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Keratinocyte Secretion of Cyclophilin B via the Constitutive Pathway Is Regulated through Its Cyclosporin-Binding Site

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Cyclophilin B (CypB) is an endoplasmic reticulum (ER)-resident member of the cyclophilin family of proteins that bind cyclosporin A (CsA). We report that as in other cell types, CypB trafficked from the ER and was secreted by keratinocytes into the media in response to CsA. Concentrations as low as 1 pM of CsA induced secretion of CypB. Using brefeldin A, we showed that CypB is secreted from keratinocytes via the constitutive secretory pathway. We defined that substitution of tryptophan residue 128 in the CsA-binding site of CypB with alanine resulted in dissociation of CypB^{W128A}-green fluorescent protein (GFP) from the ER. Photobleaching studies revealed a significant reduction in the diffusible mobility of CypB^{W128A}-GFP compared with CypB^{WT}-GFP, consistent with redistribution of CypB^{W128A}-GFP into secretory vesicles disconnected from the ER/Golgi network. Furthermore, CsA significantly decreased the mobility of CypB^{WT}-GFP but not CypB^{W128A}-GFP. These studies demonstrate that therapeutically relevant concentrations of CsA regulate secretion of CypB by keratinocytes, and that a key residue within the CsA-binding site of CypB controls retention of CypB within the ER and regulates entry into the secretory pathway. As keratinocytes express CypB receptors (CD147) and CypB exhibits chemotactic properties, these data have implications for the therapeutic effects of CsA in inflammatory skin disease.

Journal of Investigative Dermatology (2011) **131,** 1085–1094; doi:10.1038/jid.2010.415; published online 27 January 2011

INTRODUCTION

Cyclophilins are highly conserved and ubiquitously expressed proteins that are characterized by peptidyl-prolyl *cis-trans* isomerase activity, and were first identified as binding partners of cyclosporin A (CsA) (Handschumacher *et al.*, 1984). The cyclophilin/CsA complex inhibits T-cell activation by inhibiting the phosphatase calcineurin. Downstream targets for calcineurin include the transcription factor NFAT (nuclear factor of activated T cells), which is required for transcriptional activation of proinflammatory cytokines such as IL-2 (Liu *et al.*, 1991, 1992). CsA also inhibits the peptidyl-prolyl *cis-trans* isomerase activity of cyclophilins, but this is unrelated to the immunosuppressive effects of the drug.

Despite binding to and mediating the effects of CsA, the physiological role of cyclophilins remain incompletely understood, although they have been implicated in a wide variety of cellular processes including viral infectivity, chaperone activity, mitochondrial functions, apoptosis, and regulation of trafficking and signaling (Meunier *et al.*, 2002; Machida *et al.*, 2006; Sokolskaja *et al.*, 2006; Kim *et al.*, 2008). Cyclophilin B (CypB) was the second CsA-binding protein to be identified, has a 10 times higher affinity for CsA than cyclophilin A, and is distinguished from the latter by the presence of a cleavable N-terminal signal sequence that directs the protein to the endoplasmic reticulum (ER) following translation (Hasel *et al.*, 1991; Price *et al.*, 1991; Spik *et al.*, 1991). Schumacher *et al.* (1994) confirmed that this N-terminal signal sequence was necessary for delivery of CypB to the ER. Despite its ER localization, CypB lacks the classical KDEL sequence necessary for retention of proteins within the ER (Munro and Pelham, 1987; Bose *et al.*, 1994). A C-terminus motif (VEKPFAIAKE) appeared sufficient for ER localization, as attaching this peptide to a secretory protein

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Abbreviations: CsA, cyclosporin A; CypB, cyclophilin B; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; MF, mobile fraction; NHEK, normal human epidermal keratinocyte

Received 16 April 2010; revised 15 October 2010; accepted 16 November 2010; published online 27 January 2011

resulted in colocalization with CypB (s-cyclophilin) within the ER (Arber *et al.*, 1992). However, molecular modeling studies suggest that this is unlikely to be the site of action of CsA (Carpentier *et al.*, 1999), and to date this region has not been implicated in the function of CypB (Klappa *et al.*, 1995; Horibe *et al.*, 2002; Meunier *et al.*, 2002). Previous studies in HeLa cells demonstrated that CsA depleted CypB from the ER, increased the association of CypB with the plasma membrane, and caused secretion of CypB by an undefined mechanism (Price *et al.*, 1994). The secretion of CypB into biological fluids such as human milk and plasma has also been reported (Mariller *et al.*, 1996; Billich *et al.*, 1997), but the molecular mechanisms regulating secretion of CypB remain ill defined. CypB may be secreted through the constitutive secretory pathway; the default mechanism for secretion of newly synthesized proteins in eukaryotic cells that involves co-translational translocation of the nascent protein into the ER followed by vesicle-mediated transfer via the Golgi apparatus to the cell surface. Alternatively, CypB may be secreted via the regulated secretory pathway in which proteins are diverted from the constitutive route into storage vesicles that are only released in response to a defined cellular signal.

In vitro, extracellular CypB induces chemotaxis and integrin-mediated adhesion of T cells to the extracellular matrix by way of interaction with two classes of receptors (Allain *et al.*, 2002). CD147 (also known as basigin or EMMPRIN) was initially identified in T cells, but is now known to be expressed by epidermal keratinocytes, human skin mast cells, and melanoma cells (Denys *et al.*, 1997; Ghannadan *et al.*, 1998; Kanekura *et al.*, 2002). Cell surface heparan sulfate was identified as an additional class of binding site for CypB, which is thought to be important for the adhesive properties of T cells (Allain *et al.*, 2002; Pakula *et al.*, 2007).

CsA is a powerful immunosuppressant drug that is an effective treatment for many inflammatory diseases including skin diseases such as psoriasis and eczema; however, its widespread and longer-term use is limited because of serious side effects that include nephrotoxicity and hypertension. In addition to the immunosuppressive action of CsA, there is evidence that CsA exerts effects on nonimmune cells such as epidermal keratinocytes. For example, CsA inhibits proliferation of keratinocytes and inhibits cutaneous inflammation in severe combined immunodeficient mice that lack functioning T cells (Fisher *et al.*, 1988; Reynolds *et al.*, 1998).

The proinflammatory properties of CypB, together with its high affinity for CsA, make it an interesting candidate for mediating some of the non-T-cell effects of CsA in skin. We now report that CypB is secreted by keratinocytes and that CsA enhances this secretion. We define that secretion in keratinocytes occurs via the constitutive secretory pathway. We demonstrate that mutation of amino-acid residue W128, which lies in the CsA-binding site of CypB, is sufficient to release CypB from the ER, thereby identifying a molecular ER retention mechanism that to our knowledge has not previously been reported.

