medium was changed at day 0; culture medium containing EGF (50 ng/ml) in the presence of serum was then added (see Figure 2). The cells were harvested after 1–5 days as indicated and total RNA was isolated. The clusterin mRNA was analysed by Northern blot, as described in the legend to Figure 2. Abbreviation: d, day(s).

sites in the upstream sequence revealed that several consensus sequences were present in the clusterin promoter. The upstream sequence contained similarities to AP-1- and AP-2-binding sites in close vicinity to the promoter, and a similarity to an SP-1 site

further upstream (Figures 4 and 5). A sequence comparison of the 5' region up to approx. position –200 bp revealed that the promoter region was well conserved, with approx. 85 % identity between mouse and rat, and 79 % identity between mouse and human [33] (Figure 6). The AP-1 motif at –77 and the presumptive AP-2 site at –57 were present in all three species. Interestingly, an apolipoprotein E B1 motif, which was originally found in the promoter of the gene for apolipoprotein E, was present at position –132 in the mouse and human promoters [34], and was conserved with eight out of ten matches in the rat gene.

To investigate whether the promoter fragment is regulated by NGF or EGF, a 2.5 kbp fragment of the upstream sequence (–2480 to –21) was inserted into the luciferase expression vector pGL2-Basic (Figure 7, upper panel). The resulting plasmid, named pGL2-2500nt, was transfected into PC12 cells by lipofection and the cells were incubated in the absence or presence of NGF (100 ng/ml) or EGF (50 ng/ml). At 48 h after the addition of growth factor, the cells were harvested and luciferase activity was determined. The clusterin promoter in pGL2-2500nt was induced by both growth factors, with 2.1-fold (NGF) and 2.0-fold (EGF) increases in luciferase activity (Figure 7). Both NGF and EGF had no effect on the promoterless control vector pGL2-Basic (results not shown). This finding demonstrates that the regulatory sequences for the induction of NGF and of EGF are located on the 2500 bp clusterin promoter.

Potential growth factor regulatory elements in the clusterin promoter are the AP-1 motifs that are distributed over the entire

Figure 4 Structure of the clusterin promoter

Schematic presentation of the mouse clusterin promoter upstream region. A number of potential transcription-factor-binding sites are given in the top panel; a list of these sites is shown in Figure 5. 5' deletions of the promoter were constructed and the upstream fragments were inserted into the luciferase expression plasmid pGL2-Basic; the exact length of each promoter fragment is given in the Experimental section. The plasmid pGL2-500nt contains a 493 bp fragment with three AP-1 motifs that was inserted upstream of the SV40 promoter in the enhancerless vector pGL2-Promoter.

Figure 7 NGF and EGF induction of clusterin promoter 5'-deletion clones

Upper panel: three clusterin promoter constructs were used to transfect PC12 cells; the plasmid constructs are shown schematically in Figure 4. The luciferase expression was normalized to the control transfections for each plasmid, which were set to 1; the relative expressions of the three plasmid control transfections (without NGF or EGF) were 1.0 (pGL2-2500nt), 1.2 (pGL2-1500nt) and 0.9 (pGL2-300nt). The induction numbers of luciferase expression of the NGF- and EGF-treated cell cultures are given (EGF, central bar; NGF, right-hand bar, in each group); they were determined from nine transfections (three experiments with triplicate transfections each). The changes observed for EGF- and NGF-treated cells respectively compared with control cells are statistically significant (for pGL2-2500nt, $P < 0.01$ and $P < 0.01$; for pGL2-1500nt, $P < 0.05$ and $P < 0.02$ with EGF and NGF respectively; for pGL2-300nt, $P < 0.01$ and $P < 0.01$ with EGF and NGF respectively); the differences in the induction values observed with pGL2-300nt between EGF- and NGF-treated cells, and the higher induction compared with the longer constructs, are not statistically significant as judged from several independent experiments (results not shown). Lower panel: regulation of the upstream fragment in pGL2-500nt. Two plasmids, pGL2-Promoter, which carries the SV40 promoter (without enhancer), and pGL2-500nt, which has inserted into it the 493 bp clusterin promoter fragment upstream of the SV40 promoter in pGL2-Promoter (see Figure 4), were transfected into PC12 cells. The cells were treated with NGF (right-hand bar of each group) or EGF (middle bar of each group) or had no added growth factor (control). The relative expressions are shown and the induction numbers are given above each panel. The weak inductions of pGL2-500nt by NGF and EGF were not statistically significant; however, the constitutive increase in luciferase expression was statistically significant ($P < 0.01$). The transfection mixtures included DOTAP (see the Experimental section) and 4.5 μ g of luciferase plasmid; a cytomegalovirus- β -galactosidase plasmid was co-transfected as a control for transfection efficiency.

Moreover, the basal expression of the mutant promoter was 29% of that in the wild type. This finding demonstrates that the putative AP-1 site in the clusterin gene is important for basal expression and is, furthermore, required for the induction of gene expression by NGF and EGF.

