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In vitro and in vivo studies on CCR10 regulation by Annexin A1

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Abstract The mode of action of annexin A1 (ANXA1) is poorly understood. By using rapid subtraction hybridization we studied the effects of human recombinant ANXA1 and the N-terminal ANXA1 peptide on gene expression in a human larynx cell line. Three genes showed strong downregulation after treatment with ANXA1. In contrast, expression of *CCR10*, a seven transmembrane G-protein coupled receptor for chemokine CCL27 involved in mucosal immunity, was increased. Moreover the reduction in CCR10 expression induced by ANXA1 gene deletion was rescued by intravenous treatment with low doses of ANXA1. These findings provide new evidence that ANXA1 modulates gene expression.
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1. Introduction

Annexin A1 (ANXA1) is a 37 kDa member of a family of Ca^{2+}-regulated lipid-binding proteins [1]. Functional studies have identified roles for ANXA1 and its N-terminal domain [2,3] in the regulation of inflammation [4], the cell cycle [5] and as a mediator of glucocorticoid action on the release of corticotrophin (ACTH) from the anterior pituitary gland [6,7]. Many of the regulatory actions of ANXA1 in these systems have been shown to emerge promptly, i.e., within minutes to hours of contact with the protein [6]. Whilst some have been ascribed to the ability of ANXA1 to act intracellularly to disrupt signalling cascades [8], others appear to reflect interactions with cell surface receptors [9–11]. Recent studies suggest that members of the formyl peptide receptor (FPR) family of G-protein coupled receptors are particularly important in this regard [12,13]. Three members of this receptor family have been identified in human, termed FPR, FPRL1 and FPRL2. Receptor-specific ligands are not yet available, but FPR is highly sensitive to the bacterial peptide formyl-Met-Leu-Phe (fMLP), while FPRL1 shows some selectivity for lipoxin A4, a lipid metabolite, in addition to responding to high concentrations of fMLP [14].

Despite the evidence that ANXA1 might influence cellular responsiveness, the possibility that ANXA1 regulates gene expression has received scant attention. Interestingly however, the recently generated ANXA1 knockout mouse shows significant alterations in the expression of a number of gene products associated with inflammation [15,16], a finding that concurs with earlier reports that ANXA1 reduces the expression of inducible nitric oxide synthase [17].

We have used rapid subtraction hybridisation (RaSH) [18] to investigate the effects of $ANXA1_{Ac2-26}$ on gene expression in a human cell line of epithelial origin. In addition, we have compared the effects of hrANXA1 and $ANXA1_{Ac2-26}$ with those of fMLP (a bacterial peptide) and the stable analogue of lipoxin A4 [15-epi-16-(*para*-fluoro)-phenoxy-LXA4-methyl ester, ATLa-ME], an arachidonic acid metabolite, in order to explore the role of the FPR family in effecting the response to ANXA1. The data obtained from the cell line in vitro have been evaluated in vivo by studies on *Anxa1* null mice. Our findings strongly support a role for ANXA1 in the regulation of gene expression, particularly those genes concerned with the inflammatory response.

2. Materials and methods

2.1. Drugs

Human recombinant annexin A1, [19] was produced, purified and tested by SDS–PAGE for the presence of cleaved forms which were not detected, by Scientific Proteins, Witterswil, Switzerland. Furthermore, the recombinant protein did not show any phosphorylation at the N-terminal as reported previously [19]. Human $ANXA1_{Ac2-26}$ (acetylated peptide at the N-terminal) was synthesized in the Advanced Biotechnology Centre, Imperial College London, UK. The peptide fMLP was purchased from Sigma (Sigma–Aldrich Corp., Poole, Dorset, UK) and 15-epi-16-(*para*-fluoro)-phenoxy-ATLA-methyl ester (ATLa, the methyl ester of lipoxin A4) was a generous gift from Professor C. Serhan (Harvard Medical School, Boston, USA). The doses of acetylated peptide ($ANXA1_{Ac2-26}$; 2 μM) were chosen on the basis of previous studies, performed both in vitro and in vivo, in which we showed that comparable biological activity were obtained when the ratio between fMLP or ATLa and $ANXA1_{Ac2-26}$ was 1:20 [13,20] furthermore such molar ratio was of 1:15 between the entire recombinant ANXA1 and the peptide Ac2–26 as described previously [21].

