The EMBO Journal Vol.18 No.9 pp.2472–2479, 1999

Impaired neutral sphingomyelinase activation and cutaneous barrier repair in FAN-deficient mice

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The WD-40 repeat protein FAN binds to a distinct domain of the p55 receptor for tumor necrosis factor (TNF) and signals the activation of neutral sphingomyelinase (N-SMase). To analyze the physiological role of FAN *in vivo*, we generated FAN-deficient mice by targeted gene disruption. Mice lacking a functional FAN protein do not show any overt phenotypic abnormalities; in particular, the architecture and cellular composition of lymphoid organs appeared to be unaltered. An essential role of FAN in the TNF-induced activation of N-SMase was demonstrated using thymocytes from FAN knockout mice. Activation of extracellular signal-regulated kinases in response to TNF treatment, however, was not impaired by the absence of the FAN protein. FAN-deficient mice show delayed kinetics of recovery after cutaneous barrier disruption suggesting a physiological role of FAN in epidermal barrier repair. Although FAN exhibits striking structural homologies with the CHS/Beige proteins, FAN-deficient mice did not reproduce the phenotype of *beige* mice.

Keywords: Beige/Chediak–Higashi syndrome/ERK/FAN/neutral sphingomyelinase

Introduction

Tumor necrosis factor (TNF) activates several signal transduction pathways that mediate cytotoxicity, antiviral activity, stimulation of cell growth and a plethora of inflammatory and immunoregulatory responses (Beutler and Cerami, 1986; Fiers, 1991; Heller and Kronke, 1994; for review see Goeddel *et al*., 1986). The cellular responses to TNF are mediated by two distinct receptors of 55 kDa (TNF-R55) and 75 kDa (TNF-R75) molecular mass, respectively. Major advances in understanding TNF-R55 signaling were achieved by identification of functionally distinct cytoplasmic domains of the receptor (Brakebusch *et al*., 1992; Tartaglia *et al*., 1993; Wiegmann *et al*., 1994; Adam *et al*., 1996). An 80 amino acid domain (death domain) is responsible for the initiation of programmed cell death (PCD), activation of c-Jun N-terminal kinases (JNK) and the transcription factor NF-κB (Tartaglia *et al*., 1993; Smith *et al*., 1994). A number of important signaling events originate from the TNF-R55 death domain involving the recruitment of distinct receptor-associated proteins. Whereas TRADD, FADD and Caspase-8 (FLICE/MACH) mediate PCD (Hsu *et al*., 1995; Rothe *et al*., 1995a,b), RIP was shown to be necessary for NF-κB activation (Kelliher *et al*., 1998). The recruitment of TRAF-2 to TRADD promotes the activation of JNK (Yeh *et al*., 1997).

TNF not only induces cytotoxic responses, but also leads to proliferation in several cell lines and tissues. The cytoplasmic domain of TNF-R55 responsible for proliferation remained elusive, yet is possibly distinct from the death domain. One other functional domain, NSD, localizes N-terminally adjacent to the death domain, and signals the transient activation of a neutral sphingomyelinase operating at the plasma membrane (N-SMase) (Adam *et al*., 1996). Activation of N-SMase leads to accumulation of ceramide, an important lipid second messenger molecule. We have recently identified a novel WD-repeat protein, FAN, which binds to the NSD and mediates the activation of N-SMase (Adam-Klages *et al*., 1996).

Structure–function analysis of FAN confined the receptor binding property of the protein to its C-terminal cluster of WD repeats. Overexpression of this domain resulted in a marked dominant-negative effect on TNF-induced activation of N-SMase, while expression of full-length FAN enhanced the TNF-dependent N-SMase activation (Adam-Klages *et al*., 1996). Besides its role in TNF-induced activation of N-SMase, possible further signaling functions of FAN remained elusive. Members of the family of WD-40 (or WD-repeat) proteins include mainly regulatory proteins, several of which are involved in signal transduction events. WD-repeat proteins, like PLAP or RACK1, were identified as activators of phospholipase A_2 (PLA_2) (Clark *et al*., 1991) and protein kinase C, respectively (Ron *et al*., 1994). Most recently, Apaf-1 has been shown to activate Caspase-3 (Zou *et al*., 1997). Despite a considerable heterogeneity with regard to size and biochemical properties among the members of the WD-repeat protein family, the tertiary structure of the WD-repeat clusters seems to be strongly conserved (Sondek *et al*., 1996).

In order to investigate the possible involvement of FAN in signaling processes and to evaluate its *in vivo* functional significance, we generated a strain of FAN-deficient mice. We show here that mice with a FAN null mutation are healthy and fertile with no gross phenotypic abnormalities.

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This is in contrast to mouse strains with null mutations of proteins associated with death domain signaling of TNF-R55, which produce lethality neonatally or already during embryogenesis (Yeh *et al*., 1997; Kelliher *et al*., 1998). Evidence is provided that cells from FAN-deficient mice are defective in TNF-induced N-SMase activation. *In vivo*, FAN-deficient mice show a delayed cutaneous barrier repair, suggesting a previously unrecognized role of FAN and/or N-SMase in epidermal homeostasis.