RESULTS

CypB localization within the ER is modulated by CsA in human keratinocytes

Immunostaining with an antibody that recognizes a single 21-kDa band in whole-cell lysates of normal human epidermal keratinocytes (NHEKs; Figure 1a) showed that CypB was distributed throughout the cytoplasm in punctate and reticular structures consistent with localization to the ER (Figure 1b), as previously reported in other cell types (Arber *et al.*, 1992), and was also found in the nucleus (Figure 1b and c) (Schumacher *et al.*, 1994). To confirm localization of CypB to the ER in NHEKs, cells were transiently transfected with the specific ER marker Ds-RED-ER and immunostained. As previous studies had shown that CsA induces trafficking of CypB away from the ER (Price *et al.*, 1994), NHEKs transfected with Ds-RED-ER were treated for 6 hours with CsA (1 μM) or vehicle. The merged confocal image and the two-dimensional fluorocytogram in Figure 1c show colocalization of endogenous CypB with Ds-RED-ER in NHEKs, indicating localization of CypB within the ER. Following CsA treatment, the degree of colocalization between CypB with Ds-RED-ER was reduced (Figure 1c), consistent with the hypothesis that CsA induces trafficking of CypB out of the ER and into the secretory pathway (Price *et al.*, 1994).

CypB is secreted through the constitutive pathway in response to CsA

To investigate whether CypB was secreted by keratinocytes and whether CsA regulated this process as reported in other cell types (Price *et al.*, 1994), we analyzed conditioned media from NHEKs cultured with or without CsA. CypB was detected in the medium under basal conditions (Figure 2a). Notably, treatment of cells with CsA dramatically increased the amount of CypB secreted into the medium in a dose-dependent manner (Figure 2a). Concentrations of CsA as low as 1 pM mobilized CypB, and the levels of secreted CypB had not reached a plateau at 1 μM (Figure 2a). A concentration of 1 μM corresponds to the concentration of CsA detectable in the epidermis of patients with psoriasis during treatment with CsA, indicating that CypB is secreted by keratinocytes exposed to clinically relevant concentrations of CsA (Fisher *et al.*, 1988; Cooper *et al.*, 1992). To control for the possibility that CsA was causing cell death, resulting in the appearance of CypB in the medium, we performed a cytotoxicity assay following treatment of NHEKs with CsA for 24 hours that confirmed that no significant cell death was induced by CsA up to 1 μM (Figure 2d).

To determine whether CsA induced CypB secretion via the constitutive secretory pathway in keratinocytes, we used brefeldin A, an inhibitor of Arf activation that blocks post-Golgi membrane trafficking (Pelham, 1991; Klausner *et al.*, 1992; Ktistakis *et al.*, 1995). Pretreatment of NHEKs with brefeldin A substantially reduced the amount of CypB secreted into the medium in response to 1 μM CsA, as shown by western blotting (Figure 2c), indicating that CypB is secreted through the constitutive pathway.

Given that calcineurin mediates the effects of CsA in various cell types, including T cells and keratinocytes

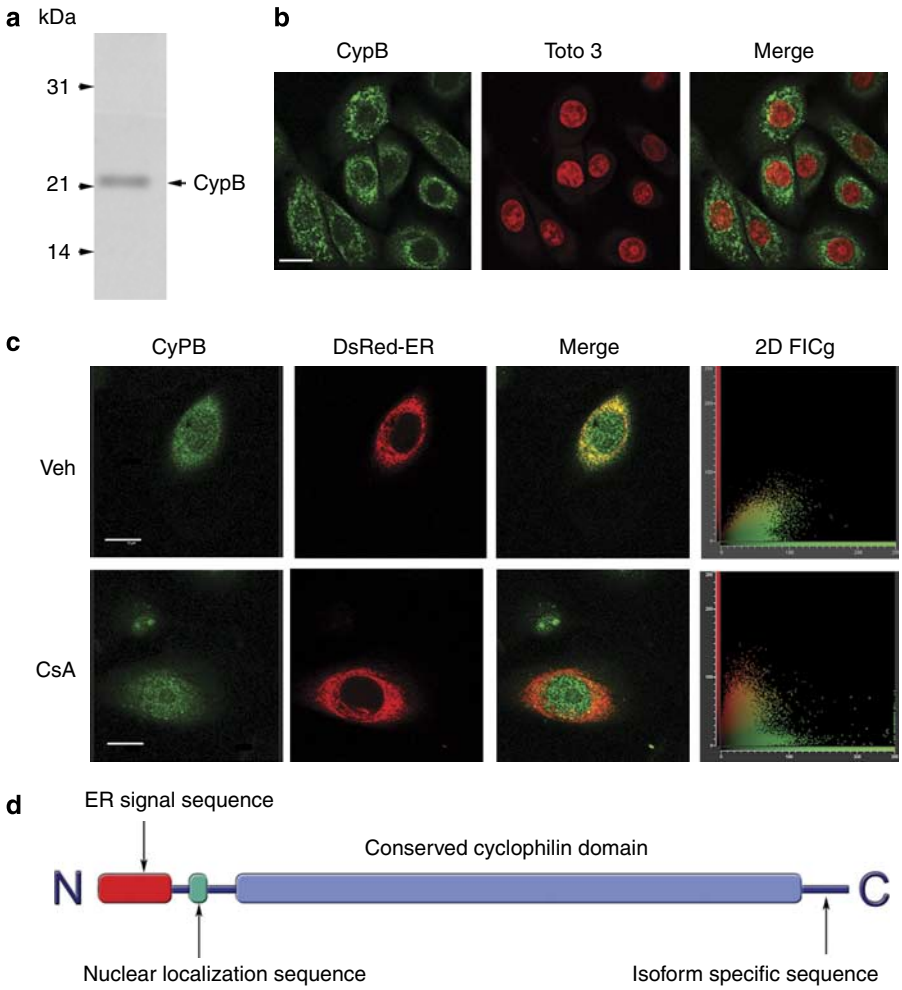


Figure 1. Expression of cyclophilin B (CypB) by human keratinocytes and modulation of endoplasmic reticulum (ER) localization by cyclosporine A (CsA). (**a**) Western blot of normal human epidermal keratinocyte (NHEK) whole-cell lysates using a polyclonal antibody to CypB shows a single band at 21 kDa. (**b**) Human keratinocytes were fixed, immunostained using anti-CypB antibody, labeled with Toto 3, and imaged by confocal microscopy. Scale bar = 46 μm. (**c**) Human keratinocytes transfected with DsRed-ER were treated with CsA (1 μM) or vehicle (DMSO) for 6 hours, fixed, immunostained for CypB, and visualized by confocal microscopy. Scale bar = 10 μm. Mid z sections are shown. Image analysis of red channel (dsRED-ER) and green channel (CypB) using two-dimensional fluorocytograms (2D FlCg) to demonstrate the degree of colocalization. (**d**) Schematic representation of CypB protein structure.

