

The Tumor-Necrosis-Factor Receptor–Associated Periodic Syndrome: New Mutations in *TNFRSF1A*, Ancestral Origins, Genotype-Phenotype Studies, and Evidence for Further Genetic Heterogeneity of Periodic Fevers

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Mutations in the extracellular domain of the 55-kD tumor-necrosis factor (TNF) receptor (*TNFRSF1A*), a key regulator of inflammation, define a periodic-fever syndrome, TRAPS (TNF receptor–associated periodic syndrome [MIM 142680]), which is characterized by attacks of fever, sterile peritonitis, arthralgia, myalgia, skin rash, and/or conjunctivitis; some patients also develop systemic amyloidosis. Elsewhere we have described six disease-associated *TNFRSF1A* mutations, five of which disrupt extracellular cysteines involved in disulfide bonds; four other mutations have subsequently been reported. Among 150 additional patients with unexplained periodic fevers, we have identified four novel *TNFRSF1A* mutations (H22Y, C33G, S86P, and c.193–14 G→A), one mutation (C30S) described by another group, and two substitutions (P46L and R92Q) present in ~1% of control chromosomes. The increased frequency of P46L and R92Q among patients with periodic fever, as well as functional studies of *TNFRSF1A*, argue that these are low-penetrance mutations rather than benign polymorphisms. The c.193–14 G→A mutation creates a splice-acceptor site upstream of exon 3, resulting in a transcript encoding four additional extracellular amino acids. T50M and c.193–14 G→A occur at CpG hotspots, and haplotype analysis is consistent with recurrent mutations at these sites. In contrast, although R92Q also arises at a CpG motif, we identified a common founder chromosome in unrelated individuals with this substitution. Genotype-phenotype studies identified, as carriers of cysteine mutations, 13 of 14 patients with TRAPS and amyloidosis and indicated a lower penetrance of TRAPS symptoms in individuals with noncysteine mutations. In two families with dominantly inherited disease and in 90 sporadic cases that presented with a compatible clinical history, we have not identified any *TNFRSF1A* mutation, despite comprehensive genomic sequencing of all of the exons, therefore suggesting further genetic heterogeneity of the periodic-fever syndromes.

Introduction

Until recently, the dominantly inherited periodic-fever syndromes represented a poorly understood group of inflammatory diseases. Clinically, these disorders are characterized by recurrent prolonged (>1 wk) attacks of fever associated with abdominal pain, myalgia, migratory erythematous skin rash, conjunctivitis, and/or periorbital edema (McDermott et al. 1997). In contrast to patients with familial Mediterranean fever (FMF [MIM

249100]), which is recessively inherited, patients with dominantly inherited periodic fever usually have a poor response to colchicine but a favorable response to corticosteroids. Dominantly inherited periodic fevers have been described mostly in families of northern-European descent (Williamson et al. 1982; Gertz et al. 1987; McDermott et al. 1997; Zaks and Kastner 1997).

In 1998, linkage studies identified, in the same region of chromosome 12p13, a candidate interval for two clinical variants—benign autosomal dominant familial periodic fever (FPF) (Mulley et al. 1998) and familial Hibernian fever (FHF [MIM 142680]) (McDermott et al. 1998). One of the several candidate genes in this interval is *TNFRSF1A*, the gene encoding the 55-kD receptor for tumor-necrosis factor (TNF) α (TNF- α). Subsequently, in 1999 we identified six mutations in the *TNFRSF1A* sequence encoding the extracellular domain of the p55 receptor in seven affected families, including

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those previously denoted as having FPF and FHF (McDermott et al. 1999). This was the first report of *TNFRSF1A* mutations in human disease.

In our initial report, all of the mutations were single-nucleotide missense substitutions, with five of the six mutations disrupting highly conserved cysteine residues involved in extracellular disulfide bonds. Although five of the first seven families were of either Irish or Scottish descent, one was Finnish and another was French Canadian. In light of the more diverse ethnic distribution, we have proposed the term “TNF receptor–associated periodic syndrome” (TRAPS) for those patients with *TNFRSF1A* mutations.

In vitro studies of leukocytes from patients bearing the C52F mutation indicated an impairment in the normal activation-induced cleavage of cell-surface p55 receptors. Receptor clearance is thought to have a negative homeostatic effect, both by preventing repeated stimulation through membrane p55 and by creating a pool of antagonistic soluble receptors, and thus a cleavage defect could at least partially account for the hyperinflammatory phenotype in TRAPS.

Four additional TRAPS mutations in the extracellular domain of *TNFRSF1A* have subsequently been reported by other groups: C30S (Dodé et al. 2000), R92P (Aganna et al. 2001), C55S (Jadoul et al. 2001), and C70Y (Simon et al. 2001). Families were of French, Dutch, Belgian, and Dutch-Indonesian descent, respectively. Individuals affected with each mutation manifested typical TRAPS inflammatory attacks, and amyloidosis was documented in patients with either the C70Y mutation or the C55S mutation.