2.2. Cell culture

The Hep-2 cell lines were derived from a human larynx epidermoid carcinoma cells (ATCC Rockville, MD, USA) and used for RaSH analysis. The PDFS cell line derived from a clinically non-functioning pituitary macroadenoma was used to confirm and compare the analysis performed on Hep-2 cells (see supplement Table 2) [22].

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2.3. Animals

Male *Anxa1*-null mice aged approximately 4 months old [15] and weighing 30 ± 4 g were used together with age- and sex-matched wild type controls. All animals were fed on a standard chow pellet diet. All procedures were carried out under licence in accordance with the UK Animals (Scientific Procedures) Act, 1986. For tissue collection the animals were killed by cervical dislocation and their lower back region was shaved. Longitudinal fragments of the dorsal skin (after shaving), trachea and ileum were obtained from each mouse (6 animals per tissue) and frozen immediately in liquid nitrogen for subsequent extraction of RNA and protein.

Anxa1 null mice were injected intravenously via the lateral tail vein with hrANXA1 (10 ng/mouse), $ANXA1_{Ac2-26}$ (14 ng/mouse) or a corresponding volume of the saline vehicle (100 μl). Mice (three animals per treatment) were killed with CO_2, 24 h after drug administration, and tissues collected exactly as described above.

2.4. Rapid subtraction hybridization (RaSH) and RT-PCR analysis

Hep-2 cells were plated at a concentration of 10^6 cells/in six well plates (Corning Incorporated, NY, USA) and 24 h later in culture, stimulated with $ANXA1_{Ac2-26}$ (2 μM; 72 h). Total RNA isolated by RNeasy (Qiagen, West Sussex, England) from control and treated cells were used for double-stranded cDNA synthesis using a standard protocol [23]. The DNA was then digested and mixed with the adaptors XPDN-14/XPDN-12 (Sigma Genosys; final concentration 20 μM) as described by Jiang et al. [18]. 100 ng *tester* cDNA (e.g., untreated cells) was mixed with 5 μg *driver* cDNA (e.g., $ANXA1_{Ac2-26}$ treated cells, see also Table 1) in hybridization solution [18,24]. After extraction and precipitation the hybridization mixtures (1 μg) were ligated to an *Xho*I digested pCDNA3 plasmid and transformed in competent bacteria. Bacterial colonies were selected randomly and amplified by PCR. The sequences of these clones were determined using the automated cycle sequencer in the Advanced Biotechnology Centre, Imperial College London. Sequences were compared with the NCBI nucleotide bank (GenBank) allowing identification of any genes up or down regulated. Experiments were then set up ($n = 3$) in the same conditions described above and RT-PCR with specific oligonucleotides (supplementary data Table 1) for the genes identified by RaSH was performed.

2.5. Semi quantitative RNA analysis

We first determined the linear range of amplification (25, 20, 35, 40 cycles) of cDNA using each of the primer sets. Amplification was within the linear range for all genes studies when 35 PCR cycles were used and this level of amplification was therefore used in all further studies. For each sample 5 μl of the PCR amplification products was analysed on 2% agarose gels and stained with ethidium bromide. The intensities of the bands were compared to GAPDH in each sample and evaluated using the Image Master Software (SYDR-1990, SYNGENE, USA).

2.6. Western blot analysis

Expression of CCR10 (*GPR2*) protein (15 μg) was examined in the cell lines and murine tissue extracts by SDS–PAGE, then transferred electrophoretically to nitrocellulose paper [25]. The blots were incubated overnight at 4 °C in incubation buffer in presence of an anti-CCR10 antiserum (diluted 1:1000, goat anti-mouse polyclonal, Abcam; or anti human CCR10 diluted 1:1000, Imgenex San Diego, CA, USA). Immunoreactive protein bands were detected by chemiluminescence (GE Healthcare, UK) after incubation with secondary antibody (rabbit anti-goat peroxidase; 1: 100000, Biomeda; or goat anti-rabbit peroxidase conjugated antiserum, 1:25000, Sigma–Aldrich Corp.). The blots were then scanned and analyzed (HP Scanjet 5200 with Adobe Photodeluxe Business Edition, version 1.1; Cupertino, CA). The intensities of the bands were compared to β-actin (mouse monoclonal 1:5000, Abcam).