Results

Targeted disruption of the murine fan gene

Genomic clones of the murine *fan* locus were obtained by screening a mouse 129/Sv genomic library using murine *fan* cDNA as a probe. The *fan* gene consists of at least nine exons encompassing a minimum of 16 kb (Figure 1A). To disrupt the murine *fan* gene in E14.1 embryonic stem (ES) cells by homologous recombination, a replacement-type targeting vector was designed, that would disrupt and partially replace exon 2 of the *fan* gene with the *neo* gene encoding neomycin phosphotransferase. Heterozygous ES clones containing a disrupted *fan* allele were aggregated with morulas obtained from superovulated CD1 females. Chimeric animals with germline transmission of the mutant allele were used to generate $FAN^{+/-}$ mice. Heterozygous animals were backcrossed to C57BL/6J animals and further interbred to obtain homozygosity.

Approximately 50% of the offspring that derived from mating heterozygous animals with wild-type C57BL/6J animals were heterozygous, as determined by Southern blot and PCR analysis of tail DNA (Figure 1B). The absence of FAN mRNA expression in $FAN^{-/-}$ mice was confirmed by RT–PCR using RNA from fibroblasts and thymocytes (Figure 1C). The progeny of heterozygous matings resulted in a normal Mendelian ratio of FAN mutants (Figure 1D). The litter size from homozygous FAN-deficient mouse matings was similar to heterozygous crosses with a male to female ratio of 1:1 (data not shown). These results demonstrate that inactivation of the *fan* gene does not cause any gross abnormalities in general health and reproduction of mice.

In TNF-R55-deficient mice, alterations of lymphoid organ development have been reported. We therefore investigated whether FAN might be necessary for the organogenic functions of TNF-R55. As judged by histological analysis of various tissues including brain, heart, lung, liver, kidney, spleen, mesenteric lymph nodes and Peyer's patches, no gross abnormalities were detectable (data not shown). In particular, Peyer's patches were present in normal numbers in FAN-deficient mice. The FAN protein therefore seems not to be essential for the development of the investigated lymphoid organs. Furthermore, the leukocyte composition of peripheral blood, peritoneal lavage, bone marrow, mesenteric lymph nodes, spleen and thymus was examined by flow cytometry. As shown in Table I, analysis of cell surface antigens revealed an inconspicuous cellular composition and distribution of $FAN^{-/-}$ tissues; in particular, T- and B-lymphocytes, and natural killer (NK) cells were present at normal frequency.

Selective requirement of FAN for TNF-induced activation of neutral sphingomyelinase

FAN has been functionally defined to mediate the activation of a TNF-responsive N-SMase (Adam-Klages *et al*., 1996). As shown in Figure 2A, N-SMase activation was completely abrogated in TNF-treated thymocytes from $FAN^{-/-}$ mice indicating a central role for the FAN protein in TNF-dependent N-SMase activation. Notably, N-SMase activation in $FAN^{+/-}$ thymocytes was significantly reduced when compared with $FAN^{+/+}$ thymocytes, suggesting that FAN represents a rate-limiting factor for the TNF-induced pathway of N-SMase activation. Similar results were obtained with splenocytes (data not shown). In contrast, the activation of A-SMase in TNF-treated $FAN^{-/-}$ thymocytes was completely intact (Figure 2B). Like N-SMase, the activation of proline-directed protein kinases, was found to emanate from a cytoplasmic region of TNF-R55 distinct from the death domain (Wiegmann *et al*., 1994). In order to investigate a possible functional connection to extracellular signal-regulated kinases (ERK), we analyzed the TNF-induced activation of ERK1/2 in $FAN^{-/-}$ embryonic fibroblasts (EF cells). As shown in Figure 2C, after treatment of wild-type and FAN-deficient fibroblasts with TNF or platelet-derived growth factor (PDGF), a significant activation of ERK1/2 was observed. Therefore, FAN does not appear to play a critical role in TNF-induced activation of ERK1/2.

Finally, we investigated a possible contribution of FAN in apoptotic signaling. The apoptotic response of $FAN^{-/-}$ thymocytes to TNF in combination with cycloheximide, anti-CD95 and dexamethasone did not reveal any differences compared with $FAN^{+/+}$ control cells (data not shown). Thus, the apoptotic pathway initiated by TNF-R55 crosslinking seems to be completely unimpaired in FAN knockout mice.

Role of FAN in cutaneous barrier repair

TNF-R55 promotes skin permeability barrier repair involving sphingomyelinases (J.M.Jensen, S.Schutze, M.Kronke and E.Proksch, submitted). To investigate a possible involvement of FAN, the permeability barrier was destroyed by tape stripping and the repair process was monitored by determinations of *trans*-epidermal water loss (TEWL). The basal level of TEWL in FAN-deficient mice was slightly increased ($FAN^{-/-}$, 13.2 $\pm$ 0.9 nmol/h/mg protein; $FAN^{+/+}$, 9.9 $\pm$0.6 nmol/h/mg protein; $p < 0.001$, $n = 18$). After artificial barrier disruption, FAN-deficient mice showed a significant delay in barrier repair 1–24 h after treatment, which was most pronounced at 7 h (32%, $p < 0.001$, $n = 6$) (Figure 3A). This delay suggests an involvement of FAN, and possibly N-SMase activation, in cutaneous barrier function and repair.

Since the repair process of the cutaneous barrier after an artificial disruption leads to epidermal proliferation, we analyzed whether FAN knockout mice show epidermal proliferation during barrier repair. As shown in Figure 3B, the fraction of bromodeoxyuridine (BrdU) positive cells after cutaneous barrier disruption was significantly reduced in FAN-deficient mice. Taken together, these data suggest an involvement of FAN and possibly stimulated N-SMase in cutaneous barrier function.