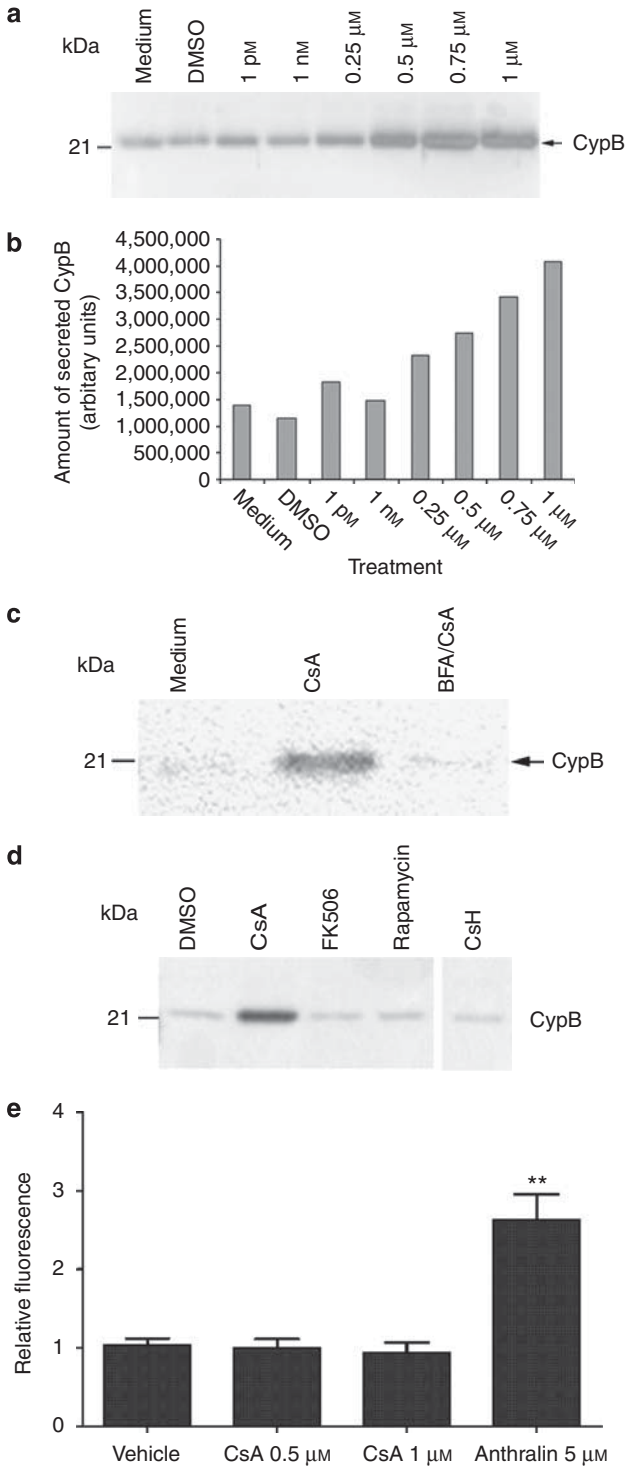
(Liu *et al.*, 1991; Al-Daraji *et al.*, 2002), we investigated the hypothesis that secretion of CypB is dependent on calcineurin inhibition. The calcineurin inhibitor FK506, which binds the immunophilin FKBP12, failed to induce secretion of CypB (Figure 2c) at doses known to suppress calcineurin activity (Enan and Matsumura, 1992; Al-Daraji *et al.*, 2002). No effect on CypB secretion was observed when NHEKs were treated with rapamycin, an immunosuppressive drug that binds to FKBP12, but whose downstream target is mammalian target of rapamycin rather than calcineurin. To control for the possibility that the effects of CsA on CypB secretion were because of a cyclophilin-independent mechanism, we tested cyclosporin H, a nonimmunosuppressive analog of CsA that lacks cyclophilin-binding activity. As predicted, cyclosporin H (1 μM) failed to promote secretion of CypB (Figure 2d).

To further characterize CypB within the secretory pathway, we made a full-length human CypB construct tagged at the C-terminus with green fluorescent protein ($CypB^{WT}$-GFP; Figure 3b, A). Western blotting following transient transfection with $CypB^{WT}$-GFP confirmed that the full-length fusion protein was expressed in NHEKs and secreted in response to CsA (Figures 3c and 4a), demonstrating that the presence of the GFP tag at the C-terminus of the protein did not interfere with CsA-induced secretion. Confocal imaging showed that $CypB^{WT}$-GFP was distributed in punctuate and reticular structures in the cytoplasm of NHEKs (Figure 3d) similar to endogenous CypB (Figure 1b), although $CypB^{WT}$-GFP showed less nuclear localization compared with the endogenous protein.

Retention of CypB in the ER is regulated in part through the CsA-binding site

We hypothesized that the localization of CypB to the ER may, in part, be mediated via the CsA-binding site. To address this, we made a, to our knowledge previously unreported, CypB-GFP construct in which alanine was substituted

for tryptophan at residue 128 in the CsA-binding site (CypB^{W128A}-GFP) (Figures 3a, 3b, B, and 4a), a mutation that reduces the interaction of CypB with CsA by 97% (Carpentier *et al.*, 1999), while only having a modest effect on its peptidyl-prolyl *cis-trans* isomerase activity (Carpentier *et al.*, 2000). We then investigated the impact of this mutation on the trafficking of CypB through the ER.