Here we report a new group of patients with TRAPS, including both familial and sporadic cases. We identified four mutations, two of which were cysteine substitutions, that were not present among a panel of ethnically matched control chromosomes. Two other substitutions, neither of which involved cysteines, were found in ~1% of control chromosomes. On the basis of their clinical presentation, their higher frequency among periodic fever patients, and functional studies of *TNFRSF1A* in vitro and in vivo, these latter two substitutions are likely to be low-penetrance mutations rather than polymorphisms. We have also investigated the carrier haplotypes associated with mutations observed in unrelated patients, the penetrance of the 16 known TRAPS mutations, and possible genotype-phenotype relationships. Our data strongly suggest that cysteine substitutions lead to a higher risk of amyloidosis—and, probably, to a higher penetrance—than do the noncysteine mutations. Finally, we report evidence for further genetic heterogeneity of periodic-fever syndromes, with one clinically consistent family not having linkage to chromosome 12p13 and with 90 sporadic cases without demonstrable mutations in the coding region of *TNFRSF1A*.

Subjects, Material, and Methods

Patients and DNA Specimens

The present study included patients referred to the National Institutes of Health for clinical and/or molecular diagnosis of unexplained periodic fevers. The study was approved by the human-experimentation committee at the National Institutes of Health, and consent was obtained from all participants. Genomic DNA was extracted from whole blood by a commercially available kit (Puregene; Gentra Systems).

TNFRSF1A Mutation Detection

Genomic DNAs from 150 patients (137 unrelated individuals) with symptoms compatible with TRAPS were sequenced for exons 2–5 of *TNFRSF1A*, encoding the extracellular domain, as described elsewhere (McDermott et al. 1999). Samples of genomic DNA from 90 patients were analyzed by the combination of denaturing high-performance liquid chromatography (DHPLC) and sequence analysis, for all 10 exons of *TNFRSF1A*. Patients from two families initially negative for a mutation in the extracellular domain of the gene were also sequenced through the complete coding region and promoter (450 bp). The forward and reverse oligonucleotide primers used to amplify regulatory sequences of the *TNFRSF1A* gene were 5'-TCACTGCTGTGATCCATCTGGG-3' and 5'-TTCTCACCAGTGGCAGCAGCAG-3'. Other exons were sequenced as described elsewhere (McDermott et al. 1999), except that exons 8 and 9 were analyzed separately, with the following primers: exon 8, forward primer 5'-TCGAGCTGCATCAGTAGCTCT-3' and reverse primer 5'-TTCTACGTGGGGTTGGGAC-3'; exon 9, forward primer 5'-TAAGTCCCAACCCACGAG-3' and reverse primer 5'-CAGCCTCCTCGTCTCCAG-3'.

Mutation detection was performed by fluorescent sequencing with dye-primer chemistry (Amersham), on an ABI 377 automated sequencer, and by DHPLC (Transgenomics). PCR was performed with *AmpliTaq Gold* (PE Biosystems) in 50- μ l reactions with 10–50 ng of genomic DNA and with the same primers, described above, that were used for exon sequencing. DHPLC analysis of amplicons was performed at temperatures modified for each of the exons, according to WAVE-MAKER 4.0 software (Transgenomics). Amplicons showing a DHPLC elution shift were confirmed in a repeat sequencing analysis. The sequencing data were analyzed by SEQUENCHER 3.0 (Gene Codes).

Microsatellite and Single-Nucleotide Polymorphism (SNP) Analysis

Genotyping in the chromosomal region of the *TNFRSF1A* locus was performed with the following

markers: *D12S99*, *D12S356*, *TNFRP55*, *CD4*, *D12S1695*, *D12S77*, and *D12S364*, as described elsewhere (McDermott et al. 1998; Mulley et al. 1998; Eskadele et al. 2000). The two intragenic SNPs utilized in the present study were recently reported (Bazzoni et al. 2000) and have been submitted to HGSDBASE, with accession numbers SNP000005194 and SNP000005195. Allele frequencies of G (.47) and of A (.53), for SNP000005194 (exon 1 SNP), and of C (.32) and of T (.68), for SNP000005195 (intron 5 SNP), were determined in 71 healthy controls. *TNFRSF1A* intragenic SNPs of our patients were determined by DNA sequencing.

Genotyping for the Muckle-Wells syndrome (MWS [MIM 191900])/familial cold urticaria (FCU [MIM 120100]) locus on distal chromosome 1q44 was performed with the *D1S2682* and *D1S423* microsatellites, which define the boundaries of the MWS/FCU critical interval (Cuisset et al. 1999; Hoffman et al. 2000; McDermott et al. 2000). Since the marker *D1S423* was not informative, we also used two other microsatellites close to the centromeric boundary of the candidate interval, *D1S1609* and *D1S547*. Microsatellite haplotypes were constructed so as to minimize the number of intrafamilial recombinations.