2.7. FACS analysis

For detection of FPR and FPRL1 on the cell surface of Hep-2 and PDFS cells, aliquots of suspensions of the cells (10^5 cells in 100 μl) were added to a 96-flat well plate in triplicate and incubated at 4 °C for 1 h in the presence or absence (controls) of either: (a) a monoclonal anti-human FPR antibody (1:100, BD Pharmingen) or mouse IgG1 clone NOPC-21 (isotype-matched control antibody 1:100, BD Pharmingen, UK) or (b) a rabbit anti-human FPRL1 polyclonal antiserum raised against the peptide TVWLKEMLFYGKYKIIDILVNP from the 3rd extracellular domain of the receptor (1:100, Neosystem Strasburg, France). Non-specific binding was minimized by the addition of human IgG (1.6 mg/ml, Roche Diagnostics, Lewes, UK). After incubation with the primary antiserum, the cells were incubated on ice for 30 min with FITC-conjugated rabbit anti-mouse or sheep anti-rabbit IgG (1:300, Serotec, Kidlington, Oxford, UK). The cell surface fluorescence was analyzed immediately by flow cytometry using a Becton Dickinson FACScan II analyzer. At least 10000 cells per sample were counted. The data were analyzed as units of fluorescence measured in the FL1 channel (mean fluorescence intensity). A similar method was used to detect and quantify expression of the cell surface receptor CCR10 (GPR2) using a goat anti-rabbit polyclonal antibody to CCR10 (GPR2, 1:100, Imgenex San Diego, CA, USA) as a probe and FITC-conjugated sheep anti-rabbit IgG (1:300, Serotec) for detection.

2.8. Statistical analysis

Statistical significance was assessed by ANOVA followed by Bonferroni's t test, and $P < 0.05$ was taken as statistically significant.

3. Results and discussion

3.1. Annexin A1 modulates gene expression in cell line

Subtractive hybridisation performed on the human cell line Hep-2, identified three genes, which were downregulated by hrANXA1 (100 nM) and $ANXA1_{Ac2-26}$ (2 μM) treatment. The first was *ITGB1BP1* (previously named *ICAP-1A*, Table 1 and Fig. 1) a protein specifically associated with the β1 cytoplasmic domain of integrins [26] containing putative phosphorylation sites. The phosphorylated form probably impairs cell adhesion and spreading [27], but over-expression of *ITGB1BP1* stimulates cell migration [28]. Thus, the ability of ANXA1 to impair monocytes on the endothelium in vitro [21] may be mediated, at least in part, through *ITGB1BP1* downregulation.

Since ANXA1 and ANXA1-derived peptides are potential ligands for members of the formyl peptide receptor (FPR) family, we explored the potential role of these receptors in

Table 1
Genes isolated by RaSH

	Symbol	Locus ID	Function
Upregulated 100 ng of *tester* ($ANXA1_{Ac2-26}$ treated cells) versus 5 μg of *driver* (untreated cells)	*CCR10* (NM_016602)	2826	A G protein coupled receptor for chemokine CCL27 mostly involved in T cell mediated skin inflammation
Downregulated 100 ng of *tester* ($ANXA1_{Ac2-26}$ treated cells) versus 5 μg of *driver* (untreated cells)	*TIFA* (NM_052864)	92610	A TRAF2 binding protein involved in the TNF-mediated signalling
	FARSLA (NM_004461)	2193	A tRNA synthetase family expressed in a cell cycle stage and differentiation-dependent manner
	ITGB1BP1 (NM_004763)	9270	Accessory protein for the integrin beta 1

