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Research Article

Expression, Localisation and Functional Activation of NFAT 4 in Human Skin, Cultured Keratinocytes and Cultured Fibroblasts

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Abstract:

Background: *Ciclosporin A (CsA) is widely utilized for the treatment of inflammatory skin diseases such as psoriasis. The therapeutic effects of CsA are thought to be mediated via its immunosuppressive action on infiltrating lymphocytes in skin lesions. CsA and tacrolimus block T cell activation by inhibiting the phosphatase calcineurin and preventing translocation from the cytoplasm to the nucleus of the transcription factor Nuclear Factor of Activated T cells (NFAT). NFAT compose a family of transcription factors that are turned on during T cell activation. The NFAT family is composed of five members: NFAT 1, NFAT 2, NFAT 3, NFAT 4 and the recently isolated NFAT 5.*

Aims: As calcineurin and NFAT 1 have been shown to be functionally active in cultured human keratinocytes, expression of other NFAT family members such as NFAT 4 and possible functional activation was investigated in human keratinocytes and dermal fibroblasts.

Methods: *RT-PCR and Western Analysis were used to investigate the presence of NFAT 4 mRNA and protein. Tissue culture of human keratinocytes and human fibroblasts, immunostaining of cells on coverslips and confocal microscopy were used to assess the degree of nuclear localisation of NFAT 4 in cultured cells. Keratome biopsies were taken from patients with psoriasis (lesional and non-lesional skin) and normal skin and immunohistochemistry was used to assess the NFAT 4 localisation in these biopsies in vivo using a well characterized anti-NFAT 4 antibody.*

Results: *The NFAT 4 mRNA and protein expression was demonstrated using RT-PCT and Western blotting. The expression of NFAT 4 in vivo in normal skin, non-lesional and lesional psoriasis was also investigated. A range of cell types in the skin express NFAT 2.*

For example, three members of NFAT (NFAT 1, NFAT 2 and NFAT 4) were shown to be present in the cytoplasm of human muscle cells at all stages of myogenesis. However, in cultured human skeletal muscle cells each NFAT undergoes nuclear translocation at a different stage of myogenesis, suggesting that each NFAT may regulate different subsets of genes necessary for muscle cell physiology. Therefore, expression of NFAT 4 in human keratinocytes and dermal fibroblasts and response to different agonists provides perhaps a unique opportunity to examine the regulation, subcellular localization and kinetics of translocation of different NFATs in primary cultured human cells.

In these experiments the author assessed the expression, localization of NFAT 2 in cultured human keratinocytes and dermal fibroblasts and measured the degree of nuclear localisaion of NFAT 4 using immunoflourescence and confocal microscopy and whether CsA and tacrolimus inhibits NFAT 4 nuclear translocation. As with NFAT 1, differentiation-promoting agents that increase intracellular calcium concentration induced nuclear translocation of NFAT 4 in cultured keratinocytes but with different kinetics. On the other hand, human dermal fibroblasts expressed NFAT 2 showing different effects to different agonists.

Conclusion: *These data provide the first evidence of that NFAT 4 is expressed in normal skin, psoriasis and that NFAT 4 functionally active in human keratinocytes and dermal fibroblasts and that nuclear translocation of NFAT 4 in human skin cells has different kinetics than NFAT 1 suggesting that NFAT 4 may play an important role in regulation of keratinocytes proliferation and differentiation at a different stage. Inhibition of this pathway in human epidermal keratinocytes many account, in part for the therapeutic effects of CsA and tacrolimus in skin disorders such as psoriasis. Thus, supporting our previous work data that calcineurin/NFAT is functionally active not only in T cells, but in skin cells.*

Key words: *Human keratinocytes, dermal fibroblasts, intracellular calcium, psoriasis, NFAT 4, signal transduction.*

Introduction

Psoriasis is a common skin condition characterized by hyperproliferative epidermis, abnormal keratinocyte differentiation and inflammation. CsA and tacrolimus are effective treatments for psoriasis. IL-2 production in T cells depends on activation of phosphatase calcineurin and translocation of the transcription factor NFAT to the nucleus. CsA and tacrolimus block T cell activation by inhibiting this pathway (Figure 1).

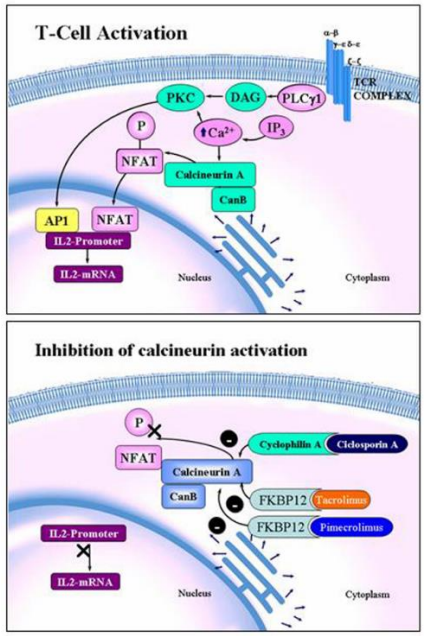

Figure 1

T cells are known to play an important role in the pathogenesis of psoriasis. However, a number of lines of evidence indicate that CsA exerts direct effects in skin, which are independent of its action on T cells. Although CsA and tacrolimus are immunosuppressive drugs that inhibit T cell activation, it is possible that these drugs may work in part through direct action on the skin, independent of their action on T-cells. A number of lines of evidence indicate direct effects of CsA on epidermal keratinocytes. However, the results obtained so far are contradictory. Several in vitro studies have reported a direct antiproliferative effect of CsA in cultures of proliferating epidermal keratinocytes (1-4). For example, CsA inhibits the proliferation of keratinocytes and fibroblasts in culture at concentrations (1-10 μg/ml) that have been in psoriatic plaques following the systemic administration of CsA (4-6). In addition, CsA and tacrolimus both induce hair growth (7-9) and topical tacrolimus stimulates hair growth in SCID mice (7). CsA also inhibits antigen presentation by Langerhans cells (10) and inhibits neutrophil chemotaxis (11). Urabe et al. (1989) reported direct in vivo antiproliferative effect of CsA on human epidermal keratinocytes grafted on to nude mice (12). Gschwendt et al. (1985, 1986, and 1987) demonstrated that CsA inhibits the induction of DNA synthesis, ornithine decarboxylase, alkaline phosphate activity, and 8-hydroxyeicosaterenoic acid in mouse epidermis, following topical application of the tumour-promoting phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (13-15). CsA was demonstrated to inhibit TPA-induced inflammatory hyperplastic response (16). In this study, inhibition of ornithine decarboxylase induction (a proliferation marker) and inhibition of membrane-

associated transglutaminase activity (a terminal differentiation marker) were observed in the skin of hairless mice (16). CsA also inhibits TPA induced-cutaneous inflammation in severe combined immunodeficient mice that lack functional lymphocytes (17).