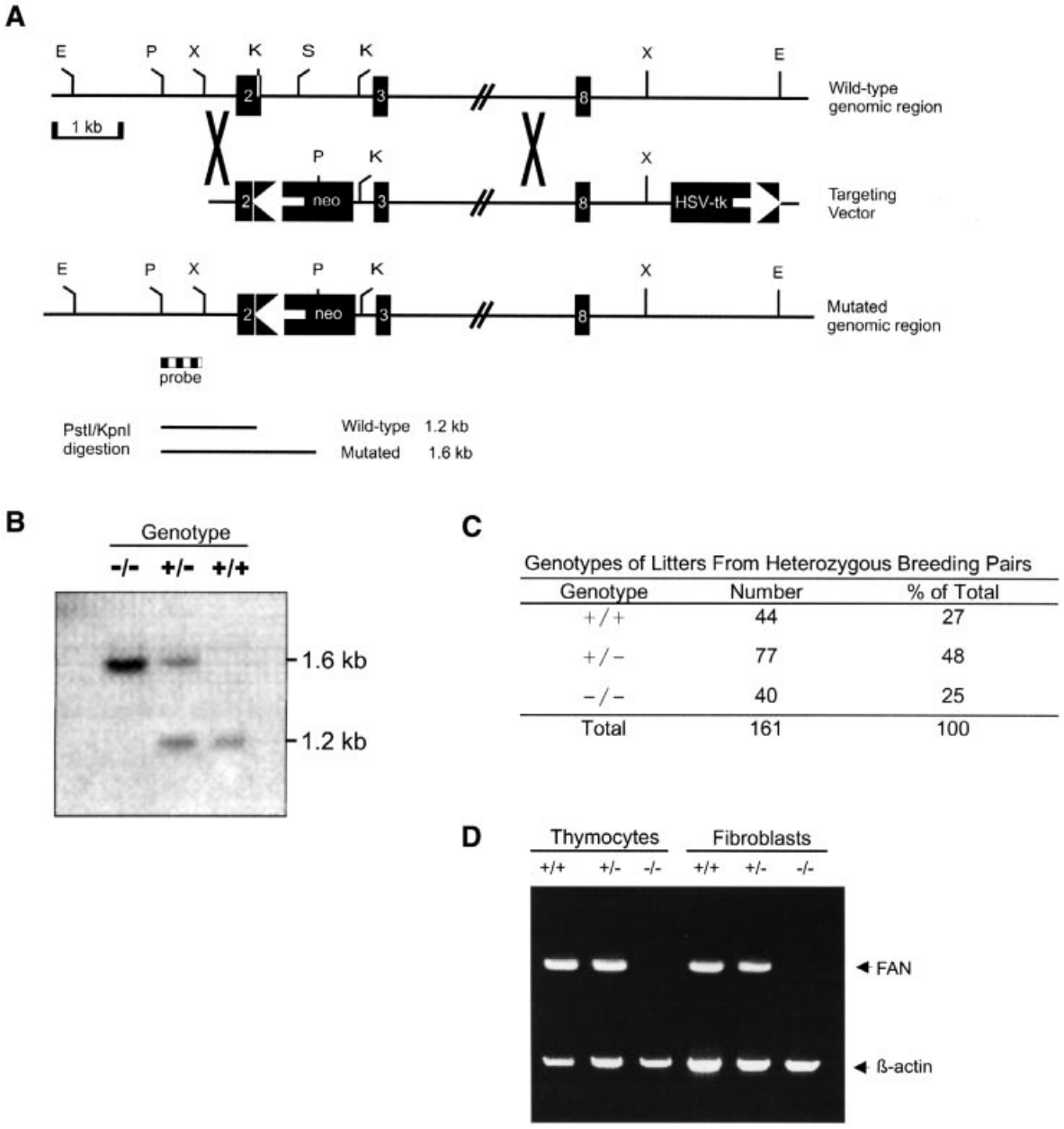

Fig. 1. Targeted disruption of the murine *fan* gene. (**A**) Strategy for inactivation of the *fan* gene by homologous recombination in ES cells. A portion of the endogenous *fan* locus containing three exons (depicted by numbered boxes, top), the targeting construct (middle) and the predicted mutated genomic locus (bottom). Introns are indicated by a solid line. The striped box indicates the DNA probe used for Southern blot analysis. 7.7 kb homology to the *fan* gene locus were disrupted by insertion of the neo-cassette into exon 2. A HSV-tk coding cassette was added for negative selection of non-specific recombination. The arrows mark direction of transcription of neo and HSV-tk cassettes. Introduction of an additional *Pst*I site by the neo-cassette and removal of a *Kpn*I site of exon 2, which was used to detect the mutant locus. The DNA probe hybridizes to 1.2 kb *Pst*I–*Kpn*I fragment in wild-type and a 1.6 kb *Pst*I fragment in mutated alleles, respectively. Restriction sites: E, *Eco*RI; P, *Pst*I; X, *Xba*I; K, *Kpn*I; S, *Spe*I. (**B**) Southern blot analysis of tail DNA from littermates from an intercross of FAN heterozygotes. DNA was harvested from tail probes, digested with *Kpn*I and *Pst*I and analyzed with the flanking probe. (**C**) Number and percentage of homozygous wild-type (+/+), heterozygous (+/–) and homozygous mutant (–/–) pups from a heterozygous breeding pair. (**D**) RT–PCR using RNA isolated from thymocytes and tail fibroblasts from $FAN^{+/+}$, $FAN^{+/-}$ and $FAN^{-/-}$ mice demonstrated absence of a functional FAN transcript in FAN-deficient mice.