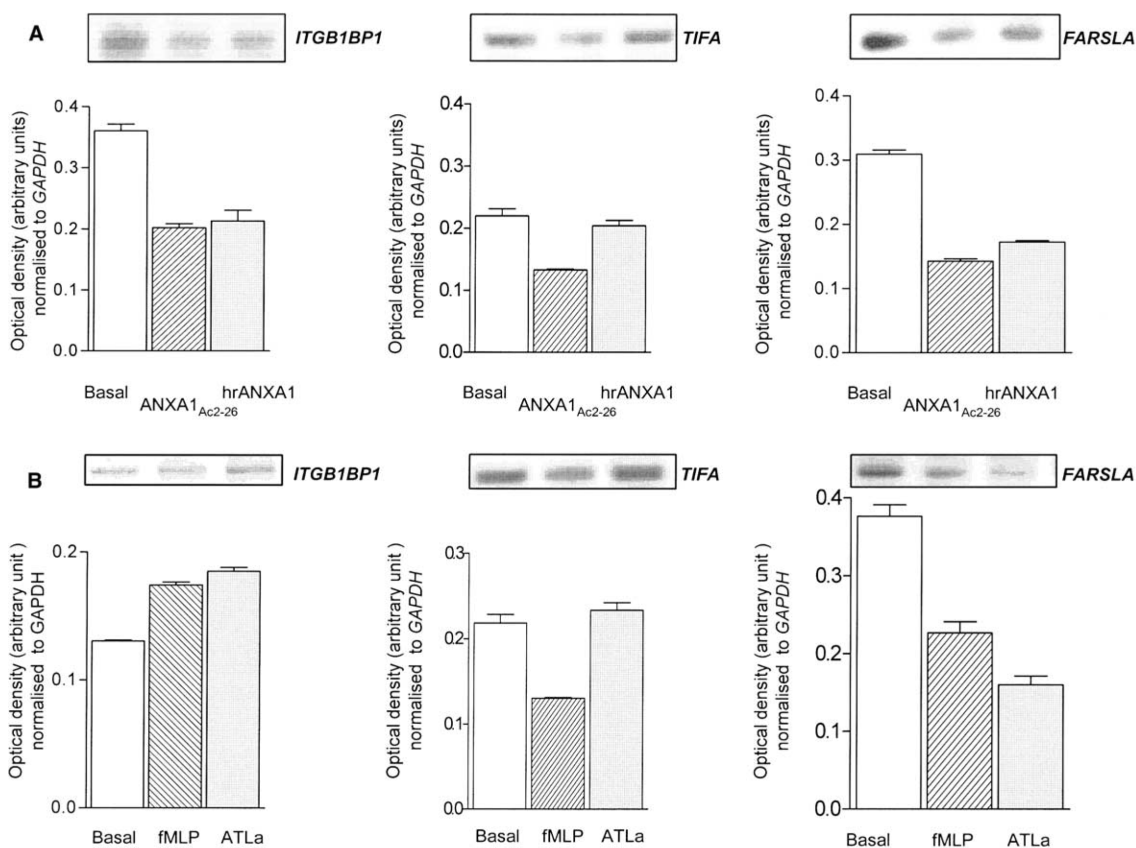

Fig. 1. Comparison of the effect of ANXA1 and FPRs ligands: fMLP and ATLa on ITGB1BP1, TIFA and FARSLA in Hep-2 genes isolated by RaSH. Cells were plated and treated as reported for RaSH analysis. After 72 h of stimulation with (A) ANXA1 (100 nM) or $ANXA1_{Ac2-26}$ (2 μM) or (B) fMLP (100 nM) and ATLa (100 nM) total RNA was extracted and RT-PCR performed using specific oligos for the above genes. Semi-quantitative normalization was performed using GAPDH expression. The data are representative of six independent experiments.

mediating the genomic effects of ANXA1 reported above. First, we used RT-PCR and FACS analysis to determine whether Hep-2 cells express FPR and FPRL1. Our data demonstrated mRNA and protein for both receptors in Hep-2 cells (Fig. 2A and B). Subsequently, we used ligands of FPR and FPRL1, fMLP and the lipoxin A4 analogue (ATLa), to explore whether activation of these receptors modulated the expression of *ITGB1BP1* (Fig. 1B and Table 3B supplement data). Neither fLMP nor ATLa, ligands for FPR and FPRL1, modified the expression of *ITGB1BP1* (Fig. 1B). Although we cannot exclude the possibility that higher concentrations of these compounds may have been effective they would be expected to act at the concentrations tested [13] and indeed have been shown to modify the expression of other genes in this study. We therefore suggest that the regulatory actions of ANXA1 and $ANXA1_{Ac2-26}$ on the expression of *ITGB1BP1*