Colocalization parameters were assessed and quantified in HaCaT keratinocytes co-transfected with either CypB^{WT}-GFP or CypB^{W128A}-GFP and DsRed-ER and treated with vehicle or 1 μM CsA (Figure 4). The mean degree of colocalization between CypB^{WT}-GFP and DsRed-ER in untreated cells was high (mean Pearson's coefficient 0.79, mean overlap 0.83, $n=23$ cells, Figure 4b and d), confirming CypB localization to the ER. Colocalization parameters between CypB^{WT}-GFP and DsRed-ER fell following 1 μM CsA treatment for 6 hours (Figure 4d, mean Pearson's coefficient 0.70, mean overlap 0.75, $n=26$ cells), confirming dissociation of CypB from the ER. Cells co-transfected with CypB^{W128A}-GFP and DsRed-ER showed significantly less colocalization than CypB^{WT}-GFP and DsRed-ER (mean Pearson's coefficient 0.66, $P<0.005$, mean overlap 0.70, $n=26$ cells, $P<0.0002$, two-way analysis of variance; Figure 4d), supporting the notion that the CsA-binding site is important for CypB retention within the ER. As expected, colocalization parameters between CypB^{W128A}-GFP and DsRed-ER did not alter appreciably after treatment with CsA (Figure 4d, mean Pearson's coefficient 0.68, mean overlap 0.72, $n=25$ cells). Moreover, colocalization parameters in CypB^{WT}-GFP/DsRed-ER and CypB^{W128A}-GFP/DsRed-ER transfected cells showed a differential response to CsA treatment (interaction term: Pearson's coefficient $P<0.019$, overlap $P<0.021$; Figure 4d).

CypB diffusional mobility is regulated via the CsA-binding site

To further investigate the role of the CsA-binding site in the trafficking of CypB within keratinocytes, we studied the dynamics of CypB^{WT}-GFP and CypB^{W128A}-GFP motility before and after treatment with CsA by fluorescence recovery after photobleaching (FRAP) in HaCaT keratinocytes. Because of the continuous nature of the ER, photobleaching of GFP-tagged luminal ER proteins results in rapid and almost complete recovery (Brough *et al.*, 2005; del Toro *et al.*, 2006), reflecting their diffusability within the organelle (Figure 5a and b). On the other hand, when proteins enter secretory vesicles, their recovery profile following photobleaching is dependent on vesicle movement rather than free protein diffusion (Handley *et al.*, 2007; Kajimoto *et al.*, 2007) and is consequently slower. Therefore, we predicted that entry of CypB-GFP into secretory vesicles or an intermediate

Figure 2. Secretion of cyclophilin B (CypB) in human keratinocytes through the constitutive pathway in a dose-dependent manner in response to cyclosporin A (CsA). (**a**) Western blotting of conditioned medium collected from normal human epidermal keratinocytes (NHEKs) treated with the indicated concentrations of CsA or vehicle for 24 hours. (**b**) Quantification of western blot in **a**. (**c**) Keratinocytes were treated with medium control, 1 μM CsA alone, or pretreated with 2.5 μg ml^{-1} brefeldin A (BFA) for 30 minutes before 1 μM CsA treatment for 2 hours. (**d**) Keratinocytes were treated for 24 hours with DMSO, CsA (1 μM), FK506 (1 μM), rapamycin (1 μM), or cyclosporine H (CsH, 1 μM) as indicated. (**e**) Keratinocytes were treated with CsA (0.5 or 1 μM), DMSO, or anthralin (5 μM) for 24 hours and protease activity released into the medium measured using a Cytotox-Fluor assay. Anthralin served as a positive control. Data represent mean ± SEM ($n=9$ wells from three independent experiments). $^{**}P<0.001$ compared with vehicle, one-way analysis of variance (ANOVA).

compartment, induced by either CsA treatment or by mutation of residue 128, would result in reduced CypB mobility and incomplete recovery following photobleaching. Consistent with this, under basal conditions, the mean time required for half-maximal recovery of CypB^{WT}-GFP fluorescence ($t_{1/2}$) (Figure 5c and e) in HaCaT keratinocytes was 3.9 ± 0.7 seconds, whereas $t_{1/2}$ of CypB^{W128A}-GFP was significantly longer at 7.5 ± 0.9 seconds ($P<0.01$; $n\geqslant 9$ cells; Figure 5c and e). Similarly, the completeness of recovery, i.e., the mean mobile fraction (MF), was significantly greater at 72.8% ± 3.9% for CypB^{WT}-GFP compared with 46.1% ± 6.2% for CypB^{W128A}-GFP; ($P<0.05$; Figure 5c and f). We determined the effect of CsA on CypB mobility in these cells. CypB^{WT}-GFP but not CypB^{W128A}-GFP showed a significantly reduced rate of recovery in response to treatment with CsA (1 μM) for 6 hours (Figure 5c and d). Thus, CsA treatment reduced the mean $t_{1/2}$ and the mean MF of CypB^{WT}-GFP transfected cells to 6.1 ± 0.7 seconds ($P<0.05$ compared with untreated control; Figure 5c and e) and 54.5% ± 7.7% ($P<0.05$ compared with control; Figure 5c and f), respectively. In contrast, CsA (1 μM) treatment did not reduce the rate of recovery of CypB^{W128A}-GFP and mean $t_{1/2}$ and the mean MF was similar to control (Figure 5d). To exclude a potential effect of the vehicle DMSO, we compared FRAP of untreated (control) and DMSO-treated CypB^{WT}-GFP and CypB^{W128A}-GFP transfected cells, and no differences were detected (Supplementary Figure S1 online). These data indicate that the presence of a mutation at a key residue within the CsA-binding site significantly reduced the mobility of CypB-GFP following photobleaching, and that CsA reduced the mobility of CypB^{WT}-GFP but not CypB^{W128A}-GFP.