RNA Extraction

Total RNA was extracted from leukocytes by use of Trizol Reagent (GIBCO-BRL), as described elsewhere (Centola et al. 2000). First-strand cDNA synthesis was performed with the GIBCO Superscript Preamplification System, according to the manufacturer's recommendations. The cDNA template was amplified by PCR with the following primers: forward primer 5'-ATTGGACTGGTCCCTCACCTAGGG-3' and reverse primer 5'-TCACACTCCCTGCAGTAC-3'.

Construction of a Minigene to Analyze Aberrant Splicing

DNA fragments comprising exon 2, intron 3, and exon 3 were obtained, by PCR amplification, from the proband and control DNA samples, by use of the following oligonucleotides: forward primer 5'-CGAGAATTCATGCGTGCTCCTGGAGCTGTTGGTG-3' and reverse primer 5'-TCGCTCGAGTCACCTTTCGGCATTTGGAGCAGCTG-3'. The forward primer contained an *EcoRI* restriction site and four nucleotides (ATGC), to create a methionine codon in-frame with the open reading frame of exon 2; the reverse primer contained an *XhoI* site followed by a stop codon. PCR amplification was performed with *AmpliTaq* Gold (PE Applied Biosystems), and cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, for 30 cycles, followed by extension at 72°C for 7 min. The amplicons were

ligated into pCR2.1 vector by use of the TOPO TA cloning kit (Invitrogen), and the resulting colonies were analyzed, by restriction digestion and dye-primer sequencing, for expected mutation-containing inserts. Verified inserts were cloned into pcDNA3 expression vector (Invitrogen) by ligation of the *XhoI/EcoRI* insert fragments from TA clones with *XhoI/EcoRI*-digested vector. Expression plasmids were transfected into Cos-7 cells by use of Lipofectamine reagent (Gibco/BRL), as described by the manufacturer. After 24 h of expression, RNA was extracted from transfected cells by use of Trizol reagent (Gibco/BRL), and bulk cDNA was prepared by the GIBCO Superscript Preamplification System, according to the manufacturer's recommendations.

Cell Activation and Flow-Cytometric Analysis

TNFRSF1A shedding assays were performed as described elsewhere (McDermott et al. 1999). In brief, blood cells were washed and incubated at 37°C for 10 min in Hanks' balanced salt solution containing 1% fetal calf serum (FCS), with or without 4- β -phorbol 12-myristate 13-acetate (PMA; Sigma). Cells were washed with HBSS/1% FCS at 4°C, and erythrocytes were lysed by use of ammonium chloride potassium lysing buffer (BioWhittaker). Cells were incubated with phycoerythrin (PE)-conjugated anti-*TNFRSF1A* monoclonal antibodies (Caltag) and isotype controls (Becton Dickinson), for 30 min at 4°C, and were washed three times with phosphate-buffered saline containing 0.5% bovine serum albumin. Expression of membrane *TNFRSF1A* on monocytes was analyzed by flow cytometry, by a FACS Calibur flow cytometer (Becton Dickinson). Soluble levels of *TNFRSF1A* were detected by solid-phase enzyme-linked immunosorbent assay, as described elsewhere (McDermott et al. 1999).

Statistical Analysis

Statistical analyses were performed by the SAS 6.12 and Epi Info 6 statistical software packages (Centers for Disease Control and Prevention [CDC]). Comparison of cysteine mutations, in patients versus a control group of healthy individuals and in patients with amyloidosis versus patients without amyloidosis, were performed by χ^2 statistics. Results were reported as odds ratios with 95% confidence intervals. All *P* values were Yates corrected.

Results

TNFRSF1A Mutational Survey

At the National Institutes of Health Clinical Center, a referral center for periodic-fever syndromes, we collected DNA samples from >800 patients suffering from periodic fever and accompanying symptoms. The 150 patients who underwent screening for mutations in *TNFRSF1A*

were selected on the basis of a clinical history suggestive of TRAPS (i.e., attacks lasting >1 wk, characteristic skin rash, and/or nonresponsiveness to colchicine). The patients were screened for mutations in exons 2–5 of *TNFRSF1A*, where all of the initially reported mutations clustered (McDermott et al. 1999). We used automated sequencing of PCR-amplified genomic DNAs, since, in our initial report, all but one of the mutations was private. Ninety patients underwent further comprehensive analysis of the entire *TNFRSF1A* coding region (i.e., 10 exons). This new cohort of patients with TRAPS with demonstrable *TNFRSF1A* mutations included several multiplex families and 10 sporadic cases of various ethnic backgrounds. Representative pedigrees are shown in figure 1.