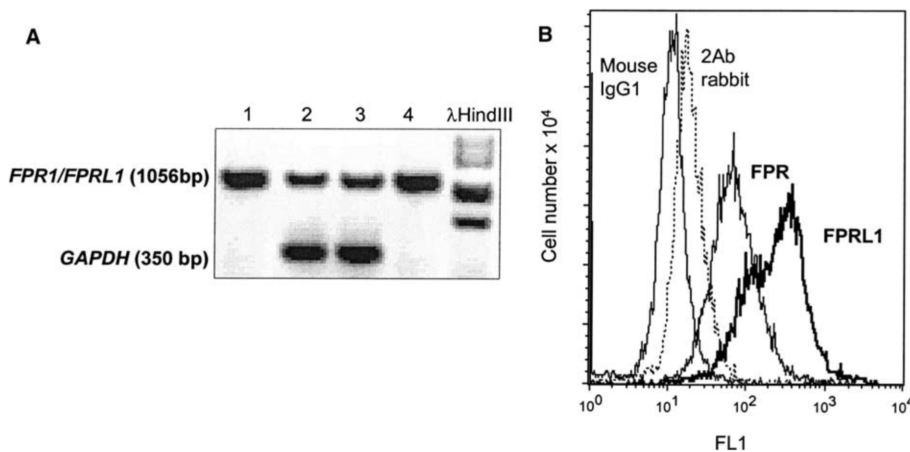

Fig. 2. Hep-2 cells express both FPR and FPRL1 receptors. (A) RT-PCR analysis lanes 1 and 4 represent the control amplified plasmids containing the entire coding sequences for FPR and FPRL1, respectively. Lanes 2 and 3 amplified product in Hep-2 cells for FPR and FPRL1 respectively. (B) FACS analysis of FPR and FPRL1 expression on the surface of the cells. The data are representative of three independent analyses.

were mediated via a mechanism independent of FPR or FPRL1.

A second gene downregulated by both hrANXA1 and ANXA1$_{Ac2–26}$ was a phenylalanine-tRNA synthetase *(FARSLA)*. Unlike ITGB1BP1 this gene was also downregulated by fMLP and ATLa, suggesting a role for the FPR family in mediating the response (Fig. 1A and B). In vivo FARSLA is expressed in a tumour-selective, cell cycle stage and differentiation-dependent manner and its expression is augmented in tumourigenic versus non-tumourigenic variant of the same cell line [29]. Interactions between ANXA1 and aminoacyl-tRNa synthase have been reported in patients with dermatomyositis [30] and between ANXA1 and tryptophanyl-tRNA synthetase [31]. Together, these results raise the possibility that ANXA1 plays a role in cell transformation as well as in protein translation and may explain why it is highly expressed in some cancers and not in others [32]. Interestingly, it has been proposed that ANXA1 and tRNA synthase share some physiological role in process related to myositis and immune response [31].

A third gene downregulated by ANXA$_{Ac2–26}$ and fMLP, but not by ANXA1 or ATLa (Fig. 1A and B) was a TRAF-interacting protein with a forkhead-associated domain termed *TIFA* or *T2BP*. Although merely speculative, as we do not know how the acetylation may affect the structure and function of the peptide, the downregulation of *TIFA* suggests that ANXA1 may modulate inflammatory processes via TNFα and *TIFA*, supporting our previous findings on the role of ANXA1 in systemic endotoxemia [33]. The data on the full protein (hrANXA1) or ATLa (ligand of FPRL1 receptor), which did not affect the expression of this gene, suggest that the full-length protein (hrANXA1) and the N-terminal peptide (ANXA1$_{Ac2–26}$) act via different mechanisms and thus support a preferential binding of ANXA1 to FPRL1 receptor [13]. We have recently published data on the differential effects of the full-length protein and its peptide in the firm adhesion of human PMNs on the endothelium under flow [20] and the results demonstrated here reinforce the dichotomy in the biological activity of the acetylated peptide versus the entire molecule.

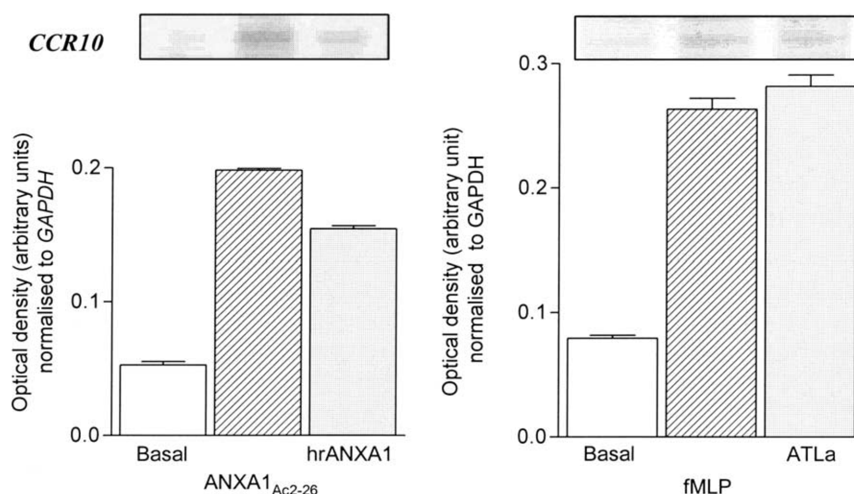

Fig. 3. CCR10 expression in Hep 2 cells. RT-PCR analysis of *CCR10* expression after stimulation with ANXA1, ANXA1$_{Ac2–26}$, fMLP and ATLa for 72 h. The data are representative of six independent experiments.

