Stress-induced transcription of the clusterin/apoJ gene

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Clusterin/apoJ is an intriguing gene frequently isolated by differential screening in laboratories from different areas of molecular biology, since it is overexpressed in numerous cases of degenerative diseases such as Alzheimer’s disease and scrapie. While the dramatic increase of clusterin expression in injured tissues is well established, the molecular basis of the gene induction remains unclear. In this study, we have focused our attention on the only DNA region strictly conserved between clusterin gene proximal promoters from different vertebrate classes. We show that this 14-bp DNA element is specifically recognized by the HSFl transcription factor and can mediate heat-shock-induced transcription in transient expression assays. Conversely, the avian clusterin proximal promoter, point-mutated at the level of this element, no longer transmits heat-shock activation. These findings provide a possible explanation for the high sensitivity of clusterin expression to environmental changes and allow the classification of clusterin as an extracellular version of heat-shock protein.

INTRODUCTION

Clusterin/apolipoprotein J (ApoJ), is a puzzling multifunctional protein [1–3], capable of interacting with a broad spectrum of molecules including itself[4], amyloid proteins [5–7], components of the complement membrane-attack complex [8,9] or lipids [10], rendering its classification problematic [1]. The clusterin protein is a disulphide-linked heterodimer derived from a single protein precursor, the structure of which is conserved among vertebrates, including myosin-like coiled-coil domains [9,11], amphipathic α helices with higher hydrophobic moments [1,11], a motif also present in the cytolytic components of the complement membrane-attack complex [3,8] and a nucleotide-binding site [11]. The amphipathic helices of clusterin should explain its propensity to stably interact with lipids and hydrophobic domains present in other proteins, but are not sufficient to predict precisely clusterin functions. Such predictions are further complicated by the variety of possible localizations of clusterin. A classical secretion signal peptide allows clusterin to be released from producing cells, to remain bound at the surface of some cells, to circulate in extracellular compartments, and even to be internalized by non-producing cells [12]. Additionally, intranuclear clusterin has also been found in some circumstances [13,14] and is endowed with a typical nuclear localization signal [13].

The clusterin gene is unique in the genome and is well conserved during evolution. It is expressed during tissue regression in many organs, including brain [15–19], retina [20], uterus [21], mammary gland [22,23], prostate [24], temporary embryonic structures [25] and kidney [26]. Therefore, it cannot be used as a marker for a particular disease but should rather be considered as a non-specific cellular response to a wide variety of tissue insults. In this respect, clusterin expression has already been compared with that of heat-shock proteins (hsp) [27].

A particularly close relationship has long been established between clusterin expression and apoptosis [22,25]. Further investigations have revealed that the clusterin gene is in fact transcribed in surviving cells located near apoptotic ones in vitro [28], as well as in vivo [29], raising the hypothesis of an anti-apoptotic role of clusterin, which is further supported by the fact that clusterin overexpression can prevent cell death [30]. Also, the circulating clusterin has been identified as a complement lysis inhibitor [8,9] and has been shown to protect the kidney from immune glomerular injury [26], and neurons against amyloid [7]. Thus, in addition to its expression features, clusterin shares cytoprotective functions with the hsp. Hence, we have examined the possibility that clusterin actually corresponds to a new class of extracellular hsp by elucidating the molecular mechanisms underlying its stress-induced expression.

MATERIALS AND METHODS

Reverse transcriptase (RT)-PCR analyses

A431 human cells were heat shocked or not for one h at 42 °C and the temperature was then reduced to 37 °C for 30 min. mRNAs were extracted and reverse-transcribed into cDNA using random hexamers as primers. Clusterin hsp70 and β-actin human cDNAs were measured by RT-PCR using the following oligonucleotides as primers: 5′-TGATGGCCCCCTCTGGGAGA-GAGT-3′, 5′-TCTCCAGAGGAGATCGTGAC-3′ for clusterin (177-bp amplified fragment); 5′-CACCTTCGACGTGTCATCCTGAC-3′, 5′-CGCTTTCTTGGGCTAGTGC-3′ for clusterin (165-bp amplified fragment) and 5′-GAGATCGAAGGAGAT-3′, 5′-TTGCTGACCATCCTTGAC-3′ for β-actin (146-bp amplified fragment), in the presence of 50 µM unlabelled nucleotides and 0.05 µCi [α-32P]dCTP. All amplification reactions were carried out using 25 cycles as follows: 95 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s. The PCR fragments were separated by electrophoresis on a 2% (w/v) agarose gel. After ethidium staining, to determine more precisely the levels of mRNA induction by phosphorimaging quantification, the gel was dried and exposed for three days on a SO230 storage phosphor screen (Kodak). Screens were read with the Storm Scanner 840 (Molecular Dynamics) and analysed.