Among these patients, we identified seven new *TNFRSF1A* mutations, one of which was recently reported by another group (Dodé et al. 2000). The mutations are listed in table 1, and corresponding DNA-sequence electropherograms are shown in figure 2. Amino acid positions are defined relative to the signal peptide–cleavage site. H22Y is a novel mutation detected in the father and three affected children in a family of Scottish/German background and in one case, of mixed northern-European background, that initially appeared to be sporadic, with lifelong unexplained periodic fevers leading to 170 hospitalizations and 13 exploratory surgeries. Subsequently, we found that the latter patient's

father, who is also of mixed northern-European background, also suffered from mild symptoms of TRAPS and bore the H22Y mutation. This substitution was not observed either in the only available healthy member (i.e., the mother) of each H22Y-positive family or in any of >100 northern-European control chromosomes screened by genomic sequencing.

C30S and C33G mutations were found at the same highly conserved cysteine residues where we previously had reported the C30R and C33Y mutations, and neither C30S nor C33G was found in any of >100 northern-European control chromosomes. The C30S mutation was observed in an Irish American family with three affected members, and has also recently been described in a French family (Dodé et al. 2000). The C33G mutation was found in a father and daughter originally from Puerto Rico, with histories of recurrent fever, abdominal pain, and arthralgia since birth. They had been treated with corticosteroids for many years. The father had developed progressive hepatic amyloidosis, eventually necessitating liver transplantation. The mother and their other three children were healthy and negative for this mutation.

S86P is a new private mutation found in a three-generation family, of Scottish/Irish descent, with four affected members (fig. 1) who experienced episodes of severe abdominal pain, high fever, and arthralgia lasting 6–8 wk. This substitution was observed in none of 100

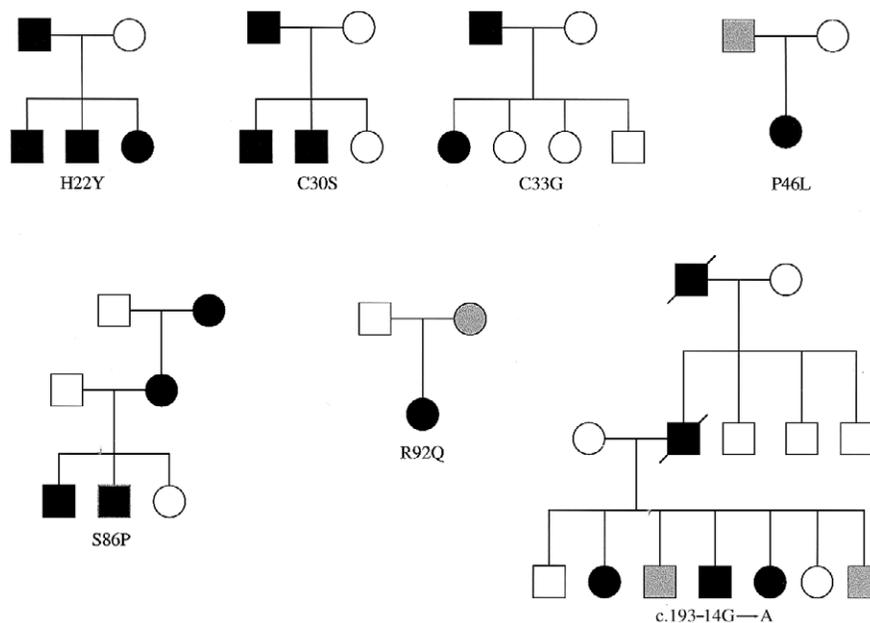


Figure 1 TRAPS pedigrees with novel *TNFRSF1A* mutations. Affected individuals are indicated by blackened symbols; individuals who are mutation positive but asymptomatic are represented by gray-shaded symbols. For the H22Y, R92Q, and c.193–14 G→A mutations, only representative families are shown.