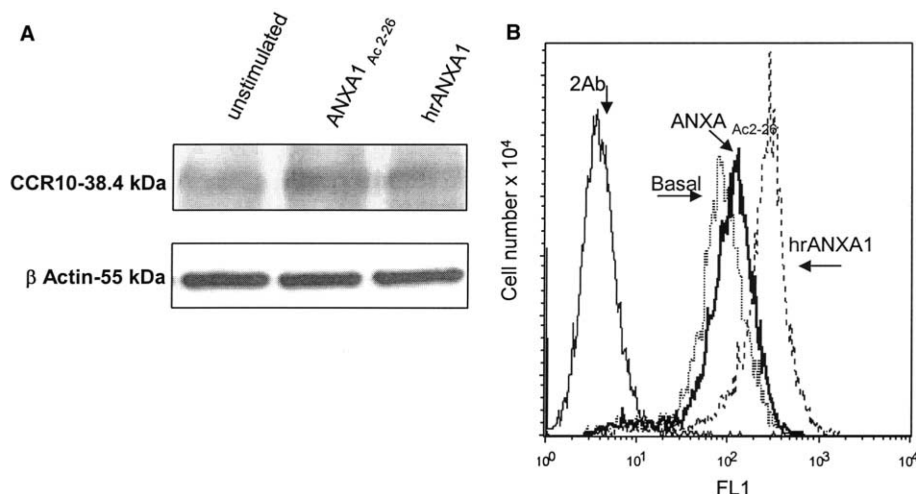

Fig. 4. Western blot and FACS analysis of CCR10 expression on Hep-2 cells treated with recombinant hANXA1 and ANXA1$_{Ac2–26}$. (A) Total protein cell extract from treated or untreated cells were analysed by Western blot. (B) FACS analysis: histogram (intensity of fluorescence versus number of cells) showing the data from one of three independent experiments which all showed similar results.

In contrast to it effects on *ITGB1BP1, FARSLA* and *TIFA*, ANXA1 (which is strongly expressed in epithelial cells and skin [34]), had a positive effect on the expression of the *CCR10* gene (Table 1), a skin homing receptor for chemokine CCL27 [35]. This effect was mimicked by $ANXA1_{Ac2-26}$, fMLP and ATLa (Fig. 3 A and B), supporting a role for the FPR