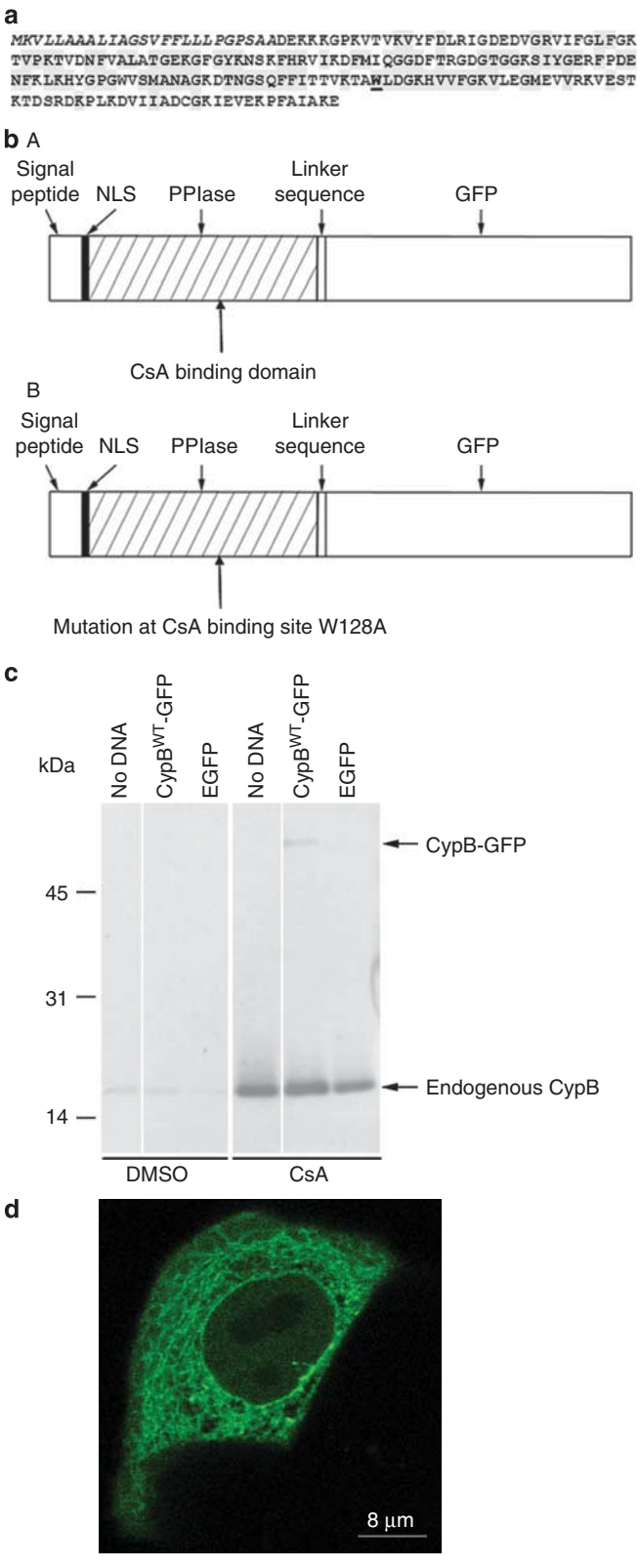


Figure 3. Localization of cyclophilin B-green fluorescent protein (CypB-GFP) and secretion in response to cyclosporin A (CsA) in keratinocytes. (**a**) The amino-acid sequence of CypB with conserved residues highlighted. The signal peptide is in italics, and W128 is underlined in bold. (**b**, A) Schematic diagram showing CypB and the location of the GFP tag. (**b**, B) Schematic diagram indicating the location of residue 128. PPIase, peptidyl prolyl *cis-trans* isomerase. (**c**) Normal human keratinocytes were transiently transfected with constructs as indicated and treated with either DMSO or CsA for 6 hours. Western blotting of conditioned medium with anti-CypB showed secretion of endogenous CypB in all samples and detection of a higher-molecular-weight band corresponding to CypB-GFP in transfected cells treated with CsA. (**d**) Keratinocytes were transiently transfected with CypB^{WT}-GFP, fixed, and then imaged by confocal microscopy. Scale bar = 8 μm. NLS, nuclear localization signal/sequence.

DISCUSSION

Along with regulating T-cell function, CypB is increasingly recognized to have an important intracellular and extracellular role in a number of tissues. In this paper we show that CsA mobilizes CypB from the ER and promotes CypB secretion in human keratinocytes, consistent with previous results in HeLa cells (Price *et al.*, 1994). As a number of cell types in skin, including keratinocytes, express CD147, our data suggest a possible paracrine role for CypB in skin and identify a further T cell-independent pathway that is regulated in keratinocytes by CsA. We confirmed that CypB localized to the ER in keratinocytes and characterized CsA-induced secretion of CypB using several approaches. First, we showed

