Functional analysis of the human annexin I and VI gene promoters

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INTRODUCTION

Annexins I and VI are members of a ubiquitous family of Ca\(^{2+}\)-dependent phospholipid-binding proteins suggested to have diverse cellular functions [1–3]. These include Ca\(^{2+}\) channel formation, vesicle trafficking in exocytosis and endocytosis, and inhibition of phospholipase A\(_2\) (PLA\(_2\)). The latter function was first suggested for annexin I, originally known as ‘lipocortin’, the first annexin to be cloned and the prototype for the family [4]. Annexin I was also discovered as a cellular substrate for phosphorylation on tyrosine by the epidermal growth factor receptor tyrosine kinase [5]. These observations led to two models for annexin I function, one being that annexin I might couple growth factor receptor activation to phospholipid turnover during signal transduction and the other proposing that, as a PLA\(_2\) inhibitor, annexin I could function as an endogenous mediator of the anti-inflammatory response. The latter model was supported by studies showing that annexin I expression seemed to increase as a consequence of steroid administration in inflammatory conditions [6]. Inhibition of PLA\(_2\) has now been shown to be a property of many of the annexin family, casting doubt on the theory that this is a specific function of annexin I. The mechanism of inhibition seems to be a consequence of the ability of all annexins to bind Ca\(^{2+}\)-dependently to acidic phospholipids, Ca\(^{2+}\) and phospholipid being co-factor and substrate respectively for the phospholipase [7].

Annexin VI is unique within the family in that it contains eight repeats of the conserved 70-residue domain that defines annexins, various lines of evidence point to the annexin VI gene having arisen through the duplication of a four-repeat annexin [8,9]. Relatively little is known about the cellular role(s) of annexin VI but recent studies suggest that it might be involved in Ca\(^{2+}\) homoeostasis. Thus the overexpression of annexin VI targeted to the cardiac myocytes of transgenic mice leads to defective mobilization of Ca\(^{2+}\) in response to stimulation, lower resting Ca\(^{2+}\) levels and general cardiac hypertrophy [10]. Other studies have identified a growth regulatory role for annexin VI, on the basis of observations that heterologous expression in A431 cells leads to a partial reversal of the transformed phenotype and less aggressive tumour growth [11,12]. In support of a tumour-suppressor role, loss of annexin VI in melanocytes was shown to be associated with the development of metastatic malignant melanoma [13]. These findings are not necessarily incompatible with the transgenic mouse studies if annexin VI acts as a negative regulator of Ca\(^{2+}\) mobilization in different cell types. Alternatively the growth-suppressive activity of annexin VI could be linked to a physical interaction with p120-GAP, which might contribute to the down-regulation of activated Ras during signal transduction [14]. Annexin VI was also proposed to form part of the molecular complex that mediates pinching-off of clathrin-coated pits, an early step in endocytosis [15]. However, the fact that endocytosis occurs in cells that lack annexin VI argues that this is unlikely to be an essential function for this protein [16].

Despite marked similarities in their biochemical properties, crystal structures and gene organization, annexins I and VI have unique patterns of tissue expression, suggesting distinct regulatory mechanisms. To examine the steroid sensitivity of the annexin I gene and identify the molecular basis for their distinct patterns of tissue expression, we performed a comparative analysis of the annexin I and VI promoters in HeLa and A431 cells. A431 cells express annexin I but not annexin VI [16], whereas our HeLa clone expresses annexin VI but not annexin I. We report that neither promoter is steroid-inducible, suggesting that if annexin I and/or VI function as anti-inflammatory mediators it is in the absence of any induction by steroids, and that the functional organizations of the two promoters are strikingly different.

EXPERIMENTAL

Materials

Tissue culture media and supplements were from Life Technologies. Tissue culture plasticware was from Nunc. Western Blue substrate, goat anti-rabbit alkaline phosphatase-conjugated antibody and the luciferase (LUC) assay kit were from Promega.

Abbreviations used: DEX, dexamethasone; LUC, luciferase; PLA\(_2\), phospholipase A\(_2\); TSS, transcription start site.