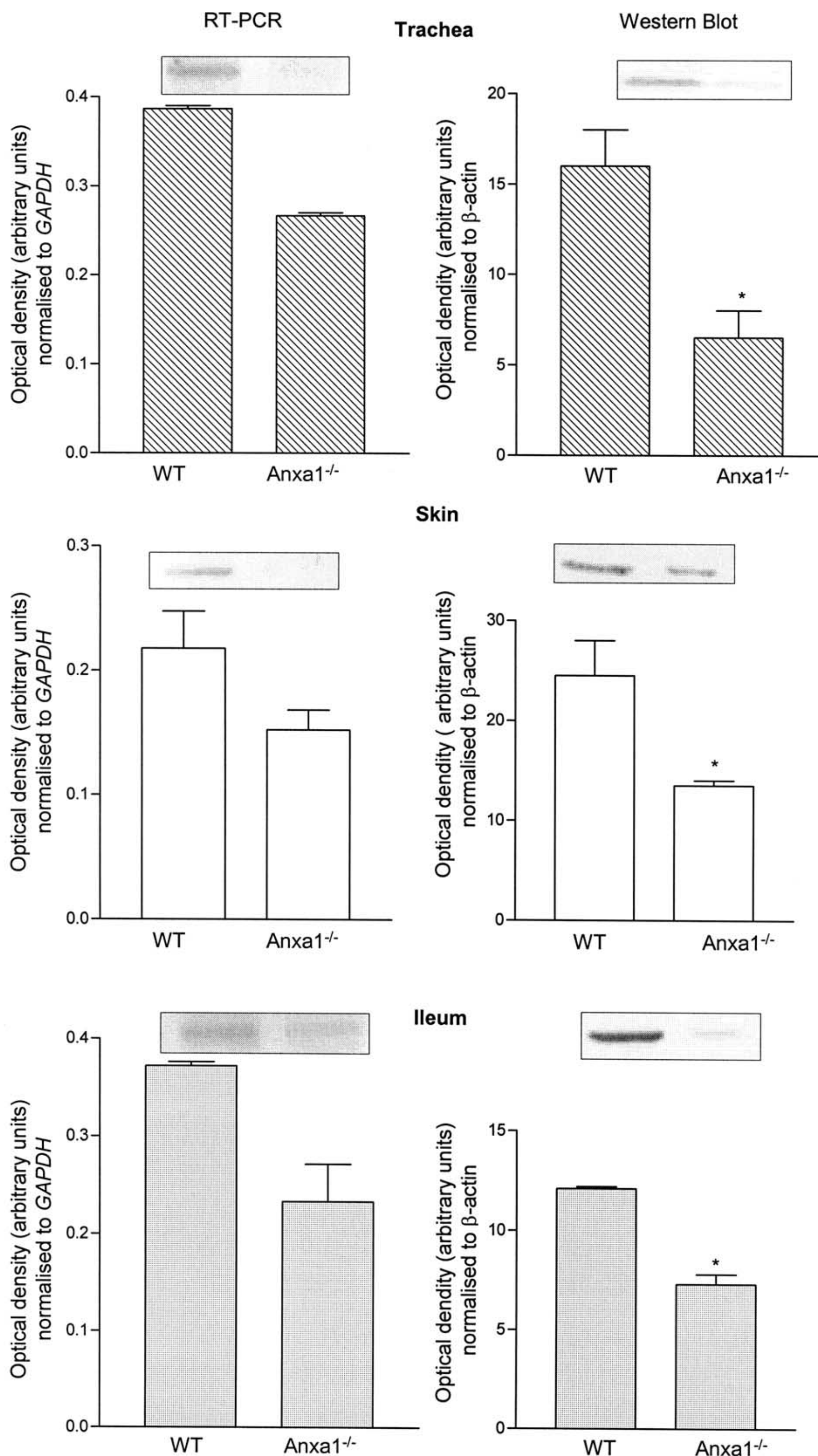

Fig. 5. CCR10 expression in Anxa 1 null mice. Left: RT-PCR analysis and right: Western blot analysis of CCR10 in trachea, skin and ileum from Anxa1 null and wild type control mice. Histograms represent a semi-quantitative analysis performed on 6 mice per genotype, * $P < 0.005$ versus relative WT.

family in mediating the positive effect of ANXA1. Support for a role of ANXA1 in CCR10 gene regulation was provided by the Western blot and FACS analysis of the protein expression and membrane localisation (Fig. 4A and B) indicating the potential for a ligand binding activity.

3.2. Analysis of CCR10 expression in vivo

Our studies on *Anxa1*-null mice and wild type controls reveal a marked reduction in CCR10 expression in the *Anxa1*-null mice versus control (Fig. 5) and show that this is reversed by treatment with hrANXA1 or $ANXA1_{Ac2–26}$ (Fig. 6). However *Anxa1* null mice did not show any phenotype that could readily be linked to downregulation of CCR10 and further studies are necessary to explore the significance of these findings. However, our own data (John et al. manuscript in preparation) and a recent report showing the LPS susceptibility of the *Anxa1* null mice [36] open new avenues on the possible significance of changes in CCR10 expression.

As ANXA1 is commonly viewed as an anti-inflammatory protein and CCL27 is pro-inflammatory, it might have been predicted that ANXA1 would downregulate CCR10 expression. However recent studies have reported that CCR10