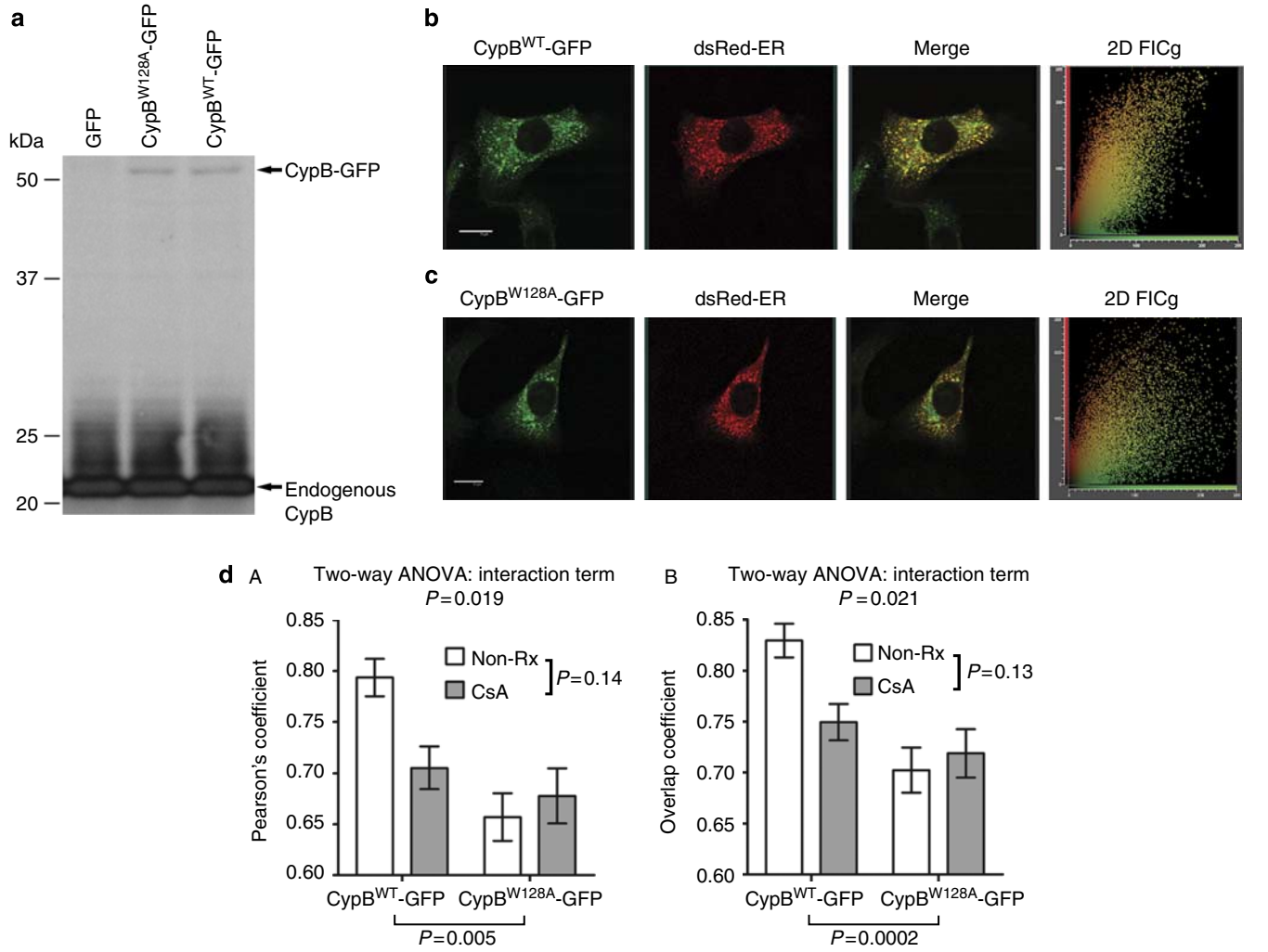


Figure 4. Retention of cyclophilin B (CypB) in the endoplasmic reticulum (ER) is regulated through the cyclosporin (CsA)-binding site. (**a**) Western blot of whole-cell lysates prepared from HaCaT keratinocytes transfected with EGFP, CypB^{W128}-GFP, or CypB^{WT}-GFP using a polyclonal antibody to CypB. (**b**, **c**) HaCaT keratinocytes were co-transfected with either (**b**) CypB^{WT}-GFP or (**c**) CypB^{W128A}-GFP and DsRed-ER, fixed, and imaged by confocal microscopy. Merge images and 2D fluocytograms (2D FlCg) indicate degree of colocalization that was greater between CypB^{WT}-GFP and DsRed-ER than between CypB^{W128A}-GFP and DsRed-ER. Scale bars = 17 μm (**b**) and 19 μm (**c**). (**d**) Colocalization parameters, (A) Pearson's coefficient and (B) overlap coefficients between DsRed-ER and CypB-GFP, were calculated using Volocity software across 10 z sections for individual cells in the CypB^{WT}-GFP ($n = 23$ cells) or CypB^{W128A}-GFP groups ($n = 26$ cells) and following treatment with CsA (1 μM) or control. Two-way analysis of variance (ANOVA) showed significant differences between colocalization parameters in CypB^{WT}-GFP and CypB^{W128A}-GFP groups. The interaction term confirms that colocalization parameters in CypB^{WT}-GFP/DsRed-ER and CypB^{W128A}-GFP/DsRed-ER transfected cells showed a differential response to CsA treatment.

that blocking ER to Golgi transport with brefeldin A prevented CsA-induced secretion of CypB. Second, we showed that CsA-induced secretion of CypB is independent of calcineurin inhibition. Next, we demonstrated that the degree of colocalization between CypB and ER markers decreased with time when the cells are treated with CsA, consistent with increased trafficking of CypB from the ER into a post-ER/Golgi compartment. Finally, using CypB^{WT}-GFP or a CypB^{W128A}-GFP construct in which a key residue at the CsA-binding site had been mutated, we showed that treatment with CsA or the W128A mutation reduced the intracellular motility of CypB. These data indicate a previously unreported role for the CsA site in retaining CypB within the ER, and suggest that disruption of the CsA-binding site either through treatment with CsA or through introduction of a mutation (W128A) leads to a redistribution of CypB from the ER compartment into secretory vesicles that are disconnected from the ER/Golgi network (del Toro *et al.*, 2006; Handley *et al.*, 2007; Kajimoto *et al.*, 2007).

CypB contains a N-terminal signal sequence (Price *et al.*, 1991) but lacks the classical KDEL ER retention motif (Munro and Pelham, 1987; Bose *et al.*, 1994). CypB forms multimeric complexes with other ER-resident proteins that include GRP94, protein disulfide isomerase, ERdj3, ERp72, BiP, and calreticulin (Klappa *et al.*, 1995; Horibe *et al.*, 2002; Meunier *et al.*, 2002; Zhang and Herscovitz, 2003), and it has been suggested that CypB is retained in the ER by direct interaction with such proteins (Arber *et al.*, 1992). Our data support the