However, Gottlieb et al. (1992) found that the predominant direct mechanism of action of CsA in vivo is a diminution of T cell activation, in biopsies from psoriatic plaques, with attendant decreased cytokine production. In addition, keratinocyte growth activation was less sensitive to the inhibitory effects of CsA (18). Furthermore, another study failed to show inhibition of epidermal growth in pig skin explant cultures by CsA at therapeutic levels (19). More research to clarify the effects of CsA on skin cells is obviously needed. We have previously shown that treatment of cultured human keratinocytes with agents that induce a sustained rise in intracellular calcium, including elevation of extracellular calcium ((20) leads to nuclear translocation of endogenous NFAT 1, which was inhibited by pre-treatment with CsA, tacrolimus(21, 22) and recently with nifedipine(23).

NFAT is known to be expressed in other cells and organs such as skin. Mouse skin tumours have been shown to express NFAT 1 mRNA (24). Verweij et al. (1990) using an oligomerized NFAT 1 binding motif that directed SV40 T-antigen expression in transgenic mice found constitutive expression only in skin. Using immunofluorescence, they reported T antigen positive cells within the dermis (25), although expression within the epidermis is also evident. Nishio et al. (2000) have also recently reported immunolocalizsation of calcineurin and FKBP12 in human epidermis using immunohistochemical methods (26). Furthermore, recent evidence indicates that UV radiation is a strong inducer of NFAT activation in mouse epidermal cells and skin (27). Santini et al. (2001) have shown that calcineurin regulates the expression of mouse keratinocyte differentiation markers and the cyclin-dependent kinase inhibitor p21WAF1 through a mechanism that appears to involve an interaction between NFAT1/NFAT2 and the Sp1/Sp3 transcription factors (28). In addition, this study showed that treatment of primary mouse keratinocytes with CsA suppressed the expression of terminal keratinocyte differentiation markers (28).

The four different NFAT proteins, their overlapping expression patterns, and the relatively mild phenotypes of mutant mice lacking single NFAT, suggest that they might be functionally redundant (29, 30). Therefore, double mutant mice were used to explore this issue. In fact, mice deficient in both NFAT 1 and NFAT 4 demonstrated more profound lymphadenopathy and increased Th2 responses (31). The striking allergic

phenotype with allergic blepharitis and interstitial pneumonitis suggests a role for NFAT 1 and NFAT 4 in suppressing the production of allergic responsible cytokines (31).In contrast, T cells deficient in both NFAT 1 and NFAT 2 were incompetent in producing Th1 and Th2 cytokines, while B cells were paradoxically hyperactive, suggesting a role for NFAT in suppressing B cell responses (32).

In lymphocytes, calcium entry regulates calcineurin activity that dephosphorylates NFAT family member unmasking the nuclear localization sequence resulting in nuclear translocation of NFAT (33, 34). Lymphocytes express NFAT 1, NFAT 2 and NFAT 4 (35) and each NFAT member translocates to the nucleus with the same kinetics in response to TPA plus ionomycin (30). Together with the relatively mild phenotypes of mutant mice lacking single NFAT, suggested that NFAT family members might be functionally redundant (29, 30). Three members of NFAT (NFAT 1, NFAT 2 and NFAT 4) were shown to be present in the cytoplasm of human muscle cells at all stages of myogenesis. However, in cultured human skeletal muscle cells each NFAT undergoes nuclear translocation at a different stage of myogenesis, suggesting that each NFAT may regulate different subsets of genes necessary for muscle cell physiology (36). In T cells and mast cells NFAT activation is mediated by calcium signals emerging from their respective antigen receptors, TCR (37) and Fc□RI respectively (38). The relevant receptors and signalling pathways that activate NFAT in other cell types have not been well studied.

As calcineurin and NFAT 1 have been shown to be functionally active in cultured human keratinocytes(21), expression of other NFAT family members and possible functional activation was investigated in human keratinocytes and dermal fibroblasts. Santini et al. (2001) showed that NFAT 1 and NFAT 2 associate with another transcription factor (Sp1) in mouse keratinocytes in a calcineurin dependent-pattern (28). The expression of NFAT 4 in human keratinocytes and dermal fibroblasts provides perhaps a unique opportunity to examine the regulation, subcellular localization and kinetics of translocation in primary cultured human cells. To determine whether nuclear translocation of NFAT 3 take place with the same kinetics in cultured keratinocytes and dermal fibroblasts, subcellular localization of these transcription factors were investigated using immunofluorescence techniques and confocal microscopy.

Materials and Methods

Material:

Ciclosporin A (CsA) and tacrolimus

CsA and tacrolimus were provided by Novartis Pharma AG, (Basle, Switzerland) and Fujisawa Pharmaceutical Co (Osaka, Japan), respectively. Tacrolimus was also obtained from Affinity Research Products Ltd (Exeter, UK).

Cell culture materials

Keratinocytes growth medium (MCDB 153) and trypsin/ethylenediamine tetra-acetic acid (EDTA) were purchased from Sigma laboratories (Poole, UK). Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium and foetal calf serum (FCS) were from Gibco BRL Life Technologies (Paisley, UK).

Primers

NFAT 4 primers were synthesized by MWG-Biotech AG (Ebersberg, Germany).

Keratinocytes differentiation agents and growth factors

TPA, ionomycin, Transforming factor β TGF-β, Retinoic Acid (RA) and Dimethyl Sulphoxide (DMSO) (vehicle control) were obtained from Sigma (Poole, UK).

Materials used in Western analysis

Precast polyacrylamide gels were purchased from Invitrogen (Paisley, UK). Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes, ECL molecular weight markers were obtained from Amersham (Buckinghamshire, UK). Prestained protein standards were provided by Bio-Rad Laboratories Ltd (Herts, UK). Anti-NFAT 4 dilution was 1:7500

Antibodies

Anti-NFAT 4 (1689) was kindly made available by Dr Nancy Rice, NCI-Frederick Cancer Research and Developmental Centre, Maryland, USA. This antibody has been previously characterized (35).

Tissue culture

The general tissue culture methods used followed those described by Freshney (39).

1. Culture medium MCDB153

The culture medium used for growing keratinocytes was the serum-free medium MCDB153 described by Boyce and Ham (40), with modifications described by Wille and Pittelkow (41, 42). Powdered MCDB153 medium was obtained from Sigma with a calcium concentration of 70 µmol/L, supplemented with ethanolamine (6.1µl/L), phosphoethanolamine (14µg/ml), hydrocortisone (0.18µg/ml), insulin (5 µg/ml),

transferrin (5µg/ml), epidermal growth factor (10ng/ml), and amino acids (42). The medium obtained is referred to as complete MCDB153. Media were filter sterilised through a 0.2µm filter (Millipore), and stored at 4°C for up to two months before use. Antibiotics were added to give a final concentration of penicillin G (5 IU/ml) and streptomycin (5 µg/ml) (Sigma; Poole, UK).

2 Collection of Biopsy Samples

Fresh human skin tissue to start each cell line, at least 2-3 cm^2, was obtained mainly from two elective surgical procedures as described (43).

- Paediatric circumcision, usually undertaken at the age of 2-5 years.
- Retro-aural skin excised during reduction of Bat Ears, usually undertaken at the age of 8-15 years. This provided one to three ellipses of skin measuring 2-3 by 4-6 cm.