Abbreviations used: ApoJ, apolipoprotein J; CLE, clusterin element; HSE, heat-shock element; HSF, heat-shock factors; hsp, heat-shock proteins; RT, reverse transcriptase; tk, thymidine kinase.

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using the ImageQuant software (Molecular Dynamics). The absence of contaminating DNA was verified by PCR on non-reverse-transcribed RNAs.

DNA Probes

The wild-type heat-shock element (HSE) probe used in binding experiments was the double stranded DNA fragment: 5'-ggccggGAGctTTcgaGAAagCTCcgg-3', obtained by concatenation of the 15-bp idealized HSE motif 5'-cGAatgTTcGAaAa-3', and then inserted into the Smal site of plasmid pBSK (Stratagene). When used as a probe, it was re-extracted from plasmids by PvuI–BamHI double digestion and labelled by using the BamHI site with Klenow polymerase in the presence of [α-32P]dCTP. Mutated HSE is the double stranded 15-bp fragment 5'-cGAatgGGAtaGAa-3', devoid of central TTC trinucleotide. The clusterin element (CLE) probe corresponds to a DNA region from the quail clusterin promoter, obtained by annealing the following oligonucleotides: 5'-ggggggactTTcgaGAAacgCTCcgg-3' and 5'-cccgGAGctTT-cGAacccggccgg-3'. Since the resulting double stranded CLE fragment presents 5' protruding ends, it was also end-labelled using Klenow polymerase and [α-32P]dCTP. Mutated CLE was obtained by annealing the following oligonucleotides: 5'-ggggggactTTcgaGAgCTCggg-3' and 5'-cccgGAGctAAGGtGA-AcGccggccgg-3'.

Electrophoretic mobility-shift assays

Protein extracts from total cells were prepared as described by Zimarino et al. [31]. A final volume of 1 ml of protein extract was obtained from 5 x 10^6 normal or heat-shocked HeLa cells. CLE probe (1.3 ng) or HSE probe (0.3 ng) were incubated for 25 min at 25 °C in a final volume of 25 μl, which contained 16 % (w/v) final protein extract, 10 mg of acetylated BSA (Biolabs), 1 μg polydl-Dc (Sigma), 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol and 5 % (v/v) glycerol. When present as unlabelled competitors, 130 ng of CLE or 30 ng of HSE were added to cell extracts before probe addition. For supershift experiments, a 1/400 dilution of control or anti-HSF1 rabbit serum (kindly provided by Dr Vincenzo Zimarino, Milan, Italy) was added to the binding reaction 5 min after complex-formation and was incubated for 20 min at 4 °C before being subjected to electrophoresis.

Plasmid constructions

Multiple copies of CLE and mutated CLE were obtained by catenation of the phosphorylated double stranded oligonucleotides and were isolated by electrophoresis on a 10 % polyacrylamide gel. They were then blunt ended using the Klenow DNA polymerase and inserted into the blunt ended AccI site of the pBLCAT2 vector, just upstream of the Herpes simplex thymidine kinase (tk) basal promoter. Point mutagenesis of the quail clusterin promoter P1-CAT plasmid [32] was achieved using the Transformer site-directed mutagenesis kit from Clontech and the mutated CLE oligonucleotide: 5'-ggggggactTTcgaGAgCTCggg-3'. All constructs were controlled by DNA sequencing.

Transient expression assays

A431 human cells were co-transfected using the calcium-phosphate–DNA co-precipitation procedure, with 10 μg of plasmids containing the CLE-tk-CAT constructions (shown in Figure 4) and 5 μg of β-actin-LacZ plasmid, used as an internal control of transfection efficiency. Cells were washed 16 h after transfection, incubated with fresh medium for 2 h, heat shocked or not for 30 min at 42 °C and harvested 3 h later for protein extraction. QT6 quail cells were co-transfected using the calcium-phosphate–DNA co-precipitation procedure, with 10 mg of plasmids containing quail clusterin promoter P1-CAT constructions mutated or not at the level of CLE (shown in Figure 5) and 5 μg of β-actin-LacZ plasmid. Cells were then incubated in the presence of DNA–calcium-phosphate precipitates for 16 h at either 37 °C (control) or at 41.5 °C (heat shock). The cells were then washed and incubated with fresh medium for 2 h either at 37 °C (control) or at 43 °C (heat shock), before protein extraction. In all cases, lacZ activities and CAT protein detection were measured using an in vitro β-galactosidase assay [33] and the CAT-ELISA kit (Boehringer) respectively.