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AC-3 forward primers were used in PCR reactions with the full-length AA-3 ethidium bromide centrifugation [17].

The 881 bp annexin I promoter was a gift from Dr. F. Russ-Marie and Dr. E. Solito (ICGM, Paris, France). Two oligonucleotide primers were constructed to amplify the annexin I promoter by PCR, and insert flanking XbaI sites to facilitate subcloning (5'-GCTCTAGATGTTGTTCAACTCTTAGG-AGGAC-3' and 5'-GCTCTAGACTGATAATTGTGAACACTAGTACCC-3'). XbaI sites underlined). Denaturation, annealing and elongation temperatures were 95, 65 and 72 °C respectively for 1 min each for 30 cycles. The PCR product was ligated directly into the TA cloning vector, excised with XbaI and ligated into Nhel-cut pGL3 Basic vector. The construct was fully sequenced to verify that no mutations had occurred.

For generation of the annexin I deletion series, the following forward primers were used in PCR reactions with the full-length promoter as template: AnxI-1, 5'-TAAAACAGTTGACACGA- AA-3'; AnxII-1, 5'-CTACAAAAGTGTGGCCCA-3'; AnxII-3, 5'-TAATGCGAGTTGAATTTG-3'; AnxII-4, 5'-CAAGTCCTCACAAGCAA-3'; AnxII-5, 5'-CTCTCTCTAGGATTTA-3'; with the reverse primer 5'-CTGATAGTTTTCATTTAG-3'. PCR products were subcloned into the pTAG cloning vector, sequenced to determine orientation, then excised with MluI and XhoI before being ligated into pGL3 Basic vector cut with the same enzymes. The 892 bp annexin VI promoter previously isolated in this laboratory [8] was fully sequenced (both strands in both directions) with the pfu-PCR sequencing kit to avoid compressions due to the high GC content. The promoter was excised with XhoI and ligated into XhoI-digested pGL3 Basic vector. For generation of the deletion series, the following forward primers were used in PCR reactions with the full-length promoter as template: AnxVI-1, 5'-ACCTTCTAAGGCGCACCC-3'; AnxVI-2, 5'-CTGGGGACAATAATTCT-3'; AnxVI-3, 5'-GGCTCTCTCCCTCTAGG-3'; AnxVI-4, 5'-GTGAGGCA-GGCCTGACTCT-3'; AnxVI-5, 5'-GCTAGAGGGTGGGGG-TGG-3'; AnxVI-6, 5'-TCGGAGCCACCGGTGT-3'; with the reverse primer 5'-CTCGAGAGCGACCGCTG-3'. PCR products were subcloned into the pTAG cloning vector, sequenced to determine orientation, then excised with MluI and XhoI before being ligated into pGL3 Basic vector cut with the same enzymes. All constructs were purified by double CsCl/ethidium bromide centrifugation [17].

Cell culture and transfections

A431 and HeLa cells were cultured in Dulbecco’s modified Eagle medium containing 10% (v/v) heat-inactivated foetal bovine serum, 25 mM l-glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin sulphate. Cells were grown in 90 mm dishes and were trypsin-treated twice weekly to maintain sub-confluence at all times. For transfection with promoter constructs, cells (70–80 % confluent) were trypsin-treated and transferred to six-well culture plates 24 h before transfection. Transfections were performed with the Bes-buffered calcium phosphate procedure [17], with 24 µg of promoter construct and 12 µg of the SV2-β-gal control vectors to normalize transfection efficiency. After incubation overnight with the calcium phosphate–DNA precipitate, cells were washed twice with complete growth medium, the medium was replaced and the cells were left for a further 24 h. For cells undergoing steroid treatment, medium was supplemented with 1 µM dexamethasone (DEX) for the times indicated. Cells were lysed into 400 µl of Promega 1 x reporter lysis buffer [125 mM bicine buffer (pH 7.6)/0.25% Tween 20/0.25% Tween 80] and extracts were centrifuged to remove unbroken cells and debris. Extracts were then used for measurement of LUC (10 µl) or β-galactosidase activity (150 µl). LUC activity was measured with the Promega Luciferase assay kit; samples were counted on a Hewlett Packard Tri-Carb liquid-scintillation analyser, ensuring that the measurements fell within the linear range of the counter (previously determined with purified LUC protein). β-Galactosidase activity was measured spectrophotometrically (at 420 nm) by the generation of o-nitrophenol from o-nitrophenyl β-D-galacto-pyranoside. Data were either normalized as relative light units per unit of β-galactosidase activity or by dot-blotting.