After removal, skin was placed in "transport medium". Universal vials containing 10ml DMEM with 5 IU/ml penicillin G, 5µg/ml streptomycin (ICN Biochemicals; Hampshire, UK) and 240 units/ml nystatin, were supplied to the surgical theatres (Royal Victoria Infirmary; Newcastle upon Tyne, UK). Samples were collected as soon as possible after excision (i.e. immediately after telecommunication from theatre staff) or stored at 4°C. Tissue was kept at 4°C, processed the same day or the following morning, and used for different applications or snap frozen in liquid nitrogen and stored at –80°C until required.

3 Separation of Epidermis from Dermis by Treatment with Dispase

The tissue was trimmed of fat and excess dermis using curved scissors, washed in sterile phosphate buffered saline (PBS), 70:30 ethanol/water (v/v) for 30-60 sec and finally in PBS. The skin was placed epidermis down and cut into small squares 2-3mm by 2-3mm (foreskin) or rectangular pieces 2-4 mm by 10 mm (retro-aural skin). Thus, subsequent removal of the epidermis was made easier. The mucosal surface of foreskin specimens was not used. Normal skin biopsy samples were rinsed in Ca2+ and Mg2+ free PBS containing 5 IU/ml penicillin G and 5µg/ml streptomycin. Protease digestion of skin leads to separation of the epidermis from the dermis (39). Epidermis was separated from dermis after overnight incubation at 4°C in Dispase II (neutral protease) (Boehinger Mannheim; Sussex, UK).

4 Culture of primary human keratinocytes

The epidermis was gently and carefully separated with fine forceps. The epidermis of retro-aural skin was thick enough to be removed in a single piece from the skin strips, but for foreskin epidermis, which is more

friable, several small squares of skin were used. The epidermis was incubated for 5-10 min at 37°C with trypsin (0.05%) and EDTA (0.02%) and shaken manually in a sealed tube to disaggregate the basal keratinocytes. Trypsin was removed by washing in Ca2+ and Mg2+-free PBS with centrifugation (2500 RPM, 5 min), cells resuspended in MCDB153 medium and an aliquot counted by the trypan blue dye exclusion method using a Niebaur counting chamber (39). Keratinocytes were cultured in a serum-free medium MCDB153 (42). Cells were plated on to plastic flasks (Costar and Corning; Netherlands) in serum-free medium MCDB153 at a density of approximately $3x10^4/cm^2$. Keratinocytes were expanded by serial passage and used for experiments between passages 2 and 3.

5 Culture of dermal fibroblasts

Human fibroblasts were established by explant culture from the dermal portion of skin specimens. Flasks were scored with a scalpel blade and small portions of dermis (3-4mm) with a small amount of medium (to prevent the tissue from floating). When the dermis had attached, medium was added and the flasks were returned to the incubator at 37°C for 2-3 days. Fibroblasts which grew out from the explanted dermis were passaged and grown in DMEM with 10% FCS. Antibiotics were added to a final concentration of penicillin G (5 IU/ml) and streptomycin (5 µg/ml). Fibroblasts were removed from flasks by incubation with trypsin (0.05%) and EDTA (0.02%) at 37°C for 10-20 min followed by gentle agitation. Fibroblasts were expanded by serial passage and used for experiments between passages 3 and 6.

6 Culture of Jurkat T cells

Jurkat T cells (44-46), a kind gift from Dr A Hall (Cancer research centre, Newcastle upon Tyne, UK), were cultured in RPMI 1640 medium with 10% FCS. Cells were incubated at 37°C with 5% CO2. RNA was extracted from Jurkat T cells in passage 4-5.

Imunofluorescence staining of cultured cells:

Coverslips preparations

Cells were trypsinized from flasks and seeded onto sterile coverslips placed in twelve well plates, so that there were $3x10^4$ cells on each coverslip. Coverslips were incubated in an incubator at 37°C in 5% CO2. Coverslips were prepared as described (21, 47).

Keratinocytes or fibroblasts were treated with specific agents, DMSO (1:1000) (vehicle control), or switched to medium containing raised extracellular calcium (1.5 mM $CaCl_2$) 15 min and 18 h. Some coverslip cultures were pre-treated with CsA or tacrolimus for 1 h. After the time of incubations, the medium was aspirated and the cells were washed three times in Ca^{2+} and Mg^{2+} -free ice cold PBS before being fixed.

Fixation method

The effects of permeabilization and fixation conditions on the subcellular localization of antigens (48) was examined carefully. Fixation methods fall generally into two categories, organic solvents and cross-linking reagents. The optimal fixation method was chosen empirically (49) and fixation in organic solvents and cross-linking agents was studied. The protocol utilised included 0.7% paraformaldehyde with 0.2% Triton X-100 and 4% paraformaldehyde as cross-linking agents. Acetone or 50% acetone/ 50% methanol for 10 min at room temperature were used as organic solvents. Cells fixed in 4% paraformaldehyde for 10 min were then permeabilised with 0.2% Triton X-100 for a further 10 min at room temperature. After fixation, the cells were washed three times in phosphate buffer saline (PBS). Cells were then immediately immunostained.

Cell staining for immunofluorescence microscopy

Non-specific binding was blocked by incubating coverslips in blocking serum (diluted 1:60 in PBS) by using serum from the species in which the secondary antibody was raised (50, 51) for 10 min. 100 µl of primary antibodies against NFAT 4 was added to each coverslip and incubated at room temperature for 45 min. Cells were washed three times in PBS. Cells were then incubated with 100 µl of FITC-conjugated anti-rabbit and FITC-conjugated anti-goat secondary antibody for 45 min at room temperature. Cells were washed three times with Ca^{2+} and Mg^{2+}-free PBS. Cells were then incubated with 50 µg/ml propidium iodide (PI) (Sigma Laboratories; Poole, UK) for 1 h at room temperature. Finally, cells were washed three times with Ca^{2+} and Mg^{2+}-free PBS. Coverslips were mounted onto slides using vectorshield fluorescence mounting medium (Vector Laboratories Ltd; Peterborough, UK) and the edges sealed with clear nail varnish.

Counting of cells showing nuclear positivity

To assess the subcellular location quantitatively, counting was done in 4 fields of each coverslip using conventional fluorescence microscopy (Carl Zeiss, Germany) using a 60x objective lens. The numbers of cells showing positive nuclear staining were counted. In practice, at least 50 cells in 3 independent experiments (150 cells in total) were assessed at each time point.

Confocal microscopy

Cells were analysed using a Bio-Rad MRC 600 confocal laser scanning microscope (BioRad; Herts, UK), mounted on a Nikon Optiphot II (Nikon UK Ltd; Surrey, UK) upright stand with a Krypton/argon laser giving 448 nm, 568 nm, and 647 nm excitation lines. Suitable areas on the slide were located with X20 na 0.4 lens, and then imaged with a 60x na 1.4 oil immersion lens. Cells were imaged utilizing 488 nm lines (FITC, Oregon Green) and 568 nm lines (Alexa 568, PI) into Photo Multiplier Tube (PMT) channel 2 and 1 respectively. Excitation using the 488 and 568 laser lines independently was necessary to reduce some effects of 'cross-talk' between the fluorochromes due to the overlap of emission spectra and were gathered stack by stack. Z-series of approximately 10 to 15 optical sections (using 1 μm Z step) were then acquired and stored on a Panasonic optical drive (1GB), later transferred to a compact disc for analysis and archival using COMOS software (Bio-Rad, version 7.0). Independent Z series images were projected and composite images merged using Confocal Assistant software (version 4.2, Todd Clark Brelje). Later processed using Adobe PhotoShop (San Jose, CA, USA). In summary, Cells were fixed in 4% paraformaldehyde, permeabilised with 0.2% Triton X-100, incubated sequentially with rabbit-polyclonal anti-NFAT 4 antibody (1:500), goat anti-rabbit FITC (Sigma laboratories; Poole), UK, PI (50 μg/ml) and visualized using a Biorad confocal microscope.