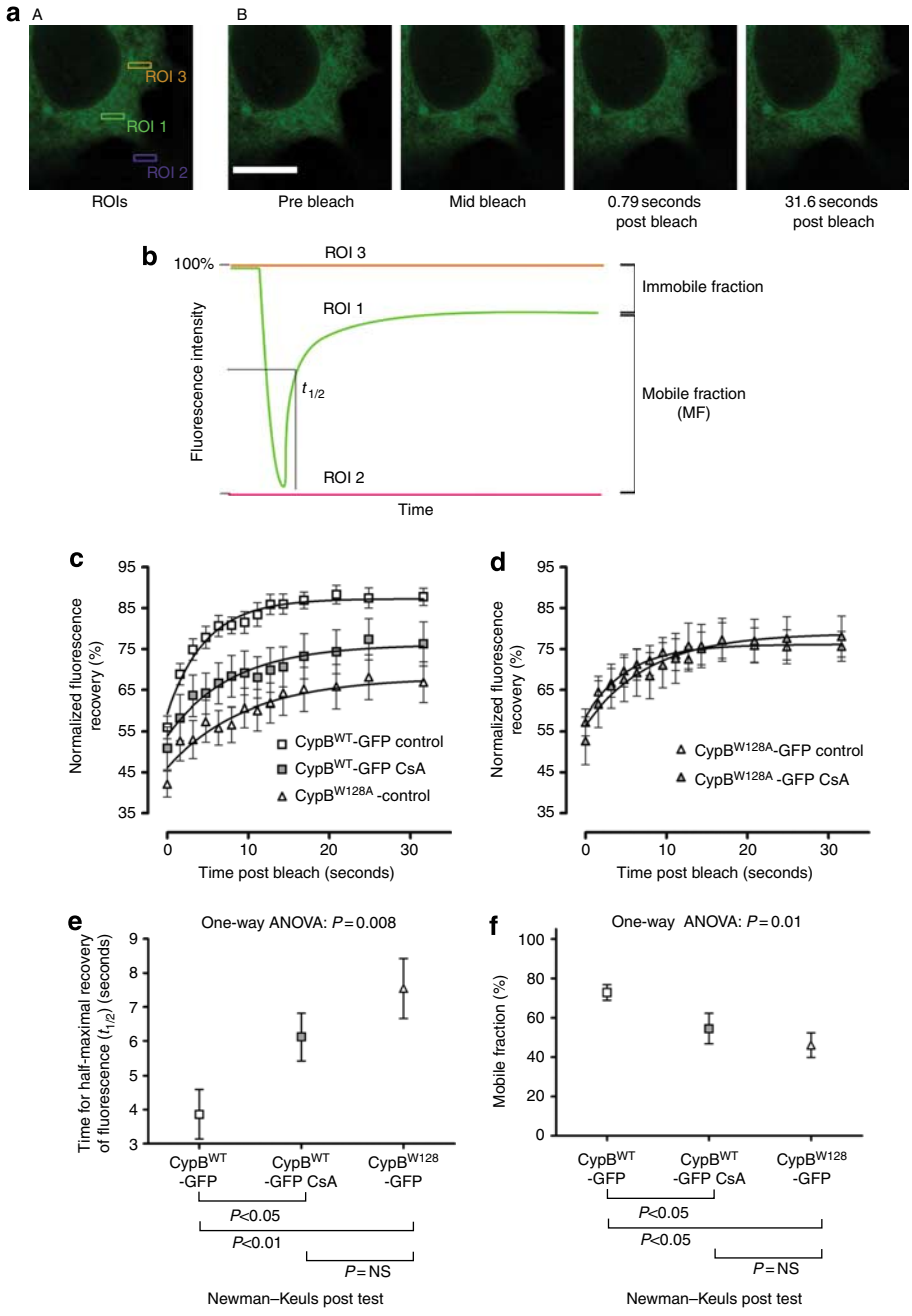


Figure 5. Cyclosporin (CsA) or mutation of the CsA-binding site alters the intracellular motility of cyclophilin B (CypB). Fluorescence recovery after photobleaching (FRAP) experiments were performed on HaCaT keratinocytes transfected with CypB^{WT}-green fluorescent protein (GFP) or CypB^{W128A}-GFP and then treated with 1 μM CsA or control for 6 hours as indicated. (**a**) (A) Region of interest 1 (ROI 1) corresponds to the 4 × 1 μm bleached area, ROI 2 corresponds to an area of background intensity, and ROI 3 is an area of the cell distant from the bleach site ROI 1. (B) Prebleach, mid-bleach, and postbleach images from FRAP experiment on HaCaT keratinocytes transfected with CypB^{WT}-GFP. Scale bar = 12 μm. (**b**) Schematic diagram to illustrate photorecovery curves for ROI 1, mobile and immobile factions, and $t_{1/2}$. (**c**, **d**) Time course of normalized photobleach recovery curves. Each graph represents experiments performed on the same day under uniform conditions. Each curve represents cumulative data from at least nine cells. (**e, f**) Data from **c** were analyzed using normalized values from the FRAP curves to derive $t_{1/2}$ and mobile fraction (MF). Data are representative of at least five independent experiments.

hypothesis that the CsA-binding site of CypB is required for retaining CypB within the ER. First, CypB-GFP containing a W128A mutation in the CsA-binding site (CypB^{W128A}-GFP) showed significantly less colocalization with an ER marker than wild-type CypB. Consistent with this, CsA reduced the colocalization of CypB^{WT}-GFP but not CypB^{W128A}-GFP with the ER. Furthermore, cyclosporin H, an optical isomer of CsA that lacks cyclophilin-binding activity (von Wartburg and Traber, 1986), failed to induce secretion of CypB, providing further evidence for the involvement of the CsA site in regulating CypB secretion. Moreover, the W128A mutation altered the speed and mobility of intracellular CypB,

suggesting that CypB had been directed out of the ER into secretory vesicles.

These data suggest that under normal physiological conditions CypB may bind other proteins via the CsA-binding site, that these interactions act to retain CypB in the ER, and that the W128A mutation disrupts the binding of CypB to its ER-binding partner(s) in a manner similar to the action of CsA. Interestingly, the decrease in $CypB^{W128A}$ mobility parallels the effect of the V66M mutation on brain-derived neurotrophic factor (del Toro *et al.*, 2006). Together, these results identify a molecular mechanism regulating localization, mobilization, and secretion of CypB within the cell that is to our knowledge previously unreported. Along with interfering with CsA binding, substitution of W128 in CypB significantly reduced the affinity of the protein for the type I receptor-binding sites on Jurkat T cells (Carpentier *et al.*, 1999), further supporting the concept that the CsA-binding sites may regulate the ability of CypB to bind other proteins, including within the ER. The specific binding partners that are disrupted by CsA or mutation of the CsA-binding site remain to be determined.

Increasing literature suggests that CypB may function as a proinflammatory mediator (Yurchenko *et al.*, 2001; Allain *et al.*, 2002; Arora *et al.*, 2005). Serum CypB levels were found to be elevated in patients with severe sepsis, and elevated peptidyl-prolyl *cis-trans* isomerase activity was associated with high mortality (Tegeder *et al.*, 1997). In addition, CypB is secreted by chondrocytes and released from cartilage explants (De Ceuninck *et al.*, 2003) and may thereby contribute to innate immune reactions. We have shown that CypB is highly expressed in the epidermis from patients with inflammatory skin diseases such as eczema and psoriasis (P. Featon and N.J. Reynolds, unpublished observations). CypB has been reported to exhibit proinflammatory properties via signaling through CD147. It may, therefore, at first sight seem paradoxical that CsA, an immunosuppressive drug, promotes secretion of a chemotactic molecule. However, CsA binds CypB with high affinity and it is likely that the CypB/CsA complex traverses the secretory pathway together, rendering CypB inactive; moreover, it has been shown that CsA inhibits the chemotactic properties of CypB *in vitro* (Allain *et al.*, 2002), so that CsA induces secretion of CypB, whereas the CypB/CsA complex may lack chemotactic activity. As extracellular levels of CypB correlate with enhanced susceptibility of patients to immunosuppression by CsA (Denys *et al.*, 1998), another possible explanation is that extracellular CypB targets CsA to lymphocytes or keratinocytes expressing CypB cell surface receptors. Further work is therefore required to establish functional links between CypB, inflammatory skin disease, and the therapeutic efficacy of CsA.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were obtained from Sigma.

Generation of GFP constructs

Full-length human CypB was amplified from keratinocyte complementary DNA and cloned into the pEGFP-N1 vector between the *Hin*dIII and *Bam*HI restriction sites to generate C-terminal fusion proteins (Clontech Laboratories, Mountain View, CA). To generate the $CypB^{W128A}$-GFP construct, a region containing the W128A mutation was subcloned from the vector $CypB^{W128A}$ (Carpentier *et al.*, 1999). The insert was amplified using the same primers used to generate full-length wild-type CypB; the PCR product was cloned into CypB-GFP between the *Apa*I and *Bam*HI sites, thus replacing the wild-type CsA-binding site with the mutated one. DsRed-ER was from Clontech Laboratories.