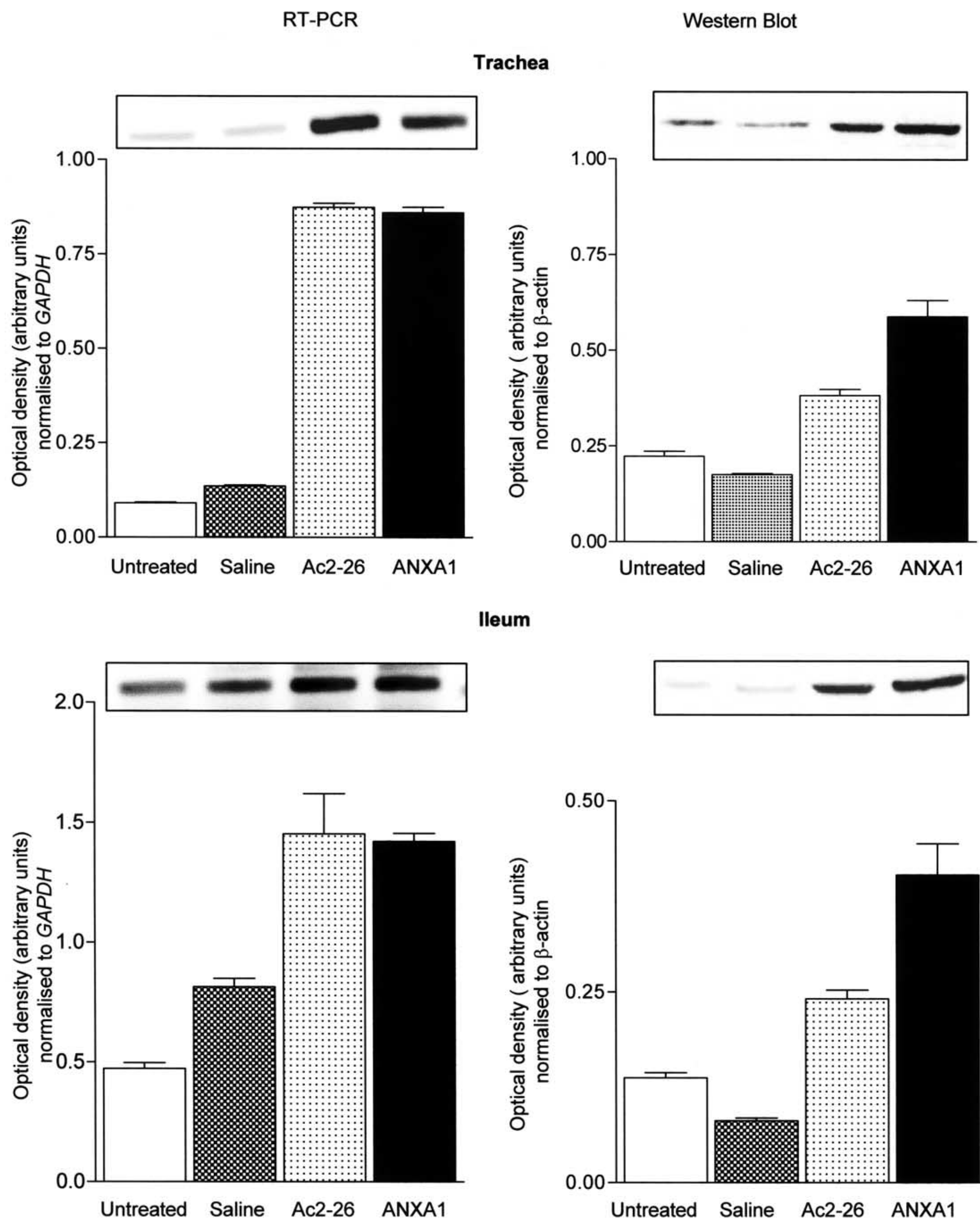

Fig. 6. Exogenous recombinant ANXA1 augments the expression of CCR10 in trachea and ileum from Anxa 1 null mice. Left panel: RT-PCR analysis Right panel: Western blot of CCR10 in trachea and ileum of wild type (WT) and *Anxa*1 null mice treated with human recombinant ANXA1 or $ANXA1_{Ac2–26}$ (10 ng/mouse, and 14 ng/ mouse, respectively, iv, 24 h before animals were killed). The data are representative of two independent experiments performed on three animals per group.

induction on pathogen-specific IgA-secreting B cells may be an important factor for successful protection following local mucosal infection [37].

4. Conclusions

Taken together, the data provide new evidence that ANXA1 modulates gene expression. The mechanisms responsible require further investigation. While our data support a role for the FPR family in mediating some of the effects, the failure of fLMP and ATLa to suppress *ITGB1BP1* suggest other mechanisms are involved. Furthermore, the dissociation between the activities of hrANXA1 and $ANXA1_{Ac2-26}$ support the premise that the actions of ANXA1, but not $ANXA1_{Ac2-26}$, are akin to those of the FPRL1 ligand LXA4.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.01.072.

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