For dot-blotting, cell extracts were treated with RNase A and proteinase K for 1 h at 37 °C in each case. Samples were then extracted first with phenol/chloroform (1:1, v/v) and then with chloroform. After denaturation (100 °C for 5 min), extracts were spotted on Hybond-N with a Hybridot Vacuum Manifold (BRL). After cross-linking, membranes were probed with the LUC cDNA randomly labelled with [γ-32P]dCTP. Spot intensity was determined with MacBas software and phosphorimaging.

Electrophoretic mobility-shifting assays

Nuclear extracts were prepared essentially as described elsewhere [18]. One 90 mm plate of subconfluent cells [approx. (1–2) x 10^6 cells] was washed twice with ice-cold PBS, lysed into 400 µl of lysis buffer [10 mM Hapes (pH 7.9)/10 mM KCl/1 mM dithiothreitol/1 mM EDTA/1 mM EGTA/0.2% (v/v) Nonidet P40/1 mM β-glycerophosphate/20 mM NaF/1 mM sodium orthovanadate/1 mM aprotinin/1 mM chymostatin/1 mM pepstatin A] and incubated on ice for 10 min. Lysates were centrifuged and nuclei were resuspended in buffer containing 20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20 mM NaF, 1 mM aprotinin, 1 mM chymostatin and 1 mM pepstatin A, then incubated on ice for 20 min. Insoluble material was pelleted by centrifugation, and the supernatant containing nuclear proteins was stored at −80 °C before use.

Three double-stranded oligonucleotide probes were designed to encompass the minimally active region of the annexin VI promoter. The ‘SP-1’ probe (5’-CTAGAGGGTTGGGTT-3’) the ‘intervening sequence’ probe (5’-GAGGGAGGGTGGG-CCG-3’) and the ‘c-ETS1’ probe (5’-CGCCGATTTGCTCTT-3’) were end-labelled with [γ-32P]dATP and polynucleotide kinase. Unlabelled competitor or random oligonucleotide (5’-AAGAGCGAGAATTTTTCAGCTGTTAAAGG-3’) (100-fold molar excess) probe was allowed to bind to nuclear proteins in the presence of poly(dI-dC) for 10 min before the addition of radiolabelled probe. Binding assays were performed in a buffer containing 8% (v/v) Ficoll, 1 mM MgCl2, 20 mM Hepes, pH 7.9, 1 mM dithiothreitol and 50 mM NaCl. Radiolabelled probe was left to bind to nuclear proteins for a further 20 min before being resolved on a 4%, (w/v) polyacrylamide gel. Radioactive bands were detected with a Fuji phosphorimagier.
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Northern and Western blotting
RNA was extracted with acid guanidinium isothiocyanate and resolved on a 1.6% (w/v) agarose gel containing 6.4% (v/v) formaldehyde as described previously [16]. This was transferred to Hybond-N before being cross-linked by irradiation with short-wavelength UV. The membrane was then hybridized to probes for human annexin I or human annexin VI, labelled with a random-primed labelling kit. Radioactive bands were detected with a Fuji phosphorimager and images were processed with MacBAS software. Western blotting was described as previously [11].

RESULTS
We previously described the partial sequence of the human annexin VI gene promoter [8] extending approx. 500 bp upstream from the transcription start site (TSS). Figure 1 shows this sequence extended in the 5′ direction. During the sequence analysis we noted several errors in our original published sequence; these have been corrected in the present paper. Database searching for similar sequences in other genes led to two unexpected observations. First, a 120 bp region from −590 to −710 exhibited a striking similarity to a sequence within the third intron of the human interleukin 4 gene as well as to other genomic DNA sequences (in the ERCC2 and NG2-6 genes). Intriguingly, interleukin 4 and annexin VI are both located on the long arm of chromosome 5. Secondly, a 100 bp region spanning the TSS was similar to part of the c-Src promoter and also elements within the long terminal repeats of the herpes simplex and pseudorabies viruses (results not shown). Analysis of the sequence for potential transcription factor-binding sites revealed numerous candidates, of which the strongest are illustrated in Figure 1.