Intact NK and CTL activities in FAN-deficient mice

We have recently reported that FAN shares with Chediak–Higashi syndrome (CHS)/Beige proteins a so-called BEACH domain (beige and Chediak–Higashi syndrome) (Adam-Klages *et al.*, 1998). As shown in Figure 4, the BEACH domain of FAN shows significant homologies to the corresponding domains of CHS and human CDC4-like (CDC4L) proteins. Mutations of the *chs1* gene represent the etiology of CHS, a rare autosomal disease in which cytotoxic T lymphocyte (CTL) and NK functions are severely impaired. The human *chs1* gene is homologous to the mouse *beige* gene, which is responsible for the beige phenotype, the murine counterpart of CHS. The homology between FAN and CHS prompted us to investigate whether FAN fulfills similar functions *in vivo*. However, the activity of NK cells isolated from $FAN^{-/-}$ mice against YAC-1 target cells was unchanged when compared with $FAN^{+/+}$ NK cells (Figure 5A). As

Table I. Cellular composition in lymphoid tissues of fan$^{-/-}$ mice

	Experiment 1		Experiment 2	
	fan$^{+/+}$	fan$^{-/-}$	fan$^{+/+}$	fan$^{-/-}$
Spleen				
CD4	20.3	19.3	21.1	21.0
CD8	11.4	8.5	6.4	6.9
B220	52.5	59.5	58.7	63.5
NK1.1	6.4	8.1	4.9	6.1
Thymus				
CD4	94.1	93.2	93.6	94.7
CD8	85.1	84.9	84.2	83.5
Lymph node				
CD4	34.2	39.4	41.2	33.0
CD8	45.1	61.3	29.8	16.1
B220	27.1	45.3	31.6	28.7

Single-cell suspensions of the tissues indicated were prepared from wild-type and FAN-deficient mice. Cells were stained using monoclonal antibodies specific for the cell surface markers indicated and analyzed by flow cytometry. Results are expressed as percentages of positive cells.

illustrated in Table I, the number of NK1.1 positive NK cells in FAN$^{-/-}$ mice was also comparable with wild-type mice. In addition, CTL exhibited normal cytotoxicity (Figure 5B and C), suggesting that FAN does not play a critical role in NK cell or CTL function.

CHS patients and beige mice show a severe coagulation disorder which manifests as prolonged bleeding after wounding. When experimental bleeding experiments or *in vitro* tests for extrinsic or exogenous phase of coagulation were performed with FAN knockout animals, no coagulation defect was detected in FAN$^{-/-}$ mice when compared with FAN$^{+/+}$ animals (data not shown). Taken together, these findings indicate that FAN-deficient mice do not share the phenotype of *beige* mice with regard to defective NK cells and cytotoxic T cells or coagulation.

Discussion

In this study we report the generation and characterization of FAN$^{-/-}$ mice. These mice develop normally, are fertile, and show no obvious signs of disease until at least 40 weeks of age. However, FAN-deficient mice displayed a functional phenotype including unresponsiveness of N-SMase in TNF-treated cells and delayed cutaneous barrier repair. This confirms our previous observation that FAN is critically involved in the activation of N-SMase in TNF-treated 293 and COS cells. When compared with wild-type or homozygous mice, cells from heterozygous FAN$^{+/-}$ mice showed an intermediate N-SMase response to TNF, suggesting that FAN might be a rate limiting factor for the N-SMase activation pathway. The indispensability of FAN for TNF-induced N-SMase activation allowed the utilization of FAN$^{-/-}$ cells to investigate the possible role of N-SMase in TNF signaling. We have previously proposed a signaling pathway consisting of the subsequent activation of N-SMase, ERK1/2 and cPLA$_2$ (Wiegmann *et al*., 1994), which was thought to be regulated by FAN (Adam-Klages *et al*., 1996). We show here, however, that the kinetics and the amplitude of ERK1/2 activation remained unchanged in TNF-treated cells from