Specificity of staining

Non-immune rabbit serum (Vector laboratories; Peterborough, UK) was included at equivalent concentrations as the primary antibodies in immunofluorescence studies as negative controls. In addition, equal dilution of secondary antibody was used with both the primary antibody and the negative control. Negative controls were scanned using the same settings (gain, black level and confocal aperture) as the positive control coverslips, thus ensuring that the pixel brightness values were due to antibody labeling rather than other factors such as autofluorescence or non-specific binding. Pixel brightness data were analyzed using COMOS software.

Reverse Transcrcription-Polymerase Chain Reaction

NFAT 4 cDNA sequences were obtained from GenBank at http://www.ncbi.nln.nih and complementary primers were designed to amplify target sequence specific for NFAT 4. Primers sequences were confirmed using the blast analysis at http://www.ncbi.nlm.nih.gov/blast. Coding sequence for NFAT 4 was aligned

using Lasergene software (DNA Star Inc., Madison; USA) and primers were designed for each calcineurin subtype or NFAT isoform in areas of low homology. Primer set for human NFAT 4 was forward: 5'CTCGCGGCCTGCAGATCTTG 3' and Backward: 5'GGCTCAAGAGGAAGATAGAG 3', resulting in amplification 375 bp

Prevention of ribonuclease (RNases) contamination

RNases are particularly stable and thus difficult to destroy. A number of precautions were taken to avoid RNase contamination (52).

Isolation of RNA

Cultured keratinocytes and fibroblasts at approximately 70% confluence were washed twice with sterile Ca2+ and Mg2+-free PBS. Keratinocytes and fibroblasts were removed from flasks by treatment with 0.05% trypsin and 0.02% EDTA. Jurkat T cells (used as a positive control) grow in suspension and can be aspirated from flasks. Total RNA was isolated using RNeasy Mini Kit (QIAGEN; West Sussex, UK) according to manufacturer's instruction.

Polymerase Chain Reaction (PCR)

3-5 μl of cDNA was amplified in 50 μl PCR reaction which consisted of 1.5 μl of 50 mM MgCl2 (Bioline; London, UK), 5 μl 10x NH4 buffer (Bioline; London, UK), 5 μl DMSO, 1.25 μl of 25 pmol forward primer, 1.25 μl of 25 pmol reverse primer and 4 μl of dNTP's (2.5 mM each dNTP). Distilled water was added to make the total reaction volume equal 50 μl. Negative controls were included in each reaction by replacing the cDNA with water. 0.2 μl of 0.625 U BioTaqTM DNA polymerase (Bioline; London, UK) was added to the reaction after heating to 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, re-annealing at 55-57°C for 1 min and elongation at 72°C for 2 min. A final cycle of 72°C for 15 min was used. Similar cycle conditions were used for each set of primers.

Agarose Gel Electrophoresis

PCR products were electrophoresed through 1.5% agarose gels to determine product size. Loaded samples were visualized on a UVP transilluminator and photographed (Mitsubishi camera/ Polaroid black and white film type 667).

Gel extraction

PCR products were gel purified using a QIAGEN kit (QIAGEN; West Sussex, UK) to obtain single fragments for sequencing. DNA was separated using agarose gel electrophoresis. The appropriate band was excised weighed and sent for sequencing.

Sequencing of PCR products

Automated sequencing was carried out by MWG-Biotech AG (Ebersberg, Germany).

Western Blotting

Cells were lyzed in 2 X Sodium Dodecyl Sulphate (SDS), sample buffer (125 mM Tris-HCl, pH 6.8, 0.05% bromophenyl blue, 4% SDS, 20% glycerol and 10% β-mercaptoethanol). Equal amounts of samples and enhanced chemiluminescence molecular weight markers (Amersham, Bucks, UK) were electophoresed through 10% polyacrylamide gels, and Western Blotting were performed as described. (53), using anti-NFAT 4 (1:7500).

Immunohistochemistry:

Subjects and skin biopsies

Skin biopsies were obtained from normal volunteers and patients with psoriasis, following local ethical committee approval. Patients with psoriasis were excluded if they had received systemic anti-psoriatic, ultraviolet B (UVB), Psoralen and UVA (PUVA) or anti-inflammatory therapy during the last 3 months. Patients discontinued topical anti-psoriatic medication apart from emollients for two weeks prior to study. Following informed consent, paired 6 mm punch biopsies were obtained from the edge of psoriatic plaques (lesional) and non-lesional (uninvolved) skin on the lower back/buttock, under local anaesthesia and embedded in optimal cutting temperature (OCT) compound, frozen and stored at –70*C until required for study. Biopsies were obtained from five normal volunteers (3 males, 2 females, mean age 36 years) and a further five patients with stable plaque psoriasis (3 males, 2 females, mean age 54 years) for NFAT 4 studies.

Immunohistochemical analysis of skin biopsies

Five μm sections were cut on a cryostat (Bright; Huntingdon, England), placed on to APES-coated slides and fixed in ice-cold acetone for 15 min. Non-specific binding was blocked by incubating skin sections in blocking serum; (diluted 1:60 in PBS). This was done by using serum from the species in which the secondary

antibody was raised (50, 51) for 20 min at room temperature. The sections were stained with anti-NFAT 4 rabbit polyclonal antibodies (1:500) in 0.1% BSA and in Ca^{2+} Mg^{2-} free PBS for 1 h at the room temperature. Sections were developed using an avidin-biotin immunoperoxidase kit (Vector Laboratories, Peterborough, UK) using Ni^{2+} plus 3,3'-diaminobenzidine as the chromagen and counterstained with methyl green as described (54).

Assessment of immunohistochemical staining

The degree of staining was assessed on a semi-quantitative scale by the author but blinding was not possible due to characteristic morphological features of lesional psoriatic biopsies. The intensity of immunostaining was evaluated by using an ordinal 0-4 scale, where 0=no staining; 1= minimal; 2=minimal-moderate; 3=moderate and 4=maximal staining. Furthermore, localization (cytoplasmic versus nuclear) of each staining was examined in basal, suprabasal and high suprabasal layers in all stained sections as described (55).

Statistical analysis:

To compare the effects of specified treatment on the number of cells showing nuclear immunostaining, Chi square analysis was used. Data were analyzed using Arcus Quickstat software (Biomedical version 1.0).