To determine whether the annexin VI promoter was functional and to examine whether this or the annexin I promoter exhibited sensitivity to DEX, both were cloned into LUC expression vectors and transiently transfected into A431 and HeLa cells (Figure 2). Although both promoters were strongly active in both cell lines, neither promoter displayed any significant change in activity in the presence of DEX over 12 h. However, because the promoters might not have contained all the regulatory elements that drive the expression of the endogenous annexin I and VI genes, we also examined the effect of DEX on annexins I and VI at both the mRNA and protein levels (Figure 3). Note that the HeLa clone used in these studies expresses annexin I but not annexin VI, whereas A431 cells express annexin I but not annexin VI. Consistent with the results obtained with the annexin I–LUC construct, DEX had no discernible effect on annexin I and VI mRNA and protein.

To identify regions of the annexin I and VI promoters involved in transcriptional regulation we used PCR to make a series of 5′ deletions of each promoter, which were cloned upstream of
LUC reporter gene and transiently transfected into A431 and HeLa cells (Figure 4). Progressive deletion of the annexin I promoter from −610 to −172 was accompanied by a slight increase in activity in both cell lines, suggesting the removal of a weakly negative regulatory region. AnxI-3 contained the minimal 5' DNA sequence necessary for full activation of the annexin I promoter. Further deletion from −172 to −13 resulted in an almost complete loss of promoter activity in both cell lines, demonstrating that the major sites for co-operative interactions between cis elements and transcription factors are located within the domain that contains the CAAT and TATA boxes, strongly suggesting that these sites form the principal regulatory locus of the annexin I gene.

A puzzling feature of the annexin VI promoter is that the CAAT and TATA boxes are located more than 200 bp upstream of the TSS. To test the functionality of these elements and to identify other regions involved in the transcriptional regulation of annexin VI, a series of annexin VI promoter–LUC deletion constructs were tested in A431 and HeLa cells. In contrast with the annexin I promoter, removal of the distal nucleotide sequences from −790 to −541 resulted in a substantial loss of activity, indicating the presence of positive regulatory sequences in this region. Further deletion of the region between −207 and −54 was accompanied by a return to full activity in A431 cells and greater than basal activity in HeLa cells. Collectively, these results suggest the presence of a strong negative regulatory element between −207 and −54 in the annexin VI promoter, and a transcriptionally active sequence immediately proximal to the TSS corresponding to the minimal active promoter.

To test the authenticity of the minimal annexin VI promoter, electrophoretic mobility-shift assays were performed on three non-overlapping double-stranded oligonucleotide probes encompassing the region from −5 to −53 (Figure 5). The most 5' probe containing the putative SP1-binding site formed a complex with a protein in nuclear extracts of both A431 and HeLa cells that was competed for by unlabelled probe but not by a 100-molar excess of an unrelated oligonucleotide. The same gel-shifted band was also competed for by a commercially obtained SP1 probe. Neither the 'c-ETS1' nor the 'intervening sequence' probe formed gel-shifted complexes. These results show that the minimally active annexin VI promoter contains sequence elements capable of forming complexes with the ubiquitous SP1 transcription factor.

FIGURE 3 Endogenous annexin I and VI genes are unaffected by DEX

(A,B) Northern blots of mRNA for annexin I (A) and annexin VI (B) after stimulation of A431 and HeLa cells respectively with 1 µM DEX for the durations indicated. The panel below each Northern blot shows an ethidium bromide-stained gel to illustrate comparative loadings. (C,D) Western blots of annexin I (C) and annexin VI (D) in A431 and HeLa cells respectively, after stimulation with 1 µM DEX for the durations indicated.