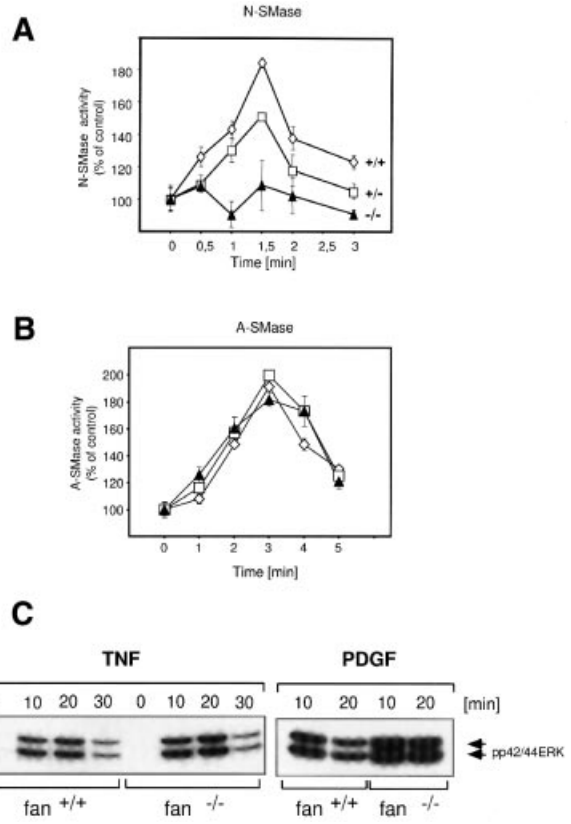

Fig. 2. Selective blockade of N-SMase activation in cells from FAN-deficient mice. (**A**) Thymocytes of homozygous wild-type (◇), heterozygous (□) and homozygous (▲) knockout mice were treated in triplicates with 100 ng/ml TNF for indicated times, and N-SMase activity was determined. TNF-induced N-SMase activities are expressed as percent of untreated controls of each genotype. The results are representative for three independent experiments. (**B**) Thymocytes of homozygous wild-type (◇), heterozygous (□) and homozygous (▲) knockout mice were treated in triplicates with 100 ng/ml TNF for the indicated times, and A-SMase activity was determined. TNF-induced A-SMase activities are expressed as a percentage of untreated controls of each genotype. The results are representative for three independent experiments. (**C**) EF cells from wild-type and homozygous FAN knockout mice were treated with 100 ng/ml TNF or 10 ng/ml PDGF for the indicated times. Cells were lysed and cellular lysates were analyzed by immunoblotting with phosphospecific anti-p42/44 ERK antibodies the presence of activated ERK.

FAN-deficient mice. Thus, the lack of FAN appears to uncouple the activation of N-SMase, but not that of ERK1/2 in TNF-stimulated cells.

One possible *in vivo* consequence is that the lack of FAN might contribute to the delayed kinetics of cutaneous barrier repair. Previously, we showed that TNF-R55 and N-SMase are involved in epidermal permeability barrier repair (J.M.Jensen, S.Schutze, M.Kronke and E.Proksch, submitted). The results of the present study reveal that the basal level of TEWL was already increased in untreated FAN-deficient mice, indicating a constitutive defect in barrier function. Thus, the FAN protein through mediating TNF-induced N-SMase activity may regulate cutaneous barrier homeostasis. After disruption of the cutaneous permeability barrier, the kinetics of the repair were significantly reduced over the entire time course monitored. More work is required to delineate the precise role of FAN for these noncytocidal effects of TNF-R55.

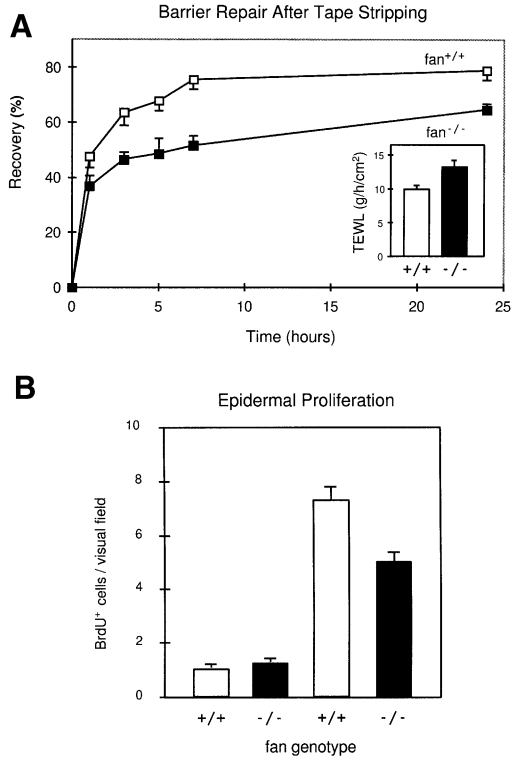

Fig. 3. FAN is important for TNF-mediated barrier repair. (**A**) The basal level of TEWL was increased in FAN-deficient mice when compared with wild-type mice (inset). The kinetic of barrier repair was measured as TEWL after tape stripping. Recovery from barrier disruption (decrease of TEWL) was significantly delayed in FAN-deficient mice with a maximal difference at 7 h. (**B**) Epidermal proliferation after tape stripping. The number of $BrdU^+$ epidermal cells increased significantly after tape stripping (right bars) when compared with basal levels (left bars). The number of $BrdU^+$ FAN-deficient epidermal cells was significantly lower than proliferation of wild-type cells.

Occasionally, the phenotype of knockout mice has been related to the disruption of the expression of neighboring genes (Olson *et al*., 1996). So far, the known examples for such a 'neighborhood effect' are derived from a multigene cluster (Olson *et al*., 1996; Pham *et al*., 1996). The exact locus of the human *fan* gene has been mapped to human chromosome region 8q12–q13 (D.Kreder and M.Kronke, unpublished results). The closest neighboring genes to human *fan* known at present are *cytochrome p450VII* and the gene for the scaffold protein PBP-1. Since the nearest relative to *fan*, the *chs1* gene, is located on chromosome 1 (Nagle *et al*., 1996), the presence of a gene cluster encoding proteins with analogous functions seems unlikely.