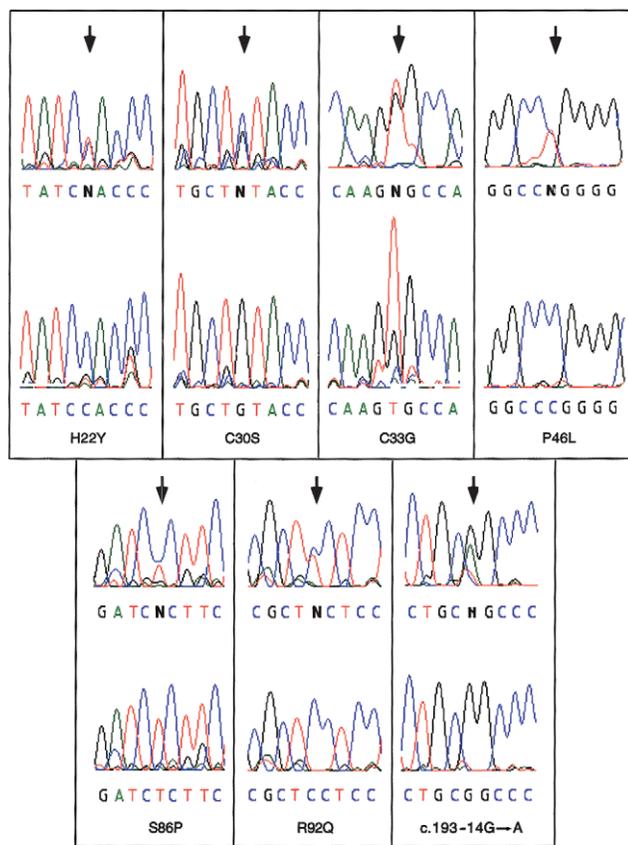


Figure 2 DNA sequence electropherograms for the seven *TNFRSF1A* mutations identified in the present study. For each sequence, the upper tracing is from a patient, and the lower tracing is from a normal control.

northern-European control chromosomes. One of the affected sibs in the third generation is symptomatic but declined genetic testing.

Screening of intronic sequences flanking the exons identified one splicing mutation. In a family of Scottish descent and in a family of Arab descent (fig. 1; Scottish family is not shown), we observed a G→A change in

intron 3 at position -14 relative to codon 193 (fig. 2). This substitution was not detected in any of 100 control chromosomes and potentially could create a new consensus splice-acceptor sequence (i.e., AG) in the presence of a preceding polypyrimidine tract (fig. 3A). We obtained mRNA from one of the patients carrying this substitution, performed reverse transcriptase-PCR, and detected a band slightly larger than the normal-size transcript. Sequencing of the cloned larger band revealed a transcript encoding an additional four amino acids, as would be expected with activation of this new splice-acceptor site in intron 3.

We also analyzed the effect of this substitution on pre-mRNA splicing, by examining Cos-7 cells transfected with a minigene construct harboring either the mutant A nucleotide or the wild-type G. Cytoplasmic RNA isolated from transfected Cos-7 cells showed that the mutant construct produced aberrantly spliced transcript with an extra four amino acids inserted between exons 2 and 3 (fig. 3B). The wild-type construct resulted in normal splicing of the *TNFRSF1A* transcript. This substitution cosegregated with affection status in both families but with reduced penetrance in two members of the Arab family and in one member of the Scottish family.

Low-Penetrance TNFRSF1A Mutations

Finally, two additional missense transitions, P46L and R92Q, were identified in patients with periodic fever and at low frequencies in the general population. P46L was detected in one sporadic patient of mixed ancestry who suffered from periodic fevers that sometimes lasted >1 mo and that were associated with severe abdominal pain, vomiting, and leg pain. We found that the patient inherited this substitution from his father, who is of mixed African American and northern-European ancestry. P46L was detected in 1 of 170 northern-European control chromosomes and in 3 of 156 African American control chromosomes, at a combined gene frequency of 1.23%. R92Q was the most common *TNFRSF1A* sequence change identified in our panel, occurring in 9 of 137 unrelated pa-

Table 1

Summary of New TRAPS Mutations

MUTATION (NUCLEOTIDE CHANGE)	LOCATION	NO. OF		ETHNICITY
		Patients	Families	
H22Y (151 C→T)	Exon 2	6	2	Scottish/German, mixed northern European
C30S (176 G→C)	Exon 2	3	1	Irish
C33G (184 T→G)	Exon 2	2	1	Puerto Rican
P46L (254 C→T)	Exon 2	1	1	African American/northern European
S86P (343 T→C)	Exon 4	4	1	Scottish/Irish
R92Q362 G→A	Exon 4	9	9	Mexican (1), Italian (2), Irish (1), English (1), Portuguese (1), Ashkenazi (1), mixed northern European (2)
Splice (c.193-14 G→A)	Intron 3	5	2	Scottish/Arab