DISCUSSION

A major aim of this study was to address the contentious issue of whether or not the annexin I promoter is sensitive to steroids. There are conflicting reports on the effects of steroids on the expression of annexin I. Several studies have reported induction of annexin I expression after exposure to DEX [19,20], but other studies find a poor correlation between increases in annexin I mRNA and increased annexin I protein [21]; some investigators have found glucocorticoids to be completely without effect on annexin I expression [22]. There are several factors that complicate the analysis of the steroid sensitivity of the annexin I gene and might account for these contradictory findings. First, in several cell types glucocorticoids seem to stimulate the secretion of annexin I [23]. If the maintenance of cellular levels of annexin I is important, the induction of annexin I to replenish normal stocks would be a response not to steroids themselves but to the depletion of the cellular pool. A second complication is that several of the model systems used to demonstrate the induction of annexin I by glucocorticoids also involve terminal differentiation of the cells under examination [24]. Clearly there is the possibility that the elevation of annexin I is a consequence of cellular differentiation rather than a direct response to steroids. Indeed, non-steroidal cell differentiation has been shown to be accompanied by significant increases in annexin I levels [25].

In this study we found the 881 bp annexin I promoter to be
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Transcript expression of serial deletions of the annexin I and VI promoters

The activity of full-length and serially deleted promoters of annexins I and VI was assayed in HeLa and A431 cells. Activities are depicted relative to those of the full-length promoters, which are assigned 100% activity and represent the mean values from six separate transfection experiments. Transfections in A431 cells were normalized against β-galactosidase and in HeLa cells by dot-blotting. For each full-length promoter, the first transcribed nucleotide is numbered +1. The relative positions of CAAT and TATA boxes and a putative glucocorticoid response element (GRE?) in the first intron of the annexin I gene are indicated [26]. The annexin VI promoter shows the relative positions of two additional elements. Towards the 5' end is a domain that has close similarity with sequences in the interleukin 4 gene (IL-4, ERCC2, NG2-6), and surrounding the transcription start site is a domain with similarity to the human c-Src gene promoter (c-Src homology). For each of the deletion series the names, for example AnxVI-2 and AnxI-3, refer to oligonucleotide primers used to generate the corresponding fragments. Primer sequences are detailed in the Experimental section.

In insensitive to DEX during 16 h of treatment, in HeLa and in A431 cells. The putative glucocorticoid response element in the human annexin I promoter [26] therefore seems to be non-functional. Consistent with these findings, there was no induction of annexin I mRNA or protein in A431 cells in response to DEX over an 8 h time course. Although fibroblasts (HeLa) and epithelial cells (A431) are not directly involved in the anti-inflammatory response, the ability of all cell types to modulate gene expression in response to steroids means that these are valid models in which to study steroid responsiveness. Our results leave open the possibility that annexin I has a role in mediating the anti-inflammatory actions of glucocorticoids; however, they strongly suggest that this is independent of any direct influence of glucocorticoids on annexin I gene expression. Analysis of a series of promoter deletion constructs revealed that the minimal annexin I promoter was equally active in both A431 and HeLa cells and slightly more active than the full-length promoter. The only cell-type-specific difference was the greater activity of fragment Axl.2 in A431 than in HeLa cells. This might indicate the existence of a weak repressor between nt −318 and −610 of the annexin I promoter that functions only in HeLa cells.

Collectively, these results lead to the unsurprising conclusion that the regulation of annexin I gene expression is mediated by conventional CAAT and TATA box-binding factors. Because gene regulation via these elements has been extremely well characterized for other genes, the minimal active annexin I promoter was not investigated further.

