Characterisation of the sarcoidosis-associated variant of annexin A11

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Abstract. Recent studies on the genetic background of sarcoidosis have resulted in the discovery of a strongly associated single nucleotide polymorphism (SNP) that switches a highly conserved arginine to a cysteine at position 230 in annexin A11. The effect of the R230C SNP on the cellular distribution and Ca^{2+}-sensitivity of annexin A11 was investigated through over-expression of GFP tagged annexin A11 in A431 cells. Cells were stimulated with calcium mobilizing agonists and changes in the cellular localisation of GFP tagged annexin A11 were recorded. Neither variant of annexin 11, nor any truncation mutants, exhibited any response to EGF. In addition, there was no relocalisation of the GFP tagged C-terminal annexin A11 variants in response to ionomycin. However, both the wild type and sarcoidosis associated variants of annexin A11-GFP relocalised to the plasma membrane and then the nuclear envelope in response to ionomycin. These observations show that the sensitivity of annexin A11 to a robust, sustained rise in intracellular calcium, is not significantly affected by the sarcoidosis associated SNP. This does not rule out functional affects in the extracellular milieu, in cytokinesis, nuclear envelope breakdown or in response to other intracellular signals.

Key words: Annexin — Sarcoidosis — Calcium

Introduction

Sarcoidosis is a multisystem immune disorder, resulting in the formation of epitheloid granulomas throughout the body, particularly within the lungs, eyes and skin. The immune systems of affected individuals exhibit significant changes in cell numbers and cell signaling, with an increase in CD3 and CD4 positive T cells in the lungs. Activated T cells within sarcoid lungs have also been shown to over-express several cytokine receptors, including the interleukin-2 receptor (IL-2R), and produce increased amounts of cytokines, including interleukin-2 (Pinkston et al. 1983) and interferon-γ (IFNγ) (Robinson et al. 1997). In addition, monocytes and macrophages are heavily involved in the formation of sarcoid granulomas and also secrete a range of cytokines that further enhance the immune response. For example alveolar macrophages secrete tumor necrosis factor α (TNFα), which is a current target for sarcoidosis therapy (Toussirot et al. 2008) and interleukin-15, which has been shown to induce T cell proliferation (Agostini et al. 1996).

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Given the complex nature of the immune pathways involved and the interplay between different immune cell types, understanding the molecular aetiology of sarcoidosis has proven difficult. Although a working hypothesis for the formation of sarcoid granulomas has been put forward (Noor and Knox 2007), the initiating antigen in this process is unknown and speculated to involve both viruses and bacteria (Ezzie and Crouser 2007). A better picture is however emerging for the genetic factors linked to susceptibility to sarcoidosis.

Familial linkage studies identifying regions encoding alleles more commonly shared between affected family members uncovered a SNP in the butyrophilin-like 2 (BTNL2) gene. BTNL2 lies within the MHC class II region and is a member of the B7 receptor family. It is thought to act as a co-stimulatory molecule for T cell activation. The SNP results in a premature stop codon, which produces a truncated protein that can no longer localize to membranes. This truncation is postulated to promote a pathologically inflammatory environment, as occurs in sarcoidosis (Valentonyte et al. 2005). Genes neighbouring BTNL2 have also been implicated as they show a high linkage disequilibrium and therefore may be inherited together, such as the human leukocyte antigen DRB1 (HLA-DRB1) (Spagnolo and du Bois 2007). There-

fore it may be the HLA class II genes, like HLA-DRB1, that increase susceptibility to sarcoidosis. Several HLA genes have been implicated in association studies, with the suggestion that these mutant HLA proteins present antigen to T cells in a manner that induces a pathological immune response. These include HLA class I genes, such as HLA-B7 and 8, as well as HLA class II genes which are known to be up-regulated on alveolar macrophages from sarcoidosis patients (Spagnolo and du Bois 2007). Thus far the focus on susceptibility genes has been restricted to MHC proteins. However a recent study has identified a non-MHC protein, namely annexin A11.

The identification of annexin A11 was the result of a genome wide association study using 500 control and sarcoidosis-presenting individuals from a German population. Several disease-associated SNPs were found, including those in HLA loci and the BTNL2 gene. Excluding these known areas of association, an individual SNP within the annexin A11 gene produced the highest association signal, alongside five other neighbouring SNPs within this haplotype that were also strongly associated with sarcoidosis (Hofmann et al. 2008).

The non-synonymous SNP in annexin A11 results in the switch of a basic arginine to a polar cysteine at position 230, within a highly conserved domain called the annexin repeat in the C-terminal domain of the protein. Immunohistochemistry of control and sarcoidosis patient lung tissue showed no difference in annexin A11 expression, and in both cases expression was nuclear in epithelial and mononuclear cells and within the cilia of bronchial epithelial cells. Annexin A11 mRNA is particularly abundant in CD4, CD8, CD14 and CD19 positive immune cells. However, in normal individuals annexin A11 mRNA expression was significantly reduced in stimulated CD8 and CD19 positive cells compared to those at rest (Hofmann et al. 2008). Whether this would also be true of the SNP variant is currently unknown.