The TNF family of ligands (TNF, LTα and LTβ) and the corresponding receptors TNF-R55 and LTβR were all shown to be involved in the ordered organization of lymphoid development (Pfeffer and Mak, 1993; Alimzhanov *et al*., 1997; Koni *et al*., 1997; Pasparakis *et al*., 1997; von Boehmer, 1997; Futterer *et al*., 1998). With regard to lymphoid organ development, the role of TNF-R55 seems to be confined to the formation of Peyer's patches (Neumann *et al*., 1996). To date, little is known about the relevant signaling pathways underlying this developmental function of TNF-R55. The question arose whether FAN would act as an intracellular mediator of TNF-induced Peyer's patch formation. However, histological and flow cytometrical data obtained from FAN-deficient mice indicated that FAN is not critically involved in development of any lymphoid tissue including Peyer's patches.

CHS in humans as well as the *beige* phenotype of mice is characterized by loss of NK cell and cytotoxic T-lymphocyte function, partial oculocutaneous albinism and a bleeding tendency. Comparison with sequences from a public database previously identified FAN as a member of the WD-40 repeat family of proteins (Adam-Klages *et al*., 1996). In addition, we reported recently that FAN also contains a BEACH domain, previously described in Beige and CHS proteins (Adam-Klages *et al*., 1998). Until now, this domain has only been found in CHS/Beige, CDC4L protein and several unknown proteins predicted from ORFs of yeast and nematodal sequences. Thus, the BEACH domain may define a novel subfamily of functionally related WD-40 repeat proteins. In view of the marked similarity in protein structure, it was plausible to investigate lymphocyte function in FAN-deficient mice. However, the $FAN^{-/-}$ mice showed no impairment in the killing activity of NK cells or CTL. In contrast, in CHS/Beige, both NK cell and CTL functions are defective, which is thought to be caused by perturbations of intracellular vesicular trafficking preventing the ordered secretion of granzymes and perforin. The molecular link between the CHS/Beige protein and vesicular trafficking, however, remains unknown. Formation of giant lysosomes due to defective fission of vesicles which is eminent in fibroblasts from beige mice, was also not observed in $FAN^{-/-}$ EF cells (D.Kreder and G.Scherer, unpublished observations). Furthermore, unlike CHS patients or *beige* mice, FAN-deficient mice displayed no coagulation problems. Thus, despite the structural homology between FAN and CHS/Beige consisting of WD-40 repeats and the BEACH domain, the physiological role of FAN seems to be different from that of CHS/Beige.

Taken together, the results of this study revealed that, with regard to TNF-R55, the function of FAN seems to be limited to the regulation of N-SMase, which might be relevant for cutaneous barrier homeostasis and repair. Notably, FAN seems to be dispensable for TNF-induced activation of ERK1/2. Further work will be required to delineate a possible role of FAN and/or N-SMase in defined intracellular signaling cascades.

Materials and methods

Generation of FAN-deficient mice

fan genomic DNA was isolated from an 129/SvJ mouse genomic library (Stratagene) and subcloned into cloning vector pBlueScript. Genomic fragments were analyzed by restriction enzyme mapping, Southern blotting and DNA sequencing. One genomic subclone containing exons 2–8 was used to construct the targeting vector using pBlueScript as backbone. The upstream genomic fragment was amplified from a genomic subclone using primers for the T7 promotor and a gene-specific primer FAN-SA3 (TCA GCG GAT CCG CAG CTT TTC AGA AAC ACT TTG GAA CCT GC). A neomycin resistance cassette was prepared from pMC1neopolyA (Stratagene). A downstream genomic fragment of 7 kb fragment was isolated from a genomic clone with *Kpn*I and *Pst*I. Finally, a thymidine kinase (tk) gene was ligated to the 3′ end of the downstream genomic fragment. The targeting vector was linearized with *Kpn*I and electroporated into the ES cell line E14.1. Clones resistant to G418 and gancyclovir were screened for homologous recombination by