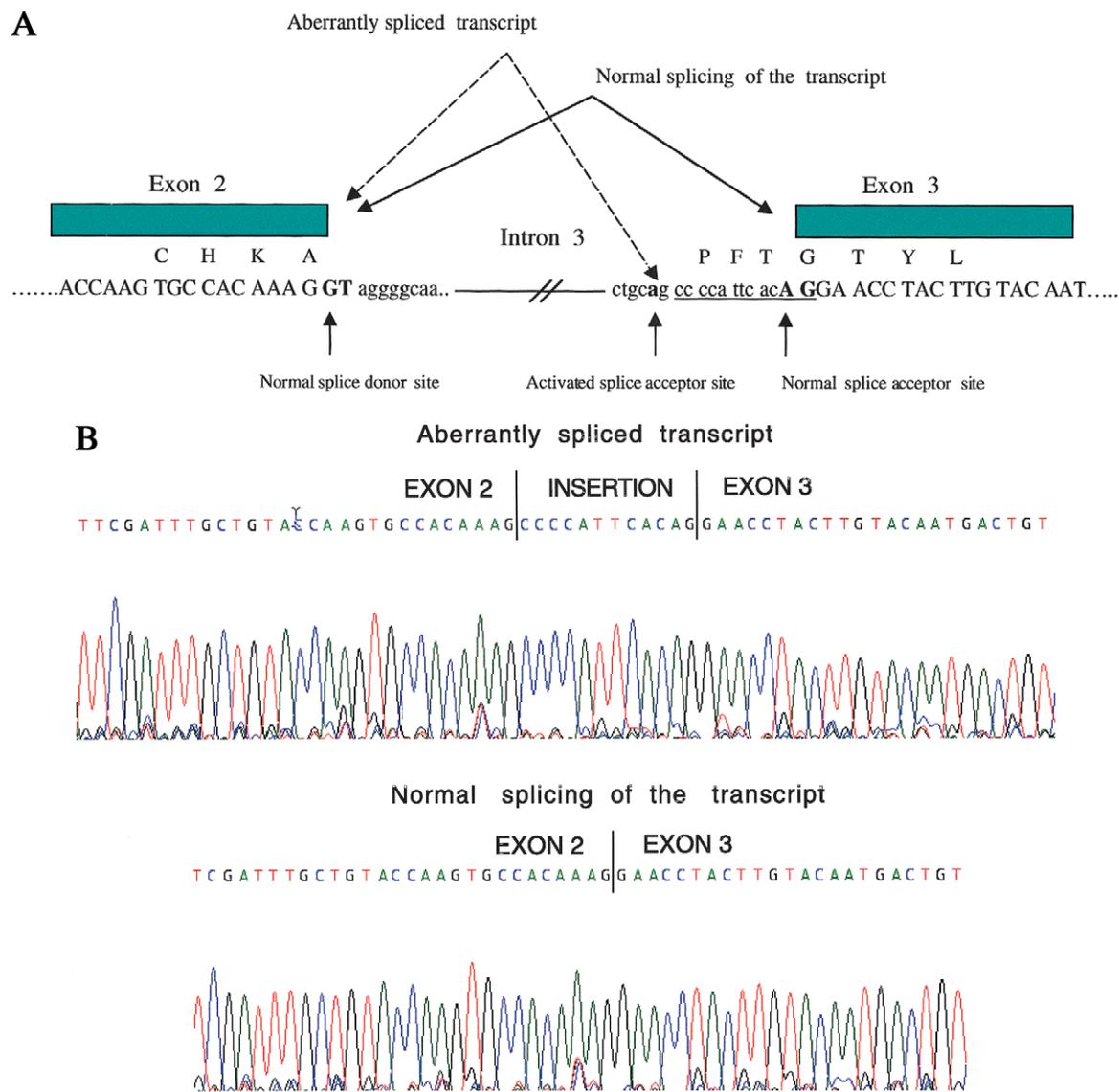


Figure 3 A, Diagram of normal and aberrantly spliced TNFRSF1A transcript. The conventional splice-donor site, splice-acceptor site, and mutated nucleotide in intron 3 are in boldface. The c.193–14 G→A mutation creates a new splice-acceptor site, as shown. Single-letter designations for amino acids are shown immediately above the first nucleotide in each codon. Four additional amino acids that are present in the alternatively spliced transcript are encoded by the nucleotides that are underlined. B, DNA-sequence electropherograms showing in vitro splicing of c.193–14 G→A transcripts (*top*) and normal transcripts (*bottom*) in Cos-7 cells.

tients. All were sporadic cases, representing a range of ethnicities. Since, in five cases, this substitution was detected in asymptomatic parents, we investigated the frequency of R92Q in the Irish and North American control populations. R92Q was detected in 2 of 132 Irish control chromosomes and in 6 of 634 North American (mostly northern-European) control chromosomes, at a combined gene frequency of 1.04% in both populations, versus 9 (3.3%) of 274 independent chromosomes in the panel with a clinical picture suggestive of TRAPS ($P = .02$).

To explore further the significance of P46L and R92Q,

we examined cell-surface TNFRSF1A in peripheral blood monocytes before and after in vitro stimulation with PMA. We have previously demonstrated that leukocytes from patients with the C52F mutation show both increased baseline levels of membrane TNFRSF1A and, after stimulation with PMA, a potent inducer of the metalloproteases mediating ectodomain cleavage, reduced receptor cleavage (shedding). Figure 4 shows TNFRSF1A expression on monocytes from patients bearing the C52F, T50M, P46L, or R92Q substitutions and from a control sample. As previously reported, unstimulated C52F