The functional significance of this SNP and so the mechanism of action of annexin A11 in sarcoidosis, is as yet unknown. Annexin A11 belongs to the family of vertebrate annexins which are calcium-dependent, phospholipid-binding proteins. Annexin A11 has been implicated in the regulation of cytokinesis (Tomas et al. 2004) and is known to bind S100A6 (calcyclin) (Tokumitsu et al. 1992) and ALG-2 (apoptosis-linked gene 2) (Satoh et al. 2002). However beyond this, little is known about the function of annexin A11. In order to better understand the effect of the sarcoidosis associated SNP on annexin A11, we have investigated the response of the wild type and the sarcoidosis-associated variant of annexin A11 to rises in intracellular calcium. We have shown that this aspect of annexin A11 biology appears unaffected by the sarcoidosis associated SNP.

Materials and Methods

Cell Culture

A431 (human epidermoid carcinoma) cells were cultured in DMEM (Dulbecco's minimal essential media, Gibco) with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine and incubated at 37°C with 5% CO_2.

Site Directed Mutagenesis

Site directed mutagenesis was carried out in plasmids containing either full-length annexin A11-GFP or C-terminal annexin A11-GFP. These wild type plasmids are as previously described (Tomas and Moss 2003). Single amino acid changes were introduced into wild type plasmids using two oligonucleotide primers that were complimentary to the target gene, but were designed to contain the appropriate mutation in the middle of the primer. (Forward primer sequence; GACTGCCTGGGGAGTTGCTCCAACAAGCAGCGG. Reverse primer sequence; GCTGCTTGTTGGAGCAACTCCCCAGGCAGTC). PCR reactions were carried out using 125 ng of each primer, 50 ng of the plasmid containing the gene insert, dNTP mix and Platinum Pfx (Invitrogen) in a 50 μl volume. The PCR reaction was performed for 18 cycles of 30 s at 95˚C (to separate template strands), 1 min at 55˚C (to anneal the primers) and finally 4 min at 68˚C (to extend from the primers). The reaction was then cooled to 4˚C and digested at 37˚C for 1 h with 1 μl Dpn1 to remove the parental methylated and hemimethylated DNA. 15 μl of this reaction was transformed into competent XL-1 Blue *E.coli*.

Transient Transfections

Cells were plated at 50% confluency on 35 mm glass bottomed microwell dishes (MatTek) in DMEM containing 10% heat-inactivated FCS without antibiotics. Following an overnight incubation at 37°C with 5% CO_2 the cells were transfected with 3 μg of plasmid DNA using 9 μl of TransIt LT1 transfection reagent (Mirus). 9 μl of TransIt LT1 transfection reagent (Mirus) was incubated with DMEM containing 10% heat-inactivated FCS without antibiotics, for 15 min at room temperature. 3 μg of plasmid DNA was then added to this solution and incubated for a further 30 min at room temperature. The volume of DMEM used in the transfection complex was such that the total volume reached 100 μl.

Cell Fixation

Cells were fixed using 4% paraformaldehyde (PFA) for 10 min at room temperature and then permeabilised for 10 min at room temperature with 0.2% Triton in PBS. Cells in 35 mm

glass bottomed microwell dishes were then mounted with Vectashield mounting medium (VectorShield Laboratories).

Live Confocal Imaging

Cells were transfected with the appropriate construct and incubated overnight at 37°C with 5% CO_2 in DMEM containing 10% heat-inactivated FCS without antibiotics. Cells were then washed once in DMEM without phenol red (Invitrogen) and imaged in DMEM without phenol red at 37°C on an inverted Leica TCS SP2 AOBS confocal microscope. Images were analysed and processed using Leica Confocal Software Version 2.6.1 and Zeiss LSM Image Browser Version 4.2.0.121.

Polyacrylamide Gel Electrophoresis and Western Blotting

Gels for SDS-PAGE were made using Acrylamide/Bis-Acrylamide (Sigma), Tris-HCl and 10% SDS and were polymerised using TEMED (Sigma) and 10% APS. 10% acrylamide was used in the resolving gel and 4% in the stacking gel. Samples were boiled in SDS-PAGE buffer before loading.

The gels were transferred onto Hybond PVDF Transfer membrane (Amersham) for Western blotting. Following transfer of the proteins, membranes were blocked with 8% milk for 1 h. Primary antibody also made up in 8% milk was then added and incubated overnight at 4°C on an orbital shaker. The membranes were washed 3 times in PBS + 0.05% Tween for 10 min each. Secondary HRP conjugated antibody made up in 8% milk was then added and incubated at room temperature on an orbital shaker for 1 h. Following 3 PBS washes for 10 min each, membranes were imaged using the ECL Western blotting detection system (GE Healthcare).

For Western blotting against phosphotyrosine-containing proteins, membranes were blocked with 5% BSA in TBS for 1 h. Primary antibody also made up in 5% BSA in TBS was then added and incubated overnight at 4°C on an orbital shaker. The membranes were washed 3 times in TBS + 0.05% Tween for 10 min each. Secondary HRP conjugated antibody made up in 5% BSA in TBS was then added and incubated at room temperature on an orbital shaker for 1 h. Following 3 TBS washes for 10 min each, membranes were imaged as above.

Antibodies

Antibodies used for Western blotting were as follows; 4G10 mouse anti-human phosphotyrosine antibody (Millipore, Upstate Biotechnology) used at 1:1000, sheep anti-human EGF receptor antibody (Fitzgerald Industries International) used at 1:2000, HRP conjugated goat anti-mouse antibody (Dako) used at 1:2000 and HRP conjugated donkey anti-sheep antibody (Dako) used at 1:2000.