RESULTS

Heat-shock-induced accumulation of clusterin transcripts

We first determined if the original stress inducer, heat shock, could lead to an accumulation of clusterin mRNAs. To this end, human A431 cells were subjected to 42 °C for 1 h. mRNAs were prepared 30 min after the temperature had been reduced to 37 °C, reverse transcribed using random primers and tested for the presence of clusterin, hsp70 and β-actin cDNAs by PCR. These measurements clearly showed that both clusterin and hsp70 mRNA accumulate after heat shock (Figure 1). Moreover,
An extracellular version of heat-shock protein

Figure 2  Search for conserved cis-elements in clusterin gene promoters

Sequence alignment between mammalian clusterin gene promoters points to very few regions of homology. Their conservation was then tested by comparison with another vertebrate class using an avian clusterin promoter. The CLE is strictly conserved, while comparison with surrounding contexts shows complete divergence between quail and mammals. DNA sequences conserved between mammals are indicated with dots and those also conserved in bird with asterisks.

the clusterin and hsp70 mRNA contents increased to a similar extent, shown by phosphorimager quantifications (Figure 1B). The molecular processes leading to this rapid clusterin mRNA accumulation were then investigated.

Identification of a heat-shock transcription cis-element in the clusterin gene promoters

Assuming that the motifs best conserved throughout evolution are the candidates of choice for having a role in important regulatory functions, we compared clusterin gene promoters from evolutionarily distant vertebrate species, in order to isolate the most conserved cis-elements. Preliminary sequence alignments between mammals have pointed to a relatively modest overall conservation [34], contrasting with the fine transcriptional control of the clusterin genes. As shown in Figure 2, the region of homology between mammalian promoters is confined within a very proximal domain, while the most upstream regions appear completely divergent. In particular, it is worth noting that the NF1 twin elements, recently shown to be important for clusterin expression during tissue involution in mouse [35] (boxed in Figure 2), are not conserved in rat or human. In the proximal promoter, an AP1 site is present in the mammalian promoters but is absent from the avian one. In fact, an AP1 site is also found in birds but is located in an alternative promoter [32]. Beside the AP1 element, too widespread to account for the specificity of clusterin expression alone, we find a longer domain not yet reported but strictly conserved between all clusterin promoters, which we named ‘CLE’ (clusterin element). Such a striking degree of conservation, restricted to a 14-bp region located at similar distances from transcription start sites, is unlikely to have occurred by chance and has led us to search for possible homologies between CLE and the cis-acting motifs identified so far. Relationships can be found with heat-shock elements (HSE), whose consensus sequence is nTTCnnGAAnnTTCn. CLE can be written: nTTCnnGAAnn-CTCn, which differs by only one base from the idealized HSE. This single change concerns the fourth base of a 5-bp repeat,
nGAAn, and thus, as suggested by Fernandes et al. [36], should not greatly affect the affinity of the heat-shock factor (HSF) binding. In addition, the central motif AGAAA corresponds precisely to the most accurate nGAAn repeat, based on the binding affinity to HSF (nucleotide A1) and on the bp frequencies in heat-shock elements of Drosophila (nucleotide A5) [36].

Heat-shock factors binding to CLE

We have tested the ability of CLE to be a specific target for HSF binding. Figure 3 shows that cellular extracts from heat shocked cells are strongly enriched in CLE- as well as HSE-binding factors. Moreover, reciprocal competition experiments using CLE and HSE alternatively as probes and as unlabelled competitors, demonstrate that CLE- and HSE-binding factors are interchangeable. As expected, the CLE probe containing only 3 substituted bases out of 27 in the central nGAAn repeat, has no competitive activity. To further identify the nature of the HSF involved in the formation of CLE complexes, anti-HSF1 antibodies were added together with the CLE probe and cellular extracts. Figure 3(B) shows that the CLE complex is converted to a slower migrating antibody-containing ternary gel-shift complex in the presence of anti-HSF1 antiserum but not with normal serum. This supershift experiment indicates that the CLE-binding cellular factor contains HSF1.