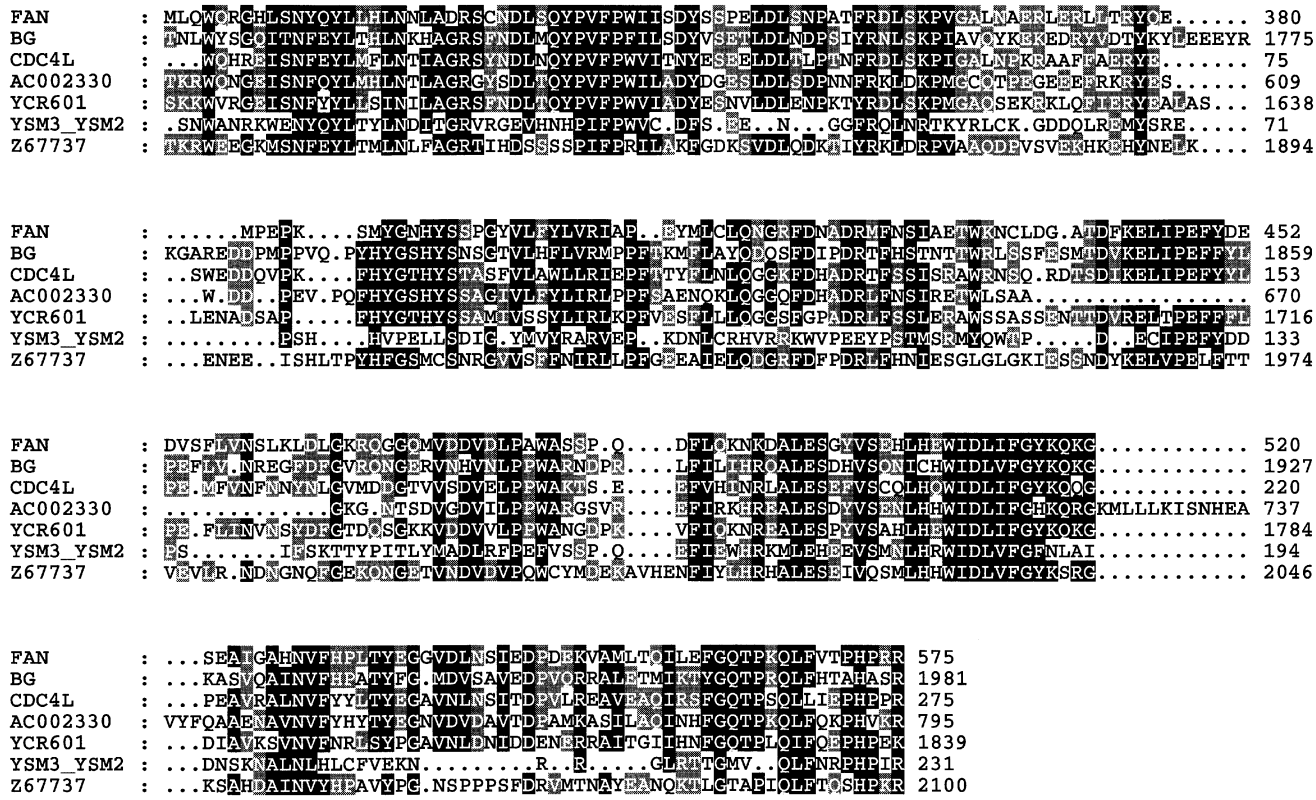

Fig. 4. Sequence homologies between FAN and CHS/Beige. Complementary DNA sequences of chs1 and bg, cdc4l genes were obtained from the DDBJ/EMBL/GenBank joint databases using the TBLASTN program. Expressed sequence tag (EST) sequences were obtained from the EST segment of GenBank. Alignments were performed with public software (Clustal W, EMBL, Heidelberg, Germany). Alignment of BEACH domains (abbreviations and DDBJ/EMBL/GenBank accession Nos given in parentheses). A BEACH domain is well conserved in murine FAN (FAN, AF013632), murine Beige (BG, U70015), human Beige-like protein (identical to CDC4L, M83822), putative protein trafficking component from *Arabidopsis thaliana* from BAC T10P11 (AC002330), gene product YCR601 from *Saccharomyces cerevesiae* (X62452), hypothetical proteins F52C9.3 and F52C9.3 from *Caenorhabditis elegans* (YSM3 and YSM2; Q10123 and Q10122), and an open reading frame from *C.elegans* cosmid T01H10 (Z67737).

PCR and confirmed by Southern blot analysis with the probe shown in Figure 1A. ES cells heterozygous for the homologous recombination were aggregated to CD1 morulas to obtain germline transmission. Heterozygous animals were interbred to obtain homozygotes.

For preparation of $FAN^{+/+}$, $FAN^{+/-}$ and $FAN^{-/-}$ EF cells, heterozygous animals were interbred. Embryos were isolated from pregnant females at day 14 of gestation. After removing head and intra abdominal organs, the carcasses were washed in DMEM and cut in small sections. After 2 days of cultivation, large pieces of tissue debris were removed by vigorous washing with phosphate-buffered saline (PBS). Adherent fibroblasts were ready for use after three to four passages.

Total RNA was prepared from tail fibroblasts and thymocytes using Trizol-reagent (Gibco-BRL) according to the protocols provided by the manufacturer. RT–PCR was performed using standard methods and employing the primers 5′-GCA TTA CAG ACA CCA GCC-3′ (located in the deleted portion of the *fan* gene) and 5′-CTC CAG TCG CTC CGC ATT TAG-3′ (located in the intact BEACH domain).

A- and N-SMase activation assays

Activation of acid and neutral SMase in thymocytes was measured as described previously (Wiegmann *et al*., 1994). Single-cell suspensions from thymi isolated from wild-type, $FAN^{+/-}$, and $FAN^{-/-}$ mice were treated with 30 U/ml interleukin-2 and 2 μg/ml concanavalin A for 3 days. Pre-activated thymocytes were treated in triplicates with 100 ng/ml TNF for the indicated times, lysed in A-SMase or N-SMase lysis buffer for 10 min at 4°C, respectively, homogenized by repeatedly squeezing through an 18 gauge needle, and centrifuged at 20 800 or 420 *g* for 10 min to obtain nuclei-free supernatants containing the cytosolic and membrane fractions. Supernatant protein contents were measured using an BCA assay (Pierce Chemical Co., Hamburg, Germany) with bovine serum albumin (BSA) as a standard. Equal amounts of protein from cellular lysates were added to 30 μl of A-SMase or N-SMase assay buffer containing [*N*-methyl-^{14}C]sphingomyelin (1.1 μCi/ml final concentration). The reaction mixtures were incubated at 37°C for 2 h. The reactions were stopped by addition of 800 μl $CHCl_3$/methanol (2:1) and 250 μl H_2O, vortexed and microfuged at 20 800 *g* for 2 min. To quantify the sphingomyelinase activity, 200 μl of aqueous phase, containing the [^{14}C]phosphorylcholine released by the enzyme was counted using a β-counter (Canberra-Packard, Downers Grove, IL).