Results

Characterisation of the Sarcoidosis-Associated Annexin A11 Single Nucleotide Polymorphism

A genome-wide study conducted in a German population, identified a single nucleotide polymorphism (SNP) within exon 6 of the human annexin A11 gene as the most highly associated SNP for sarcoidosis (Hofmann et al. 2008). Annexin A11, like other annexins, comprises two main domains, namely a variable N-terminal head and a conserved C-terminal core. Within the core domain are highly conserved sub-domains termed annexin repeats that are responsible for the calcium-binding properties of the annexins. Annexin A11 contains four of these repeats within its core domains, and the sarcoidosis associated SNP lies within the first 14 residues of the first annexin repeat (Fig. 1A). The mutation identified in the genome-wide study results in the substitution of a cytosine to a thymidine (Fig. 1B), which in turn results in a switch from a basic arginine to a polar cysteine at residue 230. Analysis of the protein sequence of annexin A11 in both vertebrates and invertebrates shows a high degree of homology at this residue and in the regions flanking the mutation, between species as distant as humans and zebrafish (Fig. 1C). First we investigated the cellular distribution of the sarcoidosis-associated variant of annexin A11, through the expression of GFP tagged proteins.

GFP tagged wild type (anx $A11^{WT}$-GFP) and mutant annexin A11 (anx $A11^{R230C}$-GFP) constructs were expressed in A431 cells. These cells endogenously express annexin A11 homozygous for arginine at residue 230, as determined by mRNA isolation and subsequent cDNA sequencing (data not shown). Cells were transfected with either the full length annexin A11 or the C-terminal core domain alone tagged to GFP, for both wild type and sarcoidosis associated variants. Full length anx $A11^{WT}$-GFP showed a nuclear and cytoplasmic distribution (Fig. 1D), in line with previous reports of endogenous expression patterns of annexin A11 in transformed cell lines (Tomas and Moss 2003). The C-terminal truncation mutant of this construct was predominantly cytoplasmic and mostly excluded from the nucleus, supporting previous studies identifying the N-terminal domain of annexin A11 as being essential for nuclear targeting (Mizutani et al. 1995) (Fig. 1D). The mutant constructs showed no difference in distribution to their wild type counterparts. Thus, anx $A11^{R230C}$-GFP was both nuclear and cytoplasmic, and the C-terminal truncation mutant of this construct was predominantly cytoplasmic with weak nuclear staining (Fig. 1D). Therefore the sarcoidosis associated SNP does not affect the subcellular localisation of annexin A11 in unstimulated A431 cells.