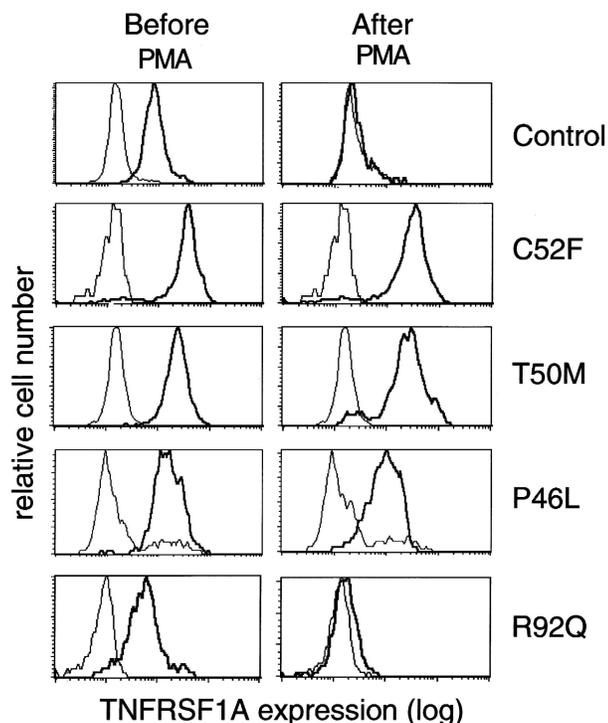


Figure 4 Defective clearance of TNFRSF1A in patients with TRAPS. Monocytes were analyzed for TNFRSF1A expression (*thicker line in each panel*), before (*left-hand panels*) or after (*right-hand panels*) PMA activation (100 ng/ml) for 10 min. Single-cell suspensions (10^6 cells) were incubated with either anti-TNFRSF1A-PE or isotype-matched IgG-PE as control (*thinner line in each panel*), were washed, and were analyzed by flow cytometry. Fluorescence histograms are shown for controls and for patients with the C52F, T50M, P46L, and R92Q TRAPS mutations.

monocytes, relative to controls, had more membrane TNFRSF1A and showed minimal receptor clearance, after PMA stimulation. Comparable results, both before and after PMA stimulation, were observed with cells bearing T50M. Consistent with the view that P46L has functional consequences, monocytes from patients bearing this substitution did not completely clear TNFRSF1A after PMA stimulation, although there was more shedding than was seen with either T50M or C52F. Variable degrees of impairment of PMA-induced TNFRSF1A shedding were also observed in monocytes from patients with the H22Y, C30S, or C33G mutations (data not shown).

In contrast, monocytes from patients bearing the R92Q substitution showed TNFRSF1A membrane staining and receptor shedding comparable to those in controls (fig. 4). Similar results were also observed for monocytes from patients with the c.193–14 G→A mutation (data not shown). Nevertheless, when we examined serum samples from a patient with R92Q that were taken during and between febrile episodes, we found that soluble p55 levels failed to increase with the attack

(3,358 pg/ml baseline, 1,997 pg/ml during attack, normal range 749–1,966 pg/ml). Similar findings have recently been reported for the C30S mutation (Dodé et al. 2000). The failure of patients’ soluble TNFRSF1A levels to increase with inflammatory episodes suggests an *in vivo* functional abnormality that, in the case of R92Q, is not detected by the *in vitro* shedding assay.

Genotype-Phenotype Studies

When the present study is included, 100 symptomatic patients positive for a total of 16 mutations in *TNFRSF1A* have been reported in the literature (McDermott et al. 1999; Dodé et al. 2000; Aganna et al. 2001; Jadoul et al. 2001; Simon et al. 2001). Because systemic amyloidosis is the most serious manifestation of TRAPS, sometimes leading to hepatic and/or renal failure and death, we first investigated the possible association of amyloidosis with specific mutations (table 2). Among the 100 patients with TRAPS, this complication has been identified in 14. Despite the fact that only 9 of 16 known mutations in *TNFRSF1A* involve cysteine residues, 13 of 14 known cases of amyloidosis occurred among patients with cysteine mutations (C30R, C33G, C33Y, C52F, C55S, C70Y, or C88Y); only 1 patient with amyloidosis had T50M. When unaffected carriers of mutations were included, amyloidosis was found in 13 of 59 persons with cysteine substitutions but in only 1 of 58 with noncysteine mutations ($P = .002$, by Fisher’s exact test; odds ratio 16.11, 95% CI 2.06–341.95).

We also analyzed family data for the 16 known

Table 2
TNFRSF1A Mutations and Development of Amyloidosis

Mutation	Frequency of Amyloidosis
H22Y	0/6
C30R	2/2
C30S	0/5
C33G	1/2
C33Y	2/17
P46L	0/1
T50M	1/18
C52F	3/5
C55S	3/3
C70Y	1/8
S86P	0/4
C88R	0/8
C88Y	1/4
R92P	0/3
R92Q	0/9
Splice	0/5
Total	14/100 ^a

^a Of the 14 cases of amyloidosis, 13 (93%) had cysteine mutations.