Heat-shock-induced transcriptional activity of CLE in transient expression assays

The ability of CLE to mediate a transcriptional response to heat shock was then investigated by inserting one or more copies of the 27-bp region from the quail clusterin promoter containing CLE, upstream of tk gene-derived proximal elements. As shown in Figure 4, even outside its original location, wild-type CLE displays a strikingly heat-shock-dependent transcriptional activity when present at two or four copies. The lack of activity of the construct containing only one copy of CLE is surprising because natural clusterin promoters contain a single CLE. Such a situation, however, is classically observed for most cis-elements when removed from their original promoter context, since they can no longer co-operate with adjacent promoter sequences. To test this possibility, we have introduced point mutations into the CLE present in the promoter of the avian clusterin gene, while maintaining unchanged the natural surrounding DNA sequences.

Figure 3 Complex formation between CLE and protein extracts from heat-shocked HeLa cells

(A) Competitive binding of HSF to the conserved CLE and to the idealized HSE. Cellular extracts (CELL EXT.) from normal (37°) or heat-shocked (42°) HeLa cells were tested for binding to (left panel) CLE or (right panel) HSE probes, as described in the Materials and methods section. The low-mobility complexes (arrow-heads) could be removed by previous addition of a 100-fold excess of wild-type but not mutated unlabelled competitors (COMPET.) corresponding to the probe. An excess of HSE, but not of mutated HSE (mutHSE), inhibited the formation of CLE complexes, while an excess of CLE, but not of mutated CLE (mutCLE) totally prevented HSE–HSF complex formation. (B) Ternary complex formation between CLE (PROBE), protein extracts from heat-shocked cells (CELL EXT. 42) and anti-HSF1 antibodies (SERUM). Anti-HSF1 or control (CONT) rabbit serum was added to CLE–heat-shock extract mixtures and incubated for 20 min before electrophoretic mobility-shift assays. HSF–CLE complex (TC) migration was retarded in the presence of anti-HSF1 antiserum.

Figure 4 Transient-expression assays using A431 human cells, either cultured in normal conditions (37°C) or heat shocked for 30 min at 42°C

The CAT expression is driven by chimeric promoters containing one, two or four copies of CLE, upstream of the tk basal promoter (TK PROM). Control experiments were done using CLE motifs mutated at the level of the central nGAAn repeat (mutCLE) and tested in the same conditions. The results represent the means of three independent transfection experiments.
Point mutagenesis of the clusterin promoter at the level of CLE

The three central bases GAA of the CLE present in the quail clusterin promoter were replaced by the triplet TCC and were subjected or not to heat shock. As shown in Figure 5, this substitution of the three central bases GAA of the CLE present in the quail clusterin gene promoter to transmit the transcriptional heat-shock induction, further demonstrate that this element, conserved in all clusterin promoters, functionally relates to a stress cis-element.

Examples of co-regulation of clusterin and hsp genes are available in the literature, for example in brain trauma [37], in the prostate cell death model, where inductions of both hsp70 [38] and clusterin [25] mRNAs were observed, or during involution of the lactating mammary gland, where clusterin and hsp27 genes are co-ordinately up-regulated [22]. The presence of a conserved and active HSE in all clusterin promoters studied so far is likely to account for these observations and shows a definitive link between clusterin and classical hsp gene expression. This finding also provides a unifying explanation for the multiplicity of biological disturbances reported to trigger clusterin mRNA accumulation, such as ischaemia [39], anti-neoplastic chemicals [25,40], neuronal injury [2], aging [41] or oncogenes [42], since all these conditions have been classified as inducers of the stress response [43]. As shown here, this list can be enlarged to the original stress inducer, heat shock. One must, in addition, notice that another cis-element with purported stress-mediating activities, namely API [44,45], is also present in clusterin regulatory regions [32,34] and may act in synergy with CLE making the clusterin gene promoters particularly sensitive to environmental changes.

While the strong overexpression of clusterin/ApoJ is well established in a wide variety of pathological states, its precise involvement in these disorders remains puzzling. Functional studies have demonstrated that the clusterin protein displays protective activities against cytolylotic hydrophobic molecules [27,46], including complement [8,9,26] and amyloid [7]. The present demonstration that clusterin gene expression can be controlled, at least in part, through a classical stress-responsive element, raises the possibility that clusterin corresponds to an extracellular atypical class of hsp, involved in membrane stabilization rather than in the protection of the protein components of the cell, as is the case for the classical hsp. Considering the purported cytoprotective and anti-apoptotic activities of clusterin, its stress-induced expression may participate favourably in the influence of heat shock on cell survival [47,48].

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REFERENCES

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