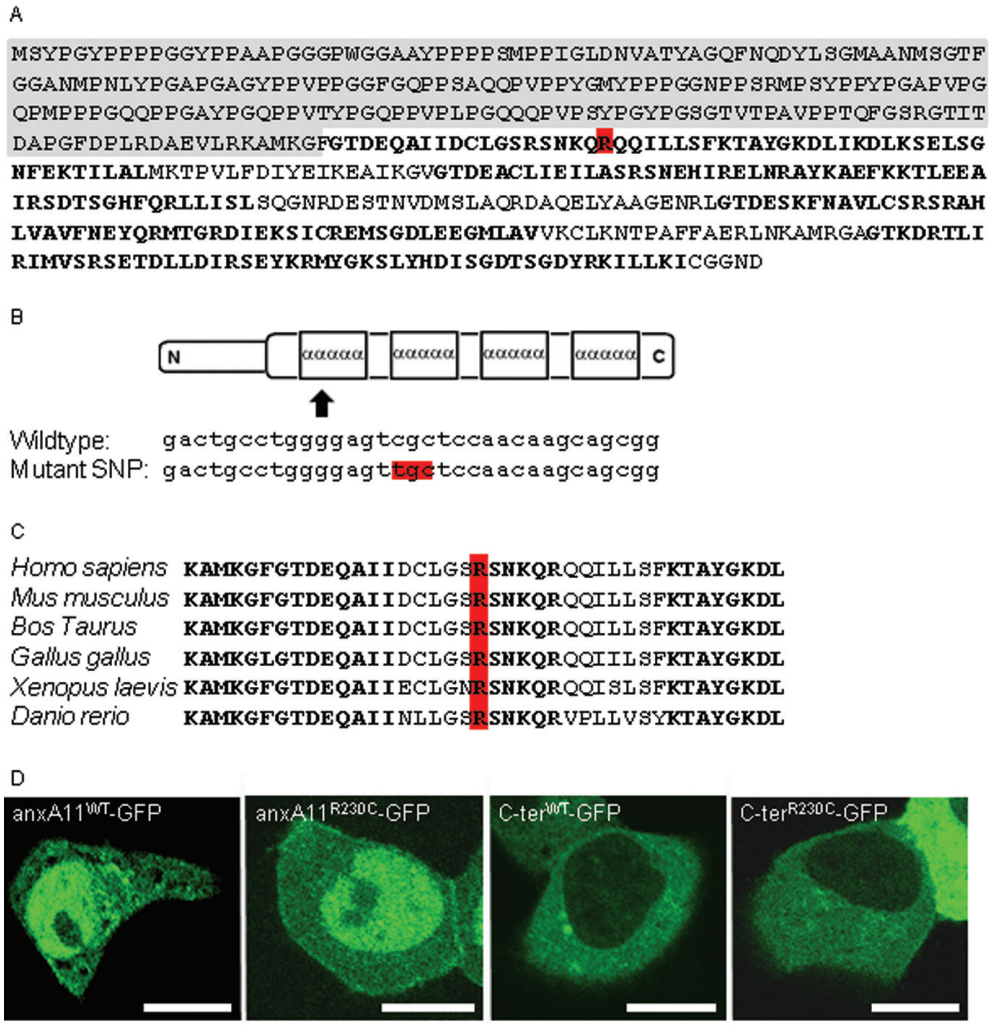

Figure 1. Annexin A11 sarcoidosis associated single nucleotide polymorphism. **A.** Full length wild type human annexin A11 protein sequence. N-terminal domain residues (grey box). Annexin repeats (bold). Sarcoidosis associated SNP results in a change in a single amino acid (red box) **B.** Schematic of annexin A11, showing that the mutation (arrow) lies within the first annexin repeat. Shown below is a section of nucleotide sequence from human annexin A11 around the sarcoidosis-associated SNP (red box) **C.** Evolutionary conservation of the amino acids surrounding the affected arginine (red box) in a range of different species. Residues conserved between all 6 species shown (bold). **D.** Expression of anx $A11^{WT}$-GFP, anx $A11^{R230C}$-GFP, anx $A11^{WT}$-Ct-GFP and anx $A11^{R230C}$Ct-GFP in A431 cells transfected with 3 µg of plasmid DNA and incubated overnight, prior to fixation in PFA. (Scale Bars 10 µm)

The Response of Wild Type and Annexin $A11^{R230C}$ to Changes in Intracellular Calcium

The SNP in annexin A11 is located within the first annexin repeat of the C-terminal domain, which as previously mentioned is responsible for the calcium-binding properties of the annexins. The change in amino acid from a basic arginine to a polar cysteine may alter the tertiary structure of the protein in a manner that could affect its calcium-binding properties. In order to investigate this possibility, the effects of elevated calcium levels on the localisation of GFP-tagged wild type and mutant annexin A11 were tested by stimulating A431 cells with ionomycin or epidermal growth factor (EGF).

The Response of Anx $A11^{WT}$-GFP and Anx $A11^{R230C}$-GFP to Ionomycin

The ionophore ionomycin was used to raise intracellular calcium levels. The addition of 1µM ionomycin to A431 cells expressing anx $A11^{WT}$-GFP resulted in a re-localisation of the tagged protein (Fig. 2A). Prior to treatment anx $A11^{WT}$-GFP was diffuse in both the nucleus and cytoplasm. Following treatment it first re-localised to the plasma