ERK activation assays

EF cells from wild-type or $FAN^{-/-}$ mice in the third passage were treated with medium, 100 ng/ml TNF or 10 ng/ml PDGF for various times and lysed in TNE (20 mM Tris pH 8.0, 140 mM NaCl, 0.5% NP-40). Equal protein amounts (20 μg) were separated on 12% SDS–PAGE and blotted on PVDF membranes. Western blots were performed using the PhosphoPlus p42/44 MAPK antibody kit (New England Biolabs, Beverly, MA).

Quantification of TEWL and epidermal proliferation

Acute disruption of the permeability barrier was induced by a tape stripping (cellophane tape, scotch type, six to eight times) until a 20- to 30-fold increase in TEWL was achieved (Meeco® electronic water analyzer, Meeco Inc., Warrington, PA; Lee *et al*., 1992). Prior to TEWL quantification the fur was carefully removed by shaving. Shaving did not result in irritation or barrier disruption. One hour before barrier disruption, 30 mg BrdU (Amersham) per kg bodyweight were injected intraperitoneally. Five micrometer skin sections were processed by standard immunohistological peroxidase technique using a monoclonal anti-BrdU antibody (Amersham) and diaminobenzidine as substrate as recommended by the supplier of the staining kit. The stained sections were examined microscopically by counting the BrdU-labeled nuclei of interfollicular keratinocytes (cells in S phase) in seven microscopic fields/sections. Labeling index in FAN-deficient and control mice was defined by numbers of $BrdU^+$ cells/mm epidermal basal membrane.

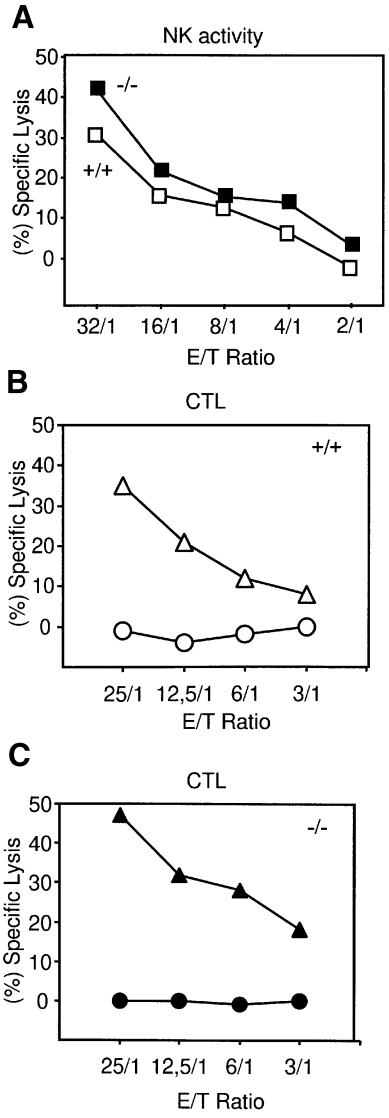

Fig. 5. Intact NK cell and CTL activity in $FAN^{-/-}$ mice. (**A**) NK cells from $FAN^{+/+}$ (□) and $FAN^{-/-}$ mice (■) were prepared from splenocytes and their lytic activity against YAC-1 target cells was analyzed. Representative results are shown from three independent experiments. (**B**) Lytic activity of cytotoxic T lymphocytes from $FAN^{+/+}$ mice against ^{51}Cr-labeled P815 cells (△) was tested after 4 h of cultivation. EL-4 cells (○) were used as specificity control. Representative results are shown from three independent experiments. (**C**) Lytic activity of cytotoxic T lymphocytes from $FAN^{-/-}$ mice against ^{51}Cr-labeled P815 cells (▲) was tested after 4 h of cultivation. EL-4 cells (●) were used as specificity control. Representative results are shown from three independent experiments.

Assays for NK and CTL cytotoxicity

NK cells were prepared from splenocytes using biotinylated pan-mouse NK cell antibody (clone DX5, Pharmingen, Hamburg, Germany) and streptavidin-coated magnetic beads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Purified NK cells were incubated with ^{51}Cr-labeled YAC-1 target cells at defined effector/target ratios. After 4 h of incubation, ^{51}Cr release was determined by γ-counting. CTL from $FAN^{-/-}$ mice were raised in bulk cultures of $FAN^{-/-}$ derived spleen cells as responder cells and irradiated BALB/c derived spleen cells as stimulator cells. Activity of cytotoxic lymphocytes was analyzed by incubation with P815-target cells at the indicated effector/target ratios. ^{51}Cr release was determined by γ-counting after 4 h of culture.

Acknowledgements

We thank D.Hartmann for excellent histological analysis, U.Huffstadt and S.Weiss for help with the SPF mouse colony, K.Mink for help with flow cytometry, and R.Schwandner, D.Adam and A.Futterer for discussions. M.K. and S.A.-K. were supported by Deutsche Forschungsgemeinschaft (SFB 415).

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Received September 10, 1998; revised and accepted March 5, 1999