Cell culture and transfection

Keratinocytes were isolated from redundant normal human skin following approval by the Newcastle and North Tyneside local ethical committee with informed written consent and cultured in low-calcium (70 μM) MCDB-153 medium as described previously (Todd and Reynolds, 1998). The spontaneously immortalized keratinocyte cell line (HaCaT) was cultured in high glucose DMEM supplemented with penicillin (5 U ml^{-1}), streptomycin (5 μg ml^{-1}), and 10% fetal calf serum (Boelsma *et al.*, 1999). Keratinocytes were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA) and HaCaTs were transfected using either FuGene 6 (Roche) or Lipofectamine Plus (Invitrogen, Paisley, Scotland), according to the manufacturers' instructions.

Immunofluorescence, antibodies, and dyes

Anti-CypB antibodies used in Figure 1b and c are as described previously (Allain *et al.*, 1995). Anti-CypB antibodies used for immunostaining in Figure 1a were from Fisher (Loughborough, UK). The secondary antibody used for immunofluorescence was Oregon Green 488 anti-rabbit (Invitrogen). To identify the nucleus, cells were incubated in propidium iodide (250 ng ml^{-1}) or Toto 3 (20 nM) with RNAse (1 mg ml^{-1}). Mouse anti-GFP was from Clontech Laboratories.

Confocal microscopy and FRAP analysis

Fluorescence micrographs were acquired with a Leica confocal scanning microscope (Leica Microsystems, Milton Keynes, UK) equipped with an argon ion laser (with lines at 458, 476, 488, and 514 nm) and two HeNe lasers (with lines at 543 and 633 nm, respectively). The images were obtained with a ×63 Plan Apo NA 1.32 oil immersion lens mounted on an inverted Leica DM IRBE microscope. Z series of images were captured line by line using sequential scanning mode to minimize cross-talk between chromophores. Mid z section images are shown. Live cell imaging was performed at 37 °C. Colocalization parameters, Pearson's coefficients, and overlap coefficients were calculated using Volocity software (Improvision, Coventry, UK). Images were processed in Adobe Photoshop 6 (Edinburgh, Scotland). FRAP studies were performed in fly-by mode on a 256 × 256 pixel region, with the scan speed set to 1,000 Hz and the zoom function set to 8. Prebleach images (3–5) were captured with the 488 nm laser attenuated to 2%. A 4 × 1 μm region was then bleached three times with the 488, 476, and 458 nm laser lines set to 100%. Fluorescence intensities, quantified/determined from three regions of each cell as described (Ferreri-Jacobia *et al.*, 2005), were collected using Leica software. Normalized fluorescence recovery (F) for bleached area (region of interest (ROI) 1) was calculated using Excel according to: $F = 100 \times ((ROI\ 1 - ROI\ 2)/(ROI\ 3 - ROI\ 2))*((ROI\ 3_i - ROI\ 2)/(ROI\ 1_i - ROI\ 2))$

where i is the initial fluorescence intensity for the ROI, ROI 2 is background fluorescence, and ROI 3 represents a control area distant from ROI 1. Normalized data were plotted against time using Prism (version 4.0; Graphpad Software, La Jolla, CA). Analysis of normalized values from the fluorescence recovery curves was performed and $t_{1/2}$ and MF derived from the curves according to: $MF = 100 \times (nROI\ 1_{\vartheta} - nROI\ 1_0)/(100 - nROI\ 1_0)$ where nROI is the normalized value for the ROI, ϑ denotes the asymptote (plateau) of the recovery curve, and 0 represents the fluorescence intensity immediately following bleaching (i.e., at time = 0); $t_{1/2} = \ln 0.5/k$ where k, the rate constant, was obtained by curve fitting and solving the nonlinear regression equation: $F = (nROI\ 1_{\vartheta} - ROI\ 1_0)*(1 - \exp(-kx)) + nROI\ 1_0$ where x = time.

Preparation of samples for western blotting

Conditioned keratinocyte medium was concentrated using Amicon concentrators (Millipore UK, Watford, UK) before western blotting. Equal quantities of proteins were separated on pre-cast 4–20% Novex Tris-Glycine gels (Invitrogen, Paisley, UK), and then transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Following incubation in primary antibody, membranes were incubated in avidin and biotinylated horseradish peroxidase macromolecular complex reagents (Vector Laboratories, Peterborough, UK) before incubation in secondary antibody. Membranes were developed using ECL Plus reagent (Amersham Pharmacia Biotech) and images collected using the Storm PhosphorImager (GE Healthcare Life Sciences, Little Chalfont, UK) using the blue fluorescence mode. Subsequent analysis of images was carried out using ImageQuant software (Version 3.3, GE Healthcare Life Sciences) and PhotoShop 6 (Adobe, San Jose, CA).

Cytotoxicity assay

We utilized the Cytotox-Fluor assay (Promega, Southampton, UK) that measures protease activity released from cells that have lost plasma membrane integrity using a fluorogenic peptide substrate (bis AAF-R110). Signal intensity was measured using a Cary Eclipse fluorometer (Varian, Southampton, UK) using an excitation wavelength of 485 nm and emission wavelengths of 520 ± 2.5 nm.

Statistical analysis

Data presented are means ± SEM. Statistical significance was taken as $P < 0.05$. Data analysis was carried out using Microsoft Excel (Microsoft, Redmond, WA); curve fits and statistical analyses were performed using Prism 4. Data from cytotoxicity assay and FRAP experiments were compared using one-way analysis of variance. If P-value summary showed statistical significance, Newman–Keuls post tests were performed. Two-way analysis of variance was used to compare colocalization parameters from all four experimental groups.

CONFLICT OF INTEREST

NJR receives research grant support from Stiefel, a GSK Company, and Astra Zeneca through a BBSRC CASE award. All other authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from the British Skin Foundation (BSF), North Eastern Skin Disease Research Fund, and the Wellcome Trust. NJR was supported by a Research Leave Fellowship to Clinical Academics from the Wellcome Trust. PF was supported by a Studentship from the BSF and AS is supported by a fellowship from the BSF. NJR's laboratory/research is supported by the NIHR-Newcastle Biomedical Research Centre. We thank the Departments of Paediatric Surgery, Plastic Surgery, and Urology, Newcastle Upon Tyne Hospitals, NHS Foundation Trust, for their help in recruiting patients.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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