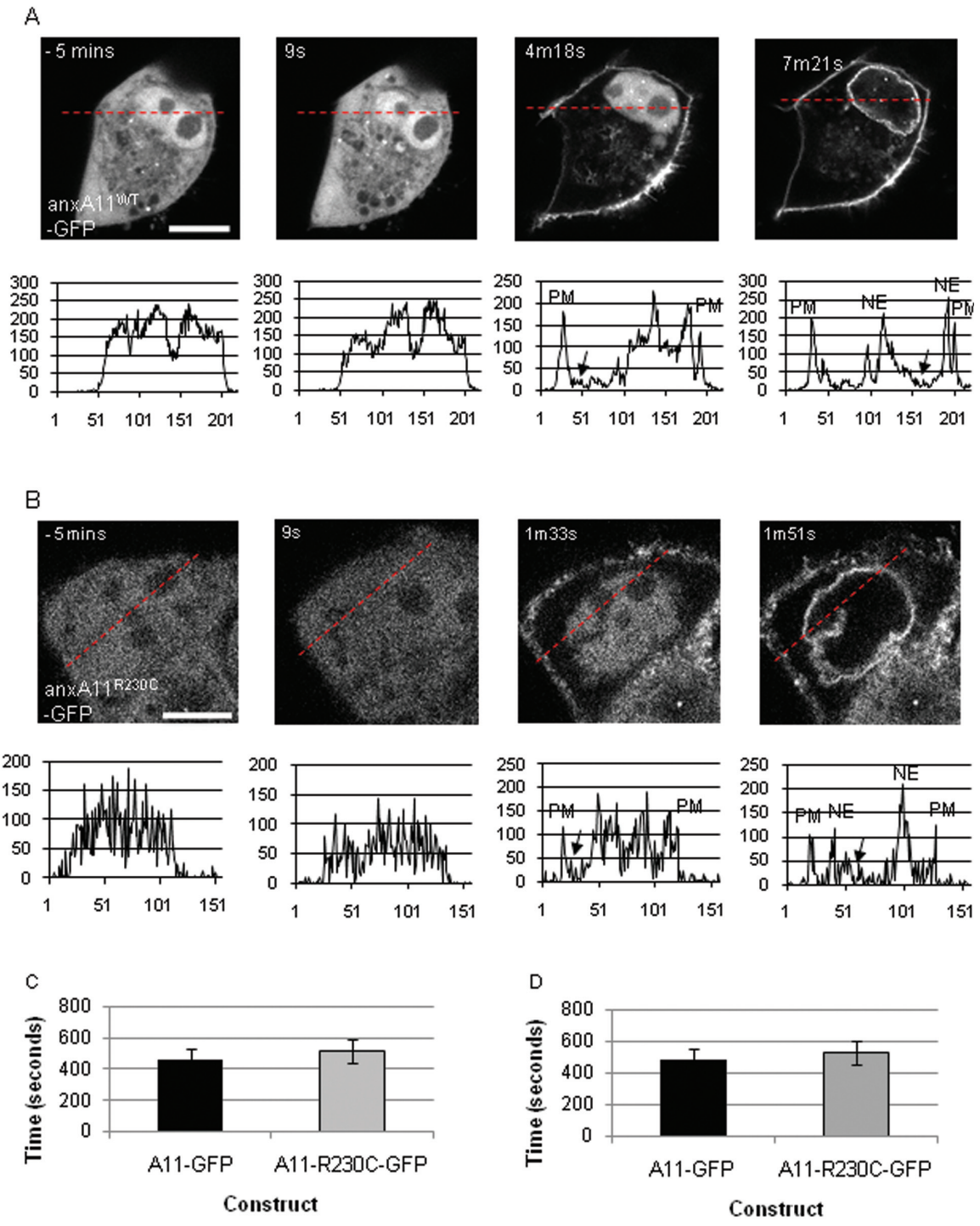

Figure 2. Full length anx A11^{WT}-GFP and anx A11^{R230C}-GFP respond to ionomycin in a similar manner. **A, B.** Real time imaging of anx A11^{WT}-GFP and anx A11^{R230C}-GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Cells were imaged in DMEM without phenol red and with treated with 1 μM ionomycin. Time pre and post treatment noted. Line scans were taken through the cell (red) and plotted in graphs below images; gray scale (y axis), position along line in pixels (x axis), plasma membrane (PM), nuclear envelope (NE), maximal cyto- or nucleo-plasmic depletion (arrow). (Scale Bars 10 μm) **C.** Bar chart of the average time taken for anx A11^{WT}-GFP and anx A11^{R230C}-GFP, expressed in A431 cells, to relocalise to the plasma membrane ($p = 0.089$) in response to 1 μM ionomycin. **D.** Bar chart of the average time taken for anx A11^{WT}-GFP and anx A11^{R230C}-GFP, expressed in A431 cells, to re-localise to the nuclear envelope ($p = 0.155$) in response to 1 μM ionomycin. Errors bars represent standard errors of the mean from 3 experiments with n = 36 cells for each construct.

membrane, depleting cytosolic GFP fluorescence whilst remaining constant in the nucleoplasm. The nucleoplasm was then also depleted of annexin A11-GFP, as the protein re-localised to the nuclear envelope. Line scans through the cell showed the appearance of clear peaks of fluorescence corresponding to these membranous accumulations; outer peaks representing the plasma membrane (PM) and inner peaks representing the nuclear envelope (NE) (Fig. 2A). The line scans also show troughs corresponding to regions of depletion of annexin A11-GFP in the cytoplasm and nucleoplasm. A similar pattern of re-localisation was observed for stimulated A431 cells expressing anx $A11^{R230C}$-GFP (Fig. 2B), with the tagged protein first becoming enriched at the plasma membrane and then the nuclear envelope, whilst concomitantly becoming depleted from the cytoplasm and nucleoplasm respectively.

The time taken for the two annexin A11 variants to re-localise to the plasma membrane and the nuclear envelope was quantified. Since the membrane accumulation of tagged protein occurred over several seconds, the time recorded for statistical analysis was defined as the point at which maximal enrichment was observed during the time course of the experiment i.e. the point at which no more GFP tagged protein is lost from the cytoplasm to the plasma membrane or the nucleoplasm to the nuclear envelope. Translocation of the tagged proteins is illustrated by line scans showing clear peaks for membrane enrichment alongside troughs of maximally depleted cytoplasm or nucleoplasm. On average the time taken for anx $A11^{WT}$-GFP to relocalise to the plasma membrane in response to ionomycin was 458s (+/- 70s, n=36) and for anx $A11^{R230C}$-GFP it was 516s (+/- 74s, n=36) (Fig. 2C). The average time taken for anx $A11^{WT}$-GFP to re-localise to the nuclear envelope in response to ionomycin was 483s (+/- 76s, n=36) and for anx $A11^{R230C}$-GFP it was 533s (+/- 75s, n=36) (Fig. 2D). Although the anx $A11^{R230C}$-GFP variant generally took approximately one minute longer than anx $A11^{WT}$-GFP to respond to the rise in Ca^{2+}, statistical analysis revealed that the difference between the wild type and mutant GFP tagged proteins fell short of significance in the time taken to re-localise to the plasma membrane (P=0.089) or the nuclear envelope (P=0.155).

Since the N-terminal domains of annexins can influence the responsiveness of these proteins to Ca^{2+}, the effects of ionomycin stimulation on mutants lacking the N-terminus was also examined. In previous work we found that removal of the N-terminus of annexin A11 rendered the protein insensitive to Ca^{2+}, despite the fact that the N-terminus lacks any Ca^{2+}-binding sites (Tomas et al. 2004). Consistent with our earlier findings, neither construct exhibited any change in subcellular localisation in response to 1μM ionomycin. Both mutant and