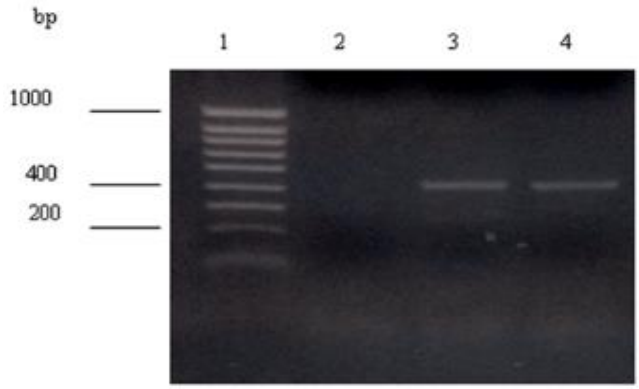

Figure 2

Results

Expression of NFAT 4 mRNA in cultured keratinocytes and cultured fibroblasts

RT-PCR of keratinocyte and fibroblast cDNA, using NFAT 4 specific primers, produced the appropriate fragments size as predicted, demonstrating the presence of NFAT 4 mRNA in human epidermal keratinocytes

and cultured dermal fibroblasts (Figure 2). Sequencing of RT-PCR products followed by BLAST analysis confirmed the identity of the products that show 100% homology with the predicted NFAT 4 (Accession# U85430) sequences. cDNA from Jurkat T cell mRNA was amplified as a positive control in these experiments.

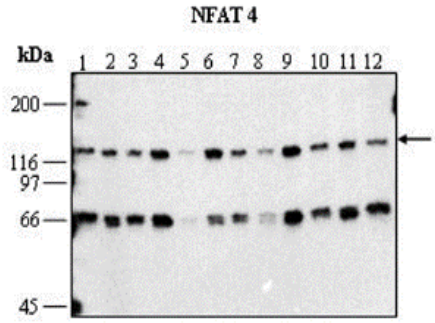

Figure 3

Confirmation of expression of NFAT 4 in cultured keratinocytes and dermal fibroblasts by Western analysis

Western blotting showed that cultured human keratinocytes and dermal fibroblasts co-express NFAT 4 at the protein level (Figure 3). These experiments also demonstrated that the antibodies used in immunostaining experiments detected the appropriate molecular weight of NFAT 4 (~120 kDa). However, an additional band of (~66 kDa) was detected in lysates prepared from cultured keratinocytes and dermal fibroblasts. This band may reflect a degradation product of NFAT 4.

Optimization of fixation method for immunfluorescence studies of NFAT 4 and negative controls for immunostaining studies

Different fixatives (see Materials and Methods) were included in studying the subcellular localization of NFAT 4. Different fixatives did not result in any significant differences in the distribution of NFAT 4 in cultured human keratinocytes (Data not shown). 0.4% paraformaldehyde followed by permeabilisation with 0.2% Triton X-100 was used in subsequent experiments. Non-immune rabbit serum, at a similar dilution to the primary antibody, was included in the immunostaining protocol as a negative control. No significant staining was detected with the non-immune in both immunofluorescence and immunohistochemical studies.

For immunofluorescence studies cells were visualised by immunofluorescence and confocal microscopy using the same settings for each experiment as described in Materials and Methods.

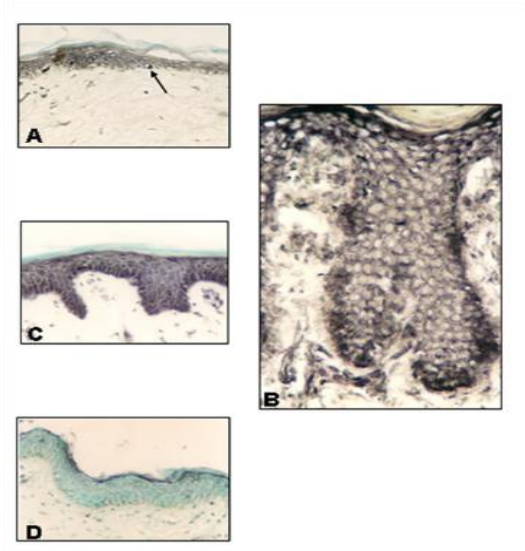

Figure 4

NFAT 4 is co-expressed in normal and psoriatic skin in vivo

Immunohistochemical studies of normal human and psoriatic skin showed prominent expression of NFAT 4 (Figure 4) in epidermal keratinocytes together with expression in dermal fibroblasts. NFAT 4 immunostaining were also observed in skin appendages in normal and psoriatic skin and within the dermal inflammatory cell infiltrate in psoriasis. NFAT 4 appeared to be expressed by melanocytes in normal skin (Figure 4). Cytoplasmic and membrane patterns of NFAT 4 expression were observed in keratinocytes (Figure 4). No consistent differences were observed between normal skin, lesional or non-lesional psoriatic skin with respect to NFAT 4 localization (Table 1).

Table 1 Distribution of NFAT 4 in normal (A) and psoriatic (B) skin.

A Normal Skin

	Basal	Suprabasal	High suprabasal
Subject 1	3 C	3 C	3 C
Subject 2	2 C	2 C	2 C
Subject 3	3 C	2 C	2 C
Subject 4	3 C	3 C	3 C
Subject 5	2 C	2 C	1 C

(B) Psoriatic skin

	Skin type	Basal	Suprabasal	High suprabasal
Subject 1	Lesional	4 C	2 C	3 C
	Non-lesional	4 C	4 C	4 C
Subject 2	Lesional	3 C	3 C	3 C
	Non-lesional	3 C	3 C	3 C
Subject 3	Lesional	2 C	1 C	1 C
	Non-lesional	3 C	2 C	2 C
Subject 4	Lesional	3 C	3 C	3 C
	Non-lesional	3 C	3 N/C	3 C
Subject 5	Lesional	2 C	3 C	2 C
	Non-lesional	3 C	3 C	3 C

The intensity of immunostaining was evaluated by using an ordinal 0-4 scale, where 0= no staining; 1= minimal; 2=minimal-moderate; 3= moderate and 4=maximal staining. N indicates that the staining was predominantly nuclear, C indicates predominantly cytoplasmic and N/C indicates an equal distribution between the two compartments.