An EMSA was used to assess the binding of PC12 nuclear factors in nuclear extracts from PC12 cells to the AP-1-responsive element of the mouse clusterin promoter. The double-stranded oligonucleotide with the AP-1 motif at position -77 of the promoter (see legend to Figure 9) was radiolabelled and incubated with nuclear extracts from PC12 cells; protein-DNA complexes

Figure 8 Requirement of an AP-1 motif for the regulation of the clusterin promoter by both NGF and EGF

A 4 bp mutation was introduced into the promoter in pGL2-300nt (see Figure 4), as indicated, which resulted in the mutant plasmid MutAP1-300nt. The transfection into PC12 cells of both wild-type and mutant plasmids gave a clear response with the wild-type plasmid but no induction with the mutant. The results shown are relative luciferase activities resulting from three transfections with triplicate culture dishes. The mutation also showed a lowered basal expression of the control cells (in the absence of growth factor). The weak induction of the mutant was not statistically significant, whereas the inductions by EGF and NGF were each significant at the $P < 0.01$ level. MutAP1-300nt was constructed by site-directed mutagenesis with PCR, as described (see the Experimental section) [28]. The transfection was performed as described in the legend to Figure 7.

Figure 9 EMSA competition of an AP-1 complex formed at the clusterin promoter

A double-stranded radioactively labelled fragment with the AP-1 motif at -77 (see Figures 4, 5 and 8) was bound to PC12 nuclear extract in the absence or presence of unlabelled competitor fragments. Specific binding to the AP-1 site was demonstrated by homologous and heterologous competition, which was reproduced in repeated experiments (results not shown). The competitors used were (see the Experimental section): lane 1, none; lane 2, homologous fragment (clusterin -77 AP-1 motif); lane 3, AP-1-binding site from human collagenase promoter; lane 4, AP-1 site from HPV16 [30]; lane 5, fragment with CACCC motif 'M'; lane 6, fragment with 'CACCC box' motif, which also contains an AP-1-related sequence (AATGTCA); lane 7, NF-1 site; lane 8, TEF1-binding site from SV40. The fragment was radioactively labelled by hybridization and filling in 5' protruding ends, resulting in the fragment GATCTTCTAGTGCCTGAGTCACGCTTGATAGATC. The protein-DNA complexes formed with PC12 nuclear extracts were separated by native PAGE [5% (w/v) gel] [30].

were separated by native PAGE. The AP-1 oligomer formed a complex with a nuclear factor (Figure 9, labelled 'AP-1'), the complex band was specific because it disappeared in the presence of a 20-fold molar excess of unlabelled AP-1 motif (Figure 9, lane 2); moreover the complex was competed for by oligonucleotides containing the AP-1 consensus sequence of the human collagenase gene (lane 3), or from human papilloma virus 16 (lane 4), with the latter fragment acting as a slightly weaker competitor. In contrast, no competition was observed with two oligomers containing CACCC-box motifs (Figure 9, lanes 5 and 6), nor with NF-1-binding sites (lane 7) or TEF-binding sites (lane 8). These results suggest that the sequence motif that is required for regulation by NGF and EGF is bound by the nuclear factor AP-1, which is known to be activated by the Ras/ERK signalling pathway, which itself responds to both NGF and EGF.

DISCUSSION

Clusterin expression is increased during the processes of tissue remodelling and apoptosis and in a number of malignancies [1,2,11]. Moreover, clusterin expression is associated with differentiation and development [35]: the gene is activated in the mouse embryo at sites of epithelial differentiation [6] and responds to proliferative stimuli in a variety of systems [26,36–38]. Only little information is available, however, on the molecular details of clusterin gene regulation in these processes. Here we present results on the regulation of the clusterin gene by differentiation and proliferation signals in a single cell line. In PC12 cells, two growth factors give a differential cellular response: NGF stimulates neuronal differentiation, whereas EGF does not induce differentiation but stimulates cell growth [17]. We demonstrate that the clusterin gene is up-regulated in these cells by treatment with both NGF and EGF, suggesting that in PC12 cells the clusterin gene is subject to regulation by differentiation-signalling and proliferation-signalling pathways.

The findings of clusterin induction by EGF in PC12 cells contrasts with previous observations that EGF repressed clusterin mRNA in the kidney-derived cell line Madin–Darby canine kidney (MDCK) [26]. Similarly, EGF was shown to down-regulate clusterin expression in the polycystic kidneys of mice [36]. These findings indicate that clusterin is regulated by EGF in a cell-type-specific manner, which results in either a repression or an induction of the gene. Other growth factors and cytokines have been reported to modify clusterin expression in a positive or negative fashion: interleukins 1 and 2 induced clusterin mRNA in astrocytes [37]; transforming growth factor β (TGF- β) repressed the clusterin mRNA in pure astrocyte cultures but induced the gene in mixed cultures of astrocytes with microglia and oligodendrocytes [38], suggesting an influence of heterotypic cell interactions on clusterin expression.