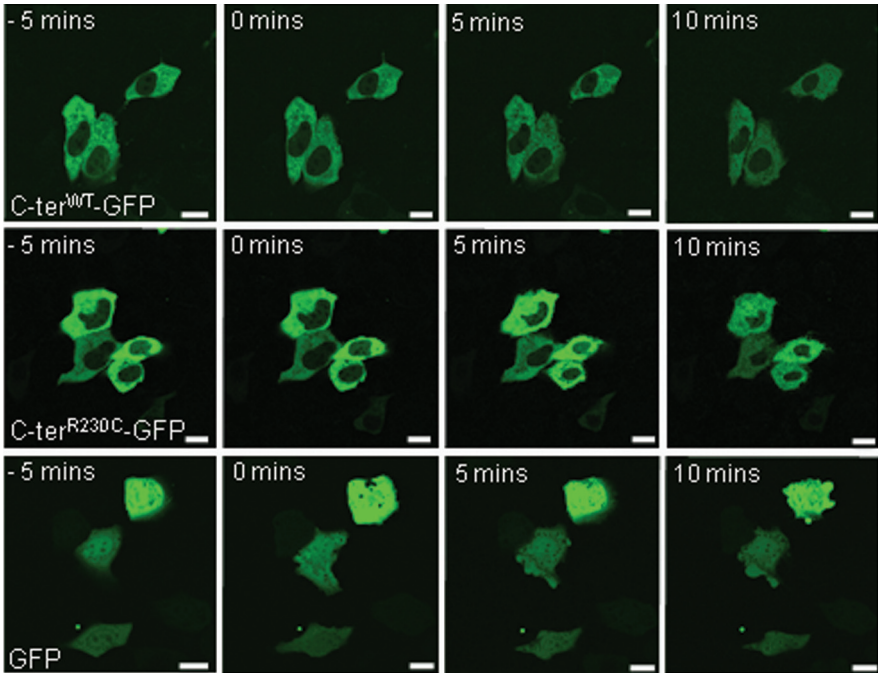

Figure 3. C-terminal wildtype and mutated annexin A11 respond to ionomycin in a similar manner. Real time imaging of anx $A11^{WT}$-Ct-GFP, anx $A11^{R230C}$Ct-GFP and GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Prior to imaging cells were loaded with Fura Red AM in DMEM without phenol red for 30 min at 30°C and then 30 min at 37°C. Cells were then imaged in DMEM without phenol red and with treated with 1 μM ionomycin. GFP construct (green), Fura Red (red). Graphs of Fura Red fluorescence over time for each experiment are shown below images. Time pre and post treatment noted. (Scale Bars 10μm).

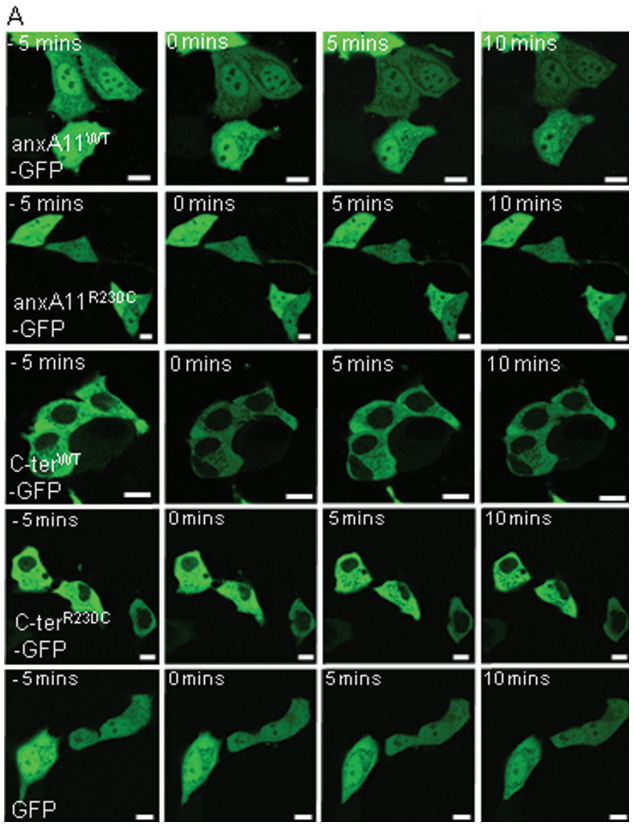

Figure 4. Wildtype and annexin A11^{R230C} do not relocalise in reponse to EGF treatment. **A.** Real time imaging of anx A11^{WT}-GFP, anx A11^{R230C}-GFP, anx A11^{WT}-Ct-GFP, anx A11^{R230C}Ct-GFP and GFP expressed in A431 cells transfected with 3 μg of plasmid DNA and incubated overnight. Cells were imaged in DMEM without phenol red and with treated with 100 ng/ml of EGF (human epidermal growth factor). Time pre and post treatment noted. (Scale Bars 10 μm). **B.** Following imaging of the transfected cells, the cells were lysed in boiling SDS-PAGE buffer and subjected to SDS-PAGE and Western blotting. EGF receptor was blotted for using a polyclonal sheep anti-EGFR antibody (1:2000 dilution) and phosphorylated proteins using a monoclonal mouse anti phosphotyrosine antibody (1:1000 dilution). A band equivalent in size to the phosphorylated EGF receptor is shown in the phosphotyrosine blot (arrow).

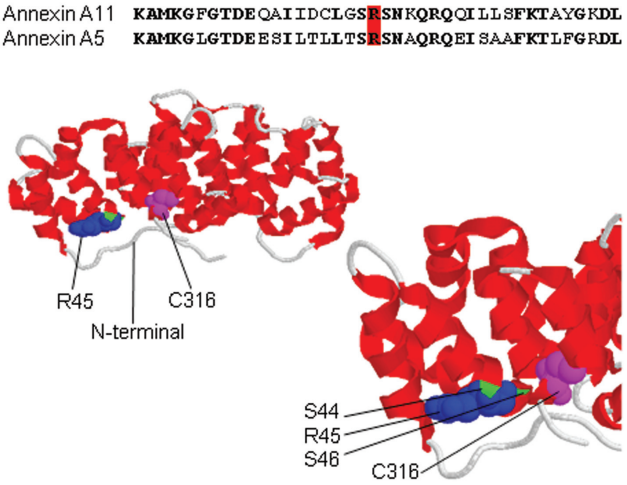

Figure 5. Structural implications of the R230C SNP in annexin A11. The crystal structure of annexin A5 was modelled in RasMol (Version 2.6). The arginine which is mutated in annexin A11^{R230C} is conserved in annexin A5 and is depicted in the crystal structure in blue. Residues either side of the arginine are shown in green. Cysteine residues which are conserved between annexin A11 and annexin A5 are shown in magenta. The crystal structure of annexin A5 highlights conserved residues S44, R45, S46 and C316. The aligned amino acid sequences of annexin A5 and annexin A11 show the conserved residues (bold), including the mutation-associated arginine (red box).