The most striking cell-specific finding for the annexin VI promoter deletion series was the vigorous activity of the minimal promoter in HeLa cells. Qualitatively, the expression of the deletion constructs was broadly similar in A431 and in HeLa cells, with evidence for repressive activity between nt −207 and −700 in both cell lines. The greater activity of all the annexin VI constructs in HeLa cells might be due to the strength of the minimal promoter in these cells. In the annexin VI promoter the CAAT and TATA boxes are located much further from the TSS than is normal in genes regulated by these elements, and although the spatial arrangement of these elements with respect to one another is consistent with a functional role, their presence was associated with decreased promoter activity. The annexin VI promoter is therefore, in functional terms, a TATA-less promoter. The regulation of such promoters can be directly
Figure 5  Electrophoretic mobility-shift assays

The minimal active annexin VI promoter was analysed as three separate components (‘SP1’, ‘c-ETS1’ and ‘intervening sequence’) to assess transcription factor binding. The first three panels show results obtained with nuclear extracts (NE) isolated from A431 and HeLa cells in the presence of competitor (comp) or non-competitor (non-comp) oligonucleotides. The right-hand panel shows the result obtained with the SP1 site oligonucleotide as probe but with a consensus SP1 oligonucleotide as competitor. The arrow indicates the position of the SP1 complex; the position of free radiolabelled oligonucleotide (oligo) is also shown. Bands were detected with a Fuji phosphorimager and images were processed with MacBAS software.

influenced by sequences covering the TSS [27]. These so-called initiator elements might function either in conjunction with a TATA box or in its absence. By analysing the activity of a number of initiator element mutants, a consensus binding site for the transcription factors TFII-I and USF has been determined [28]. The annexin VI promoter contains a perfect consensus (CACGTG) for such an initiator element. TFII-I and USF have been shown to form a co-operative complex that covers the TSS and activates transcription in specific promoters such as the adenovirus major late promoter [27]. Whether these factors are responsible for the cell-specific patterns of activity of the promoter fragments is unknown. Whereas USF has been shown to be widely expressed, TFII-I expression is less well documented, although it is present in HeLa cells [28,29].

By performing a comparative analysis of the human annexin I and VI genes, we have shown that despite being closely linked in terms of the structure and biochemical properties of the proteins they encode, they are significantly different with respect to transcriptional regulation. In particular, the annexin VI gene contains several unusual elements, most notably a sequence similar to a sequence in the third intron of the interleukin 4 gene located within the cytokine gene cluster on the same part of chromosome 5. The function of this domain is unclear: we were unable to find any evidence for enhancer activity (results not shown), yet it contains elements that allow the full-length promoter to overcome inhibitory elements located downstream. The minimal active annexin VI promoter was confined to a domain within the 53 bp proximal to the TSS in which the strongest consensus sequences for transcription factor-binding sites corresponded to those for SP1 and c-ETS1. Although we were unable to find any evidence for the binding of c-ETS1 to this region, the SP1-binding site was clearly functional and probably underlies the broad pattern of annexin VI tissue expression. Given that the expression of c-ETS1 might be restricted to T cells, the possibility remains that the putative c-ETS1 site in the annexin VI promoter is functional in lymphoid cell lineages.

There is little information on the regulation of other annexin genes. The most extensive studies have been performed by Horseman and co-workers, who compared the transcriptional regulation of the pigeon cp35 and cp37 genes [30]. These two genes are closely related in terms of their primary amino acid sequences, yet the regulation of their expression is quite distinct. The cp37 gene is constitutively expressed and is closely related to the mammalian annexin I gene [31], whereas the cp35 gene is expressed only in response to prolactin [32]. Analysis of the mechanism of prolactin induction of the cp35 gene suggests that tyrosine phosphorylation and the subsequent activation of at least one member of the STAT family of transcription factors could lead to the direct transcriptional activation of cp35 [30]. When compared with the results of this study, the work on the pigeon annexin I genes reinforces the view that diverse regulatory mechanisms underlie annexin gene expression in vertebrates. Such diversity could explain the differences in cell and tissue expression of members of the annexin family. The results described here provide the first insight into the mechanisms of regulation of the mammalian annexin gene promoters and constitute an important advance in our understanding of the relationship between the actions of glucocorticoids and annexin biology.

We thank Tim Hawkins for alerting us to interesting elements in the annexin VI promoter, and Dr. Paul Smith for advice on gel mobility-shift assays. This work was supported by the Arthritis and Rheumatism Council.
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Received 19 January 1998/25 February 1998; accepted 17 March 1998