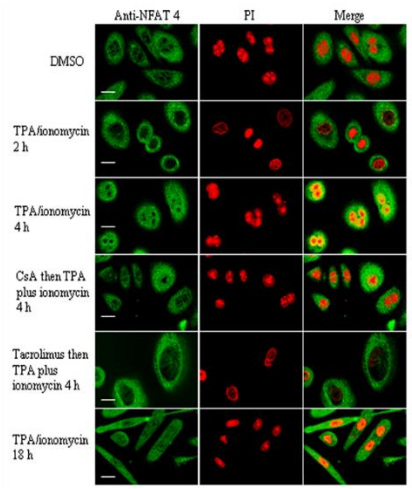

Figure 5

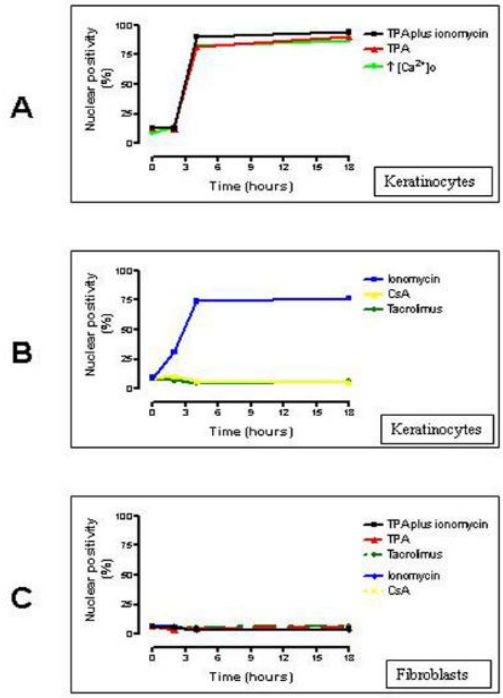

Figure 6

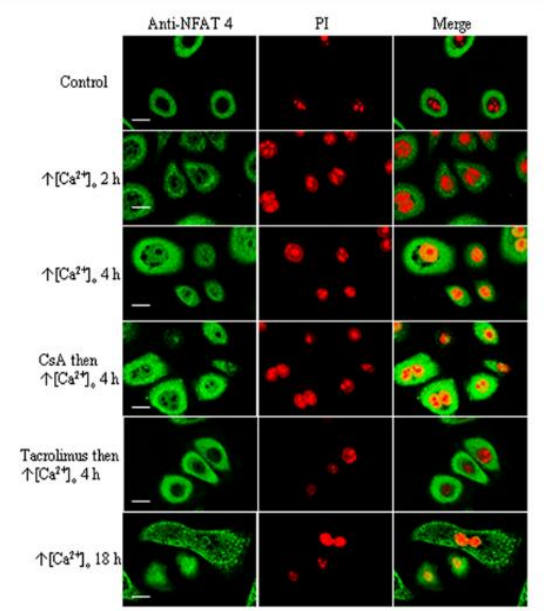

Figure 7

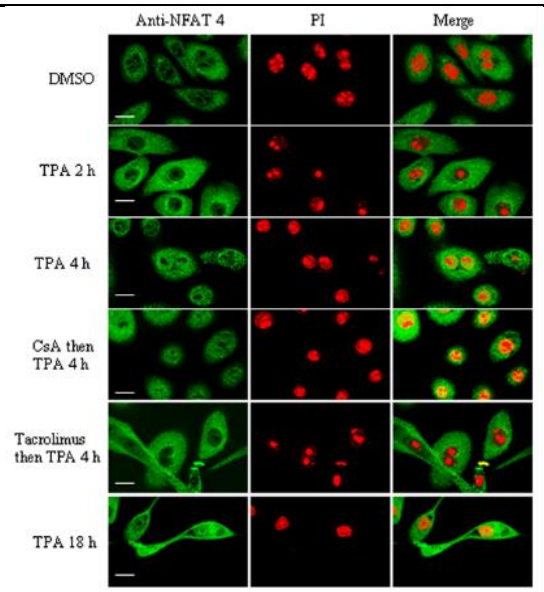

Figure 8

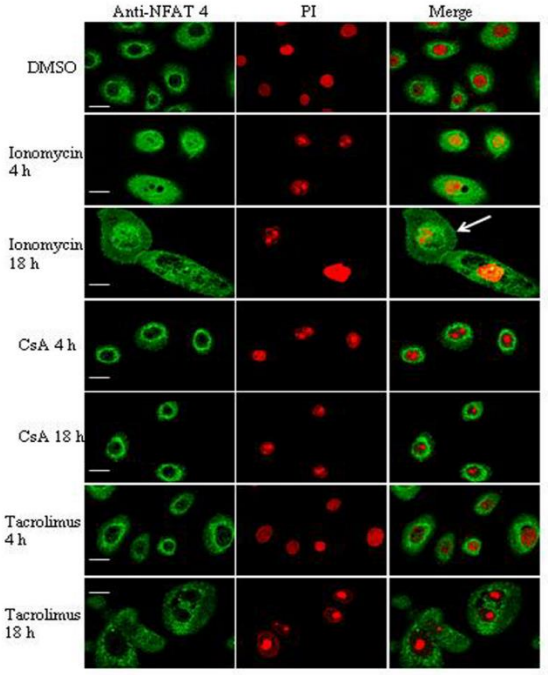

Figure 9

Nuclear translocation of NFAT 4 in normal human keratinocytes is inhibited by tacrolimus

In untreated keratinocytes or cells treated with vehicle, NFAT 4 was found predominately in the cytoplasm (9.3% and 13.3% nuclear positivity respectively, n=150 cells) (Figure 5 and Table 2). Increased nuclear localization of NFAT 4 was found after incubation of cells with TPA (50nM) plus ionomycin (1 μM) by 4 h

(90.0 % nuclear positivity, P<0.0001), which was maximal at 18 h (94.0% nuclear positivity, P<0.0001) (Figure 5, Figure 6A and Table 2). Increasing extracellular calcium from 70 μM to 1.5 mM (Figure 7), TPA (50 nM) alone (Figure 8) and ionomycin alone (Figure 9) resulted in increased nuclear localization of NFAT 4 with similar kinetics to TPA plus ionomycin (Figure 6A and B).

Table 2 Nuclear translocation of NFAT 4 in human keratinocytes

Treatment	NFAT 4	
	% cells showing nuclear positivity	Number of cells counted
Medium control*	9.3	150
DMSO control†	13.3	150
TPA/ionomycin 2h‡	12.7	150
TPA/ionomycin 4h‡	90.0***	150
CsA§ 1μM then TPA/ionomycin 4h‡	87.3	150
Tacrolimus‖ 1μM then TPA/ionomycin 4h‡	50.7††	150
TPA/ionomycin 18h‡	94.0***	150
TPA 2h¶	12.7	150
TPA 4 h¶	82.0‡‡	150
CsA§ 1μM then TPA (4h)¶	86.7	150
Tacrolimus‖ 1μM then TPA (4h)¶	54.0‡‡‡	150
TPA 18h¶	90.0‡‡	150
Increased $(20)_o$ 2h**	14.0	150
Increased $(20)_o$ 4h**	83.3§§§	150
CsA§ 1μM then increased $(20)_o$ 4h	85.3	150
Tacrolimus‖ 1μM then increased $(20)_o$ 4h	33.3§§	150
Increased $(20)_o$ 18h**	87.3§§§	150

* Low calcium (70 μM) medium,
† Vehicle control,
‡ TPA (50 nM) plus ionomycin (1 μM),
§ Pre-treatment with CsA for 1 h,
¶ TPA (50 nM),
‖ Pre-treatment with tacrolimus for 1 h,
** Raised extracellular calcium (1.5 mM),
*** P<0.0001 compared to DMSO control,

†† P=0.02 compared to TPA plus ionomycin 4 h,
‡‡ P<0.0001 compared to DMSO control,
‡‡‡ P<0.0001 compared to TPA 4 h,
§§§ P<0.0001 compared to medium control,
§§ P<0.0001 compared to increased $(20)_o$ 4h.

Whether CsA and tacrolimus inhibited translocation of endogenous NFAT 4 in human keratinocytes was determined. Tacrolimus (1 μM) significantly inhibited nuclear translocation of NFAT 4 induced by increased extracellular calcium (33.3% nuclear positivity, P<0.0001), TPA plus ionomycin (50.7% nuclear positivity; P<0.0001) and TPA alone (54.0% nuclear positivity; P<0.0001) at 4 h (Table 1). CsA (1 μM) did not inhibit nuclear translocation of NFAT 4 induced by differentiation promoting agonists (Table 2). The comparisons of the data presented in Table 2 reflect the structure of the data and the hypotheses being tested. In addition, an increase in NFAT 4 localization to the plasma membrane was also observed in one out of three experiments in response to raised extracellular calcium concentration for 18 h.