wild type proteins remained predominantly excluded from the nucleus and diffuse within the cytoplasm and showed no signs of enrichment at membranes (Fig. 3). GFP, used here as a control, also did not show any re-localisation upon ionomycin treatment. Thus, the R230C does not confer any increase in Ca^{2+}-sensitivity in the absence of the N-terminus.

The Response of Anx A11^{WT}-GFP and Anx A11^{R230C}-GFP to EGF

Ionomycin is a non-physiological agonist and is known to elicit rapid, large and sustained increases in calcium. We therefore examined the responses of the two annexin A11 variants to epidermal growth factor (EGF), which elevates intracellular calcium through activation of the EGF receptor and canonical $InsP_3$-mediated Ca^{2+}-signaling. The A431 cell line is particularly responsive to EGF as it expresses high levels of this receptor on its plasma membrane (Ullrich et al. 1984). The addition of 100ng/ml of human EGF to A431 cells transfected with anx A11^{WT}-GFP, anx A11^{R230C}-GFP, the corresponding C-terminal truncation mutants and GFP alone, failed to elicit any change in the subcellular localisation of any of these proteins during the 10 minute period imaged post-stimulation (Fig. 4A). Anx A11^{WT}-GFP and anx A11^{R230C}-GFP remained diffuse within the cytoplasm and nucleoplasm. The variants lacking N-terminal domains remained excluded from the nucleus and diffuse throughout the cytoplasm. None of the GFP tagged annexin A11 proteins or GFP alone showed membranous accumulations upon EGF treatment.

In order to verify that the cells had indeed responded appropriately to EGF, immediately following completion of the live imaging time course the cells were lysed and samples prepared for western blotting. Control samples of transfected, imaged, but unstimulated A431 cells were also prepared. Western blotting confirmed that the EGF receptor is expressed by these cells, and a phosphotyrosine blot revealed an increase in the strength of a protein band at 170kDa in stimulated cells but not in unstimulated cells (Fig. 4B). This band size corresponds to the phosphorylated EGF receptor, which upon binding EGF autophosphorylates. Therefore the stimulated A431 cells were responsive to EGF, though no re-localisation was observed of the GFP tagged annexin A11 proteins expressed.

Discussion

A recent genome-wide search for previously unknown genetic mutations associated with sarcoidosis, identified a single nucleotide polymorphism in annexin A11 as the most highly associated novel susceptibility locus for this disorder (Hofmann et al. 2008). Until this study the association of annexin A11 with autoimmune diseases was limited to the detection of autoantibodies in patients with a range of conditions including Raynaud's disease, rheumatoid arthritis and systemic lupus erythrematosos (Misaki et al. 1994). Annexin A11, like several other annexins, is widely expressed and it is therefore unsurprising that it, along with other annexins (Hayes et al. 2007; Rodriguez-Garcia et al. 1996; Salle et al. 2008) has been detected in sera from patients with autoimmune diseases. Therefore the finding of a mutation in annexin A11 that could potentially predispose an individual to developing sarcoidosis adds significant weight to the notion that annexin A11 has a causative role in autoimmune disorders.

We sought to further investigate this mutation (R230C) in A431 cells, which endogenously express the wild type variant of annexin A11 (R230). This was performed through the over-expression of wild type and sarcoidosis-associated variants of annexin A11 tagged to GFP. As the mutation is located in the C-terminal domain of the protein, both the full length and C-terminal domain alone tagged to GFP were investigated. No difference was seen in the localisation of GFP tagged wildtype annexin A11 or annexin $A11^{R230C}$ in resting A431 cells, suggesting that under unstimulated conditions annexin $A11^{R230C}$ is trafficked to the same domains as that of its wild type counterpart. This led us to investigate the effect of calcium on wild type annexin A11 and annexin $A11^{R230C}$.

In order to raise intracellular calcium levels the ionophore ionomycin was utilized, resulting in large, irreversible rises in intracellular calcium levels. This induced the re-localisation of GFP tagged annexin A11 first to the plasma membrane and then the nuclear envelope, consistent with our previous observations (Tomas and Moss 2003). The lag between these two events is most likely due to the strong calcium buffering capability of the nucleus (Badminton et al. 1998). This effect has also been observed in cells over-expressing GFP tagged annexin A7, which is the most closely related vertebrate annexin to annexin A11 (Clemen et al. 2003). The redistribution of endogenously expressed annexins in response to ionomycin has been investigated for several of the annexins, which show a variety of responses. Annexins A4 and A5 also re-localise to the nuclear envelope, whereas annexin A2 which is less similar to annexin A11 localises to granular structures (Barwise and Walker 1996). Therefore although all the annexins are capable of binding lipid membranes in response to rises in intracellular calcium, targeting to specific domains varies between different annexins.