To investigate the nature of the responses to NGF and EGF we have isolated the promoter region of the mouse clusterin gene. The clusterin promoter is well conserved and contains potential transcription-factor-binding sites present in the mouse, rat and human genes (Figure 6). We found that three promoter constructs of various lengths were responsive to both NGF and EGF, including the shortest construct pGL2-300nt (Figure 7). The inductions observed with all three constructs and with both growth factors were statistically significant; however, there were no significant differences between the induction values of the NGF-treated and EGF-treated cells (Figure 7, upper panel). The results demonstrate that NGF/EGF response element(s) are contained in the 300 bp promoter. We also tested the function of a 500 bp upstream fragment that contained several potential AP-

1 motifs. Although this fragment did not confer a significant growth factor response on the SV40 early promoter, we found a 4-fold enhancement of the SV40 promoter constitutive activity, indicating that this fragment contains positive regulatory elements. The existence of an upstream activator was not obvious when testing the three 5' deletion mutants (see legend to Figure 7). Therefore a more detailed analysis will be required for a characterization of the function of positive and negative elements modulating the activity of the clusterin promoter.

The finding that both NGF and EGF activated the 300 bp promoter suggests that both factors require the same signalling pathway(s), the best candidate being the Ras/ERK signalling cascade which leads eventually to transcription factor AP-1 activation [24]. When a 4 bp mutation in the AP-1 motif in the promoter was tested in PC12 cells, the results clearly demonstrated that this motif was required for the induction of the clusterin promoter by both NGF and EGF. Moreover, the mutant gave a weaker basal expression than the wild type, which confirms previous findings that AP-1 sites can contribute to basal promoter activity in the absence of inducers [39,40]. EMSA ('band shift') studies to identify the protein(s) interacting with the sequence motif demonstrated binding of a factor to the AP-1 motif that was competed for by the homologous sequence and by two other AP-1 sites, including the site from the collagenase promoter, but not by other fragments not containing an AP-1 site. The heterogeneous, fuzzy appearance of the AP-1 complex (Figure 9), which has also been reported with other AP-1 sites [39,41,42], might be due to the complex family of AP-1 proteins. Taken together, our findings show that an AP-1-binding site, which is conserved in mouse, rat and human genes, is required for induction of the clusterin promoter by NGF and EGF. Interestingly, it has recently been reported that the same AP-1 motif is required for the response of clusterin mRNA to TGF- β in CCL64 cells [43]. Because TGF- β can be both a growth inhibitor and a proliferation factor, depending on the cell type, it should be interesting to perform similar binding studies *in vitro* in CCL64 cells to compare the binding profiles with that from PC12 cells.

AP-1, the Jun/Fos heterodimer, is believed to be the key factor in gene regulation activated by the Ras/ERK signalling cascade, which requires both transcriptional and post-transcriptional mechanisms [24]. We tried to demonstrate an activation of AP-1-binding activity after treatment with NGF but observed no change in the amount of the complex (results not shown); this is consistent with the post-translational activation of the protein involving changes in its phosphorylation status [24]. The Ras/ERK pathway is thought to connect growth factor receptors such as the EGF and NGF receptor to AP-1 in the nucleus. AP-1 activation can also be achieved by the stimulation of protein kinase C with phorbol esters, which elicits an signalling response via the Raf-1/MEK/ERK cascade [24,44]. Most AP-1 binding sites have been functionally tested by phorbol ester stimulation of protein kinase C: only in very few cases have AP-1 sites been characterized as EGF response elements [24,40,45]. It can be assumed, however, that the stimulation of a whole set of genes by EGF, NGF and other growth factors goes through the activation of AP-1.

The Ras/ERK signalling cascade has emerged as one pathway with a central role in NGF-induced neuronal differentiation [15,20,22]. There is an interesting model that implies that the activation by NGF of a set of differentiation-specific genes leads to gene products determining the neuronal phenotype [15]. In fact, some NGF-inducible genes have been identified that do not respond to EGF [46–48]. In contrast, a second group of genes is responsive to both NGF and EGF [49,50], suggesting that

different signalling pathways are used in the two groups of genes. It should be rewarding to find out which of the known NGF-responsive elements also respond to EGF. Some of these regulatory elements do not contain AP-1-binding sites [51–53] and could therefore be activated via pathways distinct from Ras/ERK. On the basis of findings from other systems, one can assume that clusterin induction by the two growth factors uses the same signalling pathway, most probably the Ras/ERK cascade that activates AP-1. Results demonstrating the requirement of the AP-1 pathway for gene induction by both NGF and EGF has not to our knowledge previously been reported. It should therefore be of interest to examine whether it is a general phenomenon that genes responding to both NGF and EGF in neuronal systems are activated via the Ras/ERK/AP-1 signalling pathway. The clusterin gene promises to be a useful model in the elucidation of molecular mechanisms and the specificity of the NGF and EGF signalling pathways.

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