Ionomycin induces nuclear translocation of NFAT 4 in human keratinocytes

In further experiments, in untreated keratinocytes or cells treated with vehicle, NFAT 4 was found predominately in the cytoplasm (7.3% and 9.3% nuclear positivity respectively, n=150 cells). Treating cells with ionomycin, (1 μM) alone increased the proportion of keratinocytes displaying NFAT 4-immunostained nuclei between 2 h and 18 h (Figure 10, Figure 6B and Table 2). A possible increase in association of NFAT 4 with the plasma membrane was observed in one out of three experiments after 18 h incubation with ionomycin (Figure 9).

Cyclosporin A and tacrolimus do not induce nuclear translocation of NFAT 4 in human keratinocytes

The effects of CsA (1 μM) or tacrolimus (1 μM) on NFAT 4 localization in cultured keratinocytes were also assessed. CsA (1 μM) and tacrolimus (1 μM) did not significantly alter the proportion of keratinocytes displaying NFAT 4-immunostained nuclei (Figure 9, Figure 6B and Table 2).

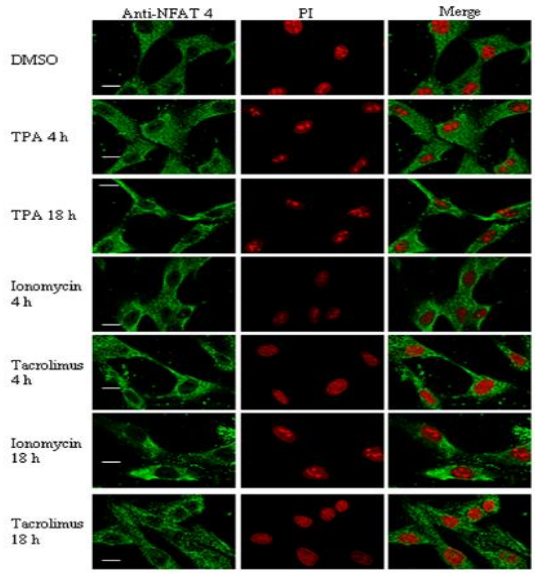

Figure 10

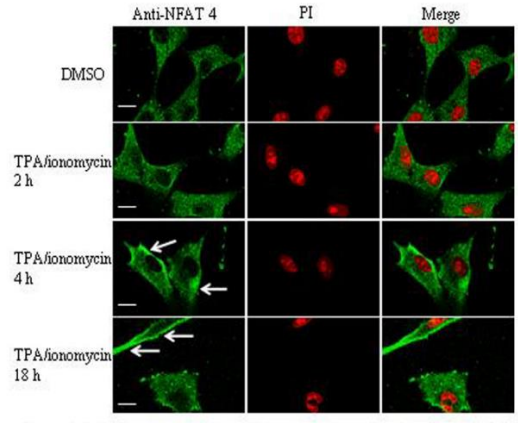

Figure 11

TPA, ionomycin or tacrolimus do not induce nuclear translocation of NFAT 4 in human dermal fibroblasts

As in human keratinocytes, NFAT 4 appeared to be predominantly in the cytoplasm of unstimulated cells (vehicle control) (6.7% nuclear positivity, n=150 cells) (Figure 11 and Table 3). Furthermore and in contrast with NFAT 1, treatment of fibroblasts with TPA, ionomycin or tacrolimus for between 2 h and 18 h did not significantly alter the cellular distribution of NFAT 4 or the proportion of cells showing nuclear NFAT 4 positivity (Figure 10, Figure 6C and Table 3).

Table 3 Nuclear translocation of NFAT 4 in human keratinocytes

Treatment	NFAT 4	
	% cells showing nuclear positivity	Number of cells counted
Medium control*	7.3	150
DMSO control†	9.3	150
Ionomycin 2h‡	31.3**	150
Ionomycin 4h‡	74.0**	150
Ionomycin 18 h‡	76.7**	150
CsA 2h¶	10.7	150
CsA 4 h¶	6.7	150
CsA 18 h¶	6.0	150
Tacrolimus 2 h‖	7.3	150
Tacrolimus 4 h‖	4.7	150
Tacrolimus 18 h‖	6.7	150

* Low calcium (70 μM) medium,
† Vehicle control,
‡ Ionomycin (1 μM),
¶ CsA (1 μM),
‖ Tacrolimus (1 μM),
** P<0.0001 compared to DMSO control.

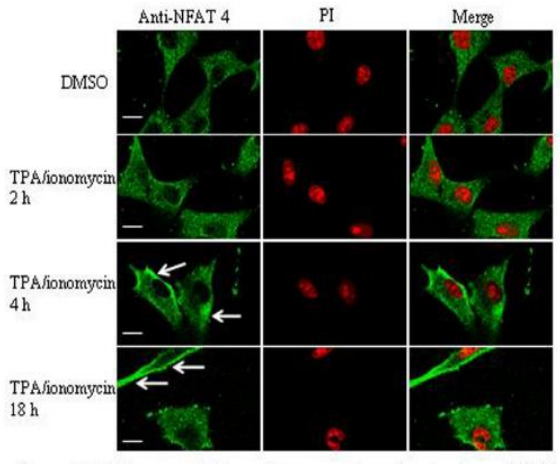

Figure 11

TPA plus ionomycin induces plasma membrane translocation of NFAT 4 in human dermal fibroblasts

Treatment of dermal fibroblasts with TPA plus ionomycin for between 2 h and 18 h induced plasma membrane translocation of NFAT 4 (Figure 11). However, TPA plus ionomycin did not significantly alter the proportion of cells showing nuclear NFAT 4 positivity (Figure 11, Figure 6C and Table 3).

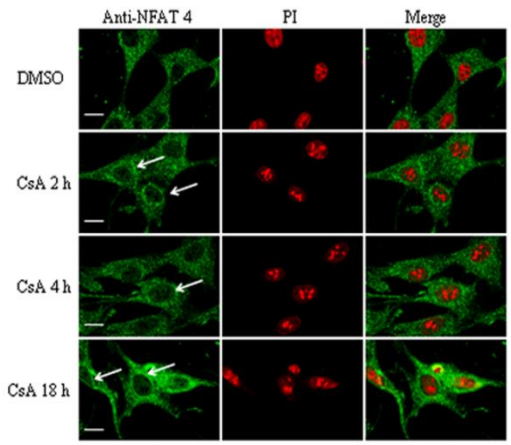

Figure 12

Cyclosporin A increases nuclear membrane localisation of NFAT 4 in human dermal fibroblasts

Treatment of dermal fibroblasts with CsA (1 μM) for between 2 h and 18 h induced nuclear membrane translocation of NFAT 4 (Figure 13). Nevertheless, CsA did not significantly alter the proportion of cells showing nuclear NFAT 4 positivity (Figure 12, Figure 7 C and Table 3).

Table 4 Alteration of NFAT 4 nuclear localisation in human dermal fibroblasts.