In cells over-expressing GFP tagged annexin $A11^{R230C}$ the same effect was observed in response to ionomycin and with the same kinetics. A subtle difference in the time taken to re-localise to these membranous compartments between these two variants cannot be entirely ruled out, without analysing many more cells. It should be noted that the GFP tagged C-terminal domain constructs showed no significant re-localisation upon ionomycin treatment for either wildtype annexin or annexin $A11^{R230C}$. This domain contains the Ca^{2+}-binding sites of annexin A11, suggesting that although Ca^{2+} is detected within the annexin A11 core, it is incapable of sustained re-localisation to the plasma membrane or nuclear envelope in the absence of the N-terminal domain.

We also investigated the responses of these constructs to EGF but in all four cases no re-localisation of the fluorescently tagged proteins was observed, although the cells were shown to be responsive to EGF from Western blots against phosphorylated proteins. It may therefore be the case that the rise in intracellular calcium induced by EGF is too transient and/or too small to stimulate the membrane translocation of annexin A11. EGF stimulation of A431 cells is known to increase calcium levels to between 400nM and 700nM, whereas ionomycin raises levels into the low micromolar range. Furthermore, calcium levels are sustained upon treatment with ionomycin, whereas the rise in EGF-stimulated Ca^{2+} levels has been shown to return to basal levels after 30s to one minute (Moolenaar et al. 1986). The need for sustained increases in calcium for annexin relocalisation has been shown to be a requirement for endogenously expressed annexin A4, which relocalises in response to ionomycin but not to transient increases in calcium induced by weaker agonists such as bradykinin (Raynal et al. 1996).

Taken together, the failure of EGF to elicit responses from annexin A11, and the long delay in response time to ionomycin, suggest that annexin A11 is generally rather

unresponsive to Ca^{2+}, as has been reported in other studies (Lecona et al. 2003). The delay in the response to ionomycin suggests that other Ca^{2+}-dependent cellular events need to occur prior to annexin A11 translocation, such as tyrosine phosphorylation of annexin A11, which we showed is stimulated by ionomycin (Tomas and Moss 2003), or binding to an accessory protein such as ALG-2 (Satoh et al. 2002). It would be of interest in future studies to investigate other physiological agonists capable of raising intracellular calcium levels to different concentrations and different durations. Ideally, this would involve the use of different cell lines, including those of the immune system, which may show differences in response to calcium, as is the case for annexin A6 which relocalises to different compartments in fibroblasts and T cells (Barwise and Walker 1996; Podszywalow-Bartnicka et al. 2007). It would be of particular interest to investigate annexin A11 in cell types known to be directly involved in sarcoid granuloma formation such as macrophages and monocytes which form part of the granuloma core (Baughman 2006). T cells are also highly involved in sarcoidosis and it would therefore be important to investigate annexin A11 in CD4 positive T cells which are increased in sarcoid lungs (Grunewald and Eklund 2007) as well as CD8 and CD19 positive T cells, which have been shown to exhibit a striking decrease in expression of annexin A11 dependent upon activation (Hofmann et al. 2008) - over-expression in these activated T cells of wild type annexin A11 or annexin $A11^{R230C}$ may uncover differences in their function.

Although no functional differences between wildtype annexin A11 and annexin $A11^{R230C}$, were detected in this study, in terms of sensitivity to Ca^{2+}, structural analysis suggests that the R230C mutation may exert a functional effect. The tertiary structures of several of the annexins have been crystallized, though not that of annexin A11. Annexin A5 however has been crystallized and shows the highest degree of similarity with annexin A11 (of all the crystallized annexins) with 53% sequence identity and 73% sequence similarity, including conservation of the arginine at residue 230 (Fig. 5). Structural analysis shows that this residue is in close proximity to the N-terminal domain and may therefore interact with it (Fig. 5). A change from a basic arginine to a polar cysteine could alter these interactions and therefore the functionality of the protein.

Furthermore, annexin A11 contains six cysteine residues, one of which is conserved in annexin A5 (C316) (Fig. 5). This could be of importance in the context of disulphide bridge formation. Structural analysis shows that residue 230 lies near the start of an alpha helix (Fig. 5) and lies opposite the conserved cysteine residue (C316). In the wild type protein the arginine at residue 230 points away from the cysteine. Upon mutation of this arginine to a cysteine it is possible that a new conformation could arise in which the two cysteine residues are facing each other, facilitating the formation of a disulphide bridge that would alter the conformation of the protein. Disulphide bridge formation would however only be likely to occur in the extracellular environment. Interestingly, annexin A11 has been shown to be secreted by activated neutrophils (Boussac and Garin 2000), and autoantibodies against annexin A11 have been detected in several autoimmune diseases (Mizutani et al. 1995).

The conformational changes postulated here are hypothetical and would require crystallization studies to be substantiated. If structural changes do occur in annexin $A11^{R230C}$, it is not clear whether these would be sufficient to result in functional effects. It is possible that although the mutation identified in annexin A11 is strongly associated with sarcoidosis, susceptibility to the disease and cellular dysfunction requires the complete haplotype to be present. This includes five other single nucleotide polymorphisms surrounding the annexin A11 R230C mutation, which were also validated as strongly associated with sarcoidosis (Hofmann et al. 2008). Alternatively, the mutation in annexin A11 alone may be sufficient, and testing these hypotheses will be the focus of future work.

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