Treatment	NFAT 4	
	% cells showing nuclear positivity	Number of cells counted
Medium control*	4.7	150
DMSO control†	6.7	150
TPA/ionomycin 2 h‡	6.0	150
TPA/ionomycin 4 h‡	4.0	150
TPA/ionomycin 18 h‡	4.7	150
TPA 2 h¶	4.0	150
TPA 4 h¶	4.7	150
TPA 18 h¶	6.0	150

Ionomycin 2 h¶¶	6.7	150
Ionomycin 4 h¶¶	4.0	150
Ionomycin 18 h¶¶	4.0	150
CsA 2 h#	4.0	150
CsA 4 h#	4.7	150
CsA 18 h#	4.7	150
Tacrolimus 2 h‖	4.0	150
Tacrolimus 4 h‖	6.0	150
Tacrolimus 18 h‖	6.7	150

* DMEM medium plus 10% fetal calf serum,
† Vehicle control,
‡ TPA (50 nM) plus ionomycin (1 μM),
# CsA (1 μM),
‖ Tacrolimus (1 μM),
¶¶ Ionomycin (1 μM),
¶ TPA (50 nM).

Discussion

This study shows for the first time that NFAT 4 mRNA and proteins are expressed in both cultured epidermal keratinocytes and cultured dermal fibroblasts by using RT-PCR and Western blotting techniques.

The NFAT 4 antibody has been previously characterised (35) but NFAT 4 antibody specificity in skin cells is supported by the single bands observed on Western blot analysis, the different staining patterns in skin, the differences in the subcellular localisation observed by immunofluorescence studies and the different kinetics and patterns of translocation observed in response to a variety of stimuli when compared to NFAT 1 (21). Cytoplasmic and membrane patterns of NFAT 4 expression were observed in keratinocytes. In addition, dermal fibroblasts and melanocytes appeared to express NFAT 4.

Keratinocytes are the main cell type in the epidermis. Although there are some differences between cell culture and human skin in vivo, cultured keratinocytes have proved useful in investigating keratinocyte growth and differentiation (53, 56). Therefore, cultured keratinocytes were used to study the role of calcineurin/NFAT pathway in keratinocyte growth and differentiation.

NFAT 4 appeared predominantly cytoplasmic in untreated subconfluent keratinocytes . Previous work in BHK fibroblasts has indicated that co-migration of calcineurin to the nucleus is required to maintain GFP-NFAT 4 in the nucleus and prevent its export (57, 58). Previous studies have shown that NFAT-dependent transactivation requires a second signal provided by protein kinase C (59-61). However, ionomycin alone induced nuclear translocation of NFAT 2 in B cells (62) and in HeLa cells (63). Furthermore, luciferase expression was stimulated when pNFAT-luc-transfected HeLa cells were treated with ionomycin alone (63). As with NFAT 1, ionomycin alone did induce nuclear translocation of NFAT 4 in cultured keratinocytes.

The relatively wide distribution of NFAT proteins, coupled with the known function of GSK-3β in developmental signalling pathways in Xenopus and Drosophila, also suggested that NFAT proteins might be important in cellular differentiation programs outside the immune system (64-66). This is because GSK-3β is required for ventral differentiation, while dorsal differentiation involves suppression of GSK-3β activity in Drosophila (64). In addition, a potential role of NFAT in the process of adipocyte differentiation has been documented (67). Indeed, nuclear translocation of endogenous NFAT 1(21) in normal human keratinocytes in response to differentiation promoting agents provides extra evidence that the calcineurin/NFAT pathway is functionally active in these cells. Together with the differentiation dependent pattern of expression of NFAT family members, suggest an essential role for these proteins in specifying the temporal and spatial pattern of gene expression during keratinocyte differentiation.

Fibroblasts produce a large number of different growth factor and cytokines that might regulate keratinocytes hyperproliferation by a paracrine mechanism. For example, fibroblasts isolated from psoriatic skin produced greater amount of glycosaminoglycans and collagen than fibroblasts isolated from normal skin (68, 69). In addition, serum from psoriatic patients can induce normal fibroblasts to hyperproliferate at a rate equivalent to psoriatic fibroblasts (70). However, fibroblasts derived from lesional and non-lesional skin stimulate keratinocyte hyperproliferation to a similar degree (70). In contrast, Saiag et al. (1985) used different components of skin from psoriatic and normal subjects to develop an in vitro-skin equivalent. It was demonstrated that fibroblasts derived from psoriatic (lesional or non-lesional) but not normal skin induced normal keratinocytes to hyperproliferate (71). Thus, fibroblasts are potent effector cells and may play a role in the pathogenesis of psoriasis. As NFAT proteins appear to play a role in keratinocytes differentiation, the role of NFAT activation in dermal fibroblasts was studied using immunofluorescence techniques.

Previous work in BHK fibroblasts has indicated that co-migration of calcineurin to the nucleus is required to maintain GFP-NFAT 4 in the nucleus and prevent its export (57, 58). In contrast to human keratinocytes, CsA but not tacrolimus increases the proportion of fibroblasts showing NFAT 4 nuclear membrane positivity. Thus, the influence of both CsA and tacrolimus on the subcellular localisation of different NFATs in dermal fibroblasts is different from their effects in keratinocytes. These data highlight that the responsiveness and regulation of the calcineurin/NFAT pathway in human skin is cell type specific. In addition, CsA and tacrolimus effects on skin fibroblasts indicate direct effects of CsA and tacrolimus in cells derived from dermal components.

The central theme of the work presented in this study was an investigation of the role of different NFATs isoforms (NFAT 4) signalling in skin, relevant to the mechanism of action of CsA and tacrolimus in psoriasis.

The work presented in this study investigated the expression of NFAT 4 in cultured keratinocytes and dermal fibroblasts and the functional activation of the phosphatase enzyme, calcineurin in these cells. TPA, TPA plus ionomycin, and raised extracellular calcium are differentiation-promoting agents in human keratinocytes. Activation of calcineurin/NFAT by these agonists suggests that this pathway may play a role in regulating keratinocyte differentiation. The differentiation dependent pattern of expression of NFAT family members in human epidermis supports the in vitro results. The different kinetics of translocation of each NFAT protein suggests that different NFAT proteins may regulate specific genes required during different stages of keratinocyte differentiation. The different subcellular localization of NFAT 4 and responses to different agents in human fibroblasts highlight that the responsiveness and regulation of calcineurin/NFAT pathway in human skin is cell type specific. CsA and tacrolimus inhibited calcineurin/NFAT translocation in cultured keratinocytes to different degrees

In summary, this study has identified an important component of the calcineurin/NFAT signalling pathway in non-immune cells within human skin and increased our understanding of the role played by calcineurin and NFAT in keratinocyte biology. The efficacy of CsA or tacrolimus in psoriasis has been used as a powerful argument to support the fundamental role of T cell in the pathogenesis of this disease. Showing that these drugs are exerting direct effects in skin cells, independent of T cells, may have important implications for our understanding of the pathophysiology of psoriasis. There is a concern about long term side effects, particularly nephrotoxicity from use of systemic CsA and tacrolimus in psoriasis. Consequently there is

currently significant interest in optimisation of topical delivery and introduction of tacrolimus and ascomycin analogues with improved absorption profiles. Since NFAT is one of a number of calcineurin substrates, drugs, which interfere selectively with NFAT regulation, might be expected to have fewer side effects than CsA and tacrolimus. Thus, future work may lead to the development of topically active drugs that specifically block NFAT signalling pathway (72, 73), and result in significant therapeutic advances.

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