Table 3

Microsatellite and Intragenic SNP Haplotypes of TRAPS T50M Carrier Chromosomes and of R92Q Carrier Chromosomes

Subject (Ethnicity)	Marker D12S99	Marker D12S356	Exon 1 SNP	Marker TNFRp55	Intron 5 SNP	Marker CD4	Marker D12S1695	Marker D12S77	Haplotype Designation
T50M									
Patient 466 (Scottish)	299/303	215/219	A/G	144/150	C/T	86/86	147/155	191/183	1
Patient 469 (Scottish), child of 466	299/297	219/219	A/A	150/150	T/T	86/112	147/155	191/173	1
Patient 594 (unknown)	303/303	215/217	A/A	148/150	T/T	91/112	157/161	173/173	1
Father of 594, unaffected	ND	ND	A/A	150/150	T/T	91/106	ND	ND	
Patient 32 (Irish)	293/297	219/223	A/G	144/152	C/T	86/86	157/159	163/163	2
Patient 510 (Irish), child of 32	293/297	219/223	A/A	150/152	T/T	86/91	155/159	ND	2
Patient 511 (Irish), child of 32	293/293	297/223	A/A	150/152	T/T	86/91	155/159	ND	2
Patient 802 (Irish)	ND	223/225	A/G	144/150	C/T	106/106	ND	ND	3
Patient 804 (Irish)	ND	?/225	A/G	144/152	C/T	101/106	ND	ND	3
Patient 805 (Irish)	ND	223/225	A/G	144/150	C/C	91/106	ND	ND	3
Patient 806 (French Canadian)	ND	219/223	G/G	144/144	C/C	86/91	ND	ND	3
Patient 807 (French Canadian), child of 806	ND	219/223	G/G	144/144	C/C	86/91	ND	ND	3
R92Q									
Patient 498 (mixed northern European)	295/297	211/211	G/G	140/144	C/C	91/91	147/155	177/177	
Mother of 498	295/295	211/219	A/G	140/152	ND	91/112	147/159	ND	
Patient 522 (Irish)	293/297	219/223	A/G	140/150	C/T	86/91	147/157	167/179	
Patient 560 (Mexican)	297/303	217/221	A/G	140/150	C/T	86/86	147/155	177/181	
Mother of 560	297/301	213/217	G/G	140/144	C/C	86/112	155/159	177/177	
Patient 618 (English)	293/301	211/213	A/G	140/150	C/T	91/112	159/161	163/181	
Patient 644 (Italian)	297/301	217/223	A/G	140/150	C/T	86/86	147/151	177/185	
Patient 657 (Portuguese)	297/?	205/221	A/G	140/150	C/T	91/121	151/155	179/?	
Father of 657	293/297	205/217	G/G	140/144	C/C	91/121	155/155	179/?	
Patient 670 (unknown)	301/?	205/211	G/G	140/150	C/C	91/112	141/155	181/183	
Father of 670	301/303	205/219	A/G	140/144	C/C	86/91	147/155	181/?	
Patient 758 (mixed northern European)	ND	221/223	A/G	140/152	C/C	91/106	ND	ND	
Patient 791 (Jewish)	ND	217/223	G/G	140/144	C/C	91/91	ND	ND	
Father of 791 (Jewish)	ND	223/225	G/G	140/144	C/C	91/91	ND	ND	

NOTE.—The genetic markers are presented in the order in which they occur on chromosome 12. Alleles representing haplotypes segregating with either T50M or R92Q within the family are in boldface italic; alleles representing a putative haplotype cosegregating with a mutation, determined on the basis of allele similarities with other defined haplotypes, are underlined. ND = not determined; ? = results not interpretable.

TRAPS mutations, to compare the penetrance of cysteine and noncysteine mutations. In our study and in data published by other groups (Dodé et al. 2000; Simon et al. 2001), 5 of 59 individuals identified with cysteine *TNFRSF1A* mutations had no reported symptoms, versus 12 of 58 individuals with noncysteine mutations ($P = .11$; Aganna et al. 2001; present study). Only 1 of 18 individuals positive for the T50M mutation was asymptomatic. Therefore, the total number of individuals positive for *TNFRSF1A* mutations was 117, with 100 of them being symptomatic carriers of various mutations and with 17 being asymptomatic carriers of various mutations.

Haplotype Analysis

We next asked whether unrelated carriers of the same mutation share a common founder haplotype. Data relevant to this issue were available for three mutations: T50M, R92Q, and c.193–14 G→A. T50M was initially observed in eight affected members of an Irish family with FHF (including patients 802, 804, and 805 in table

3) and in one available member (patient 806) of a French Canadian family (McDermott et al. 1999). Additionally, we found this mutation in 10 other patients: 3 members (patients 32, 510, and 511) of an Irish American family, 2 patients (patients 466 and 469) from a Scottish American family, 1 additional member (patient 807) of the French Canadian family, 1 patient (patient 594) from the United States who is of unknown descent, and, recently, 3 members of another Scottish American.

To test the hypothesis of common ancestry, we constructed haplotypes with both microsatellite and intragenic SNP markers. *TNFR1P55* is an intragenic microsatellite marker that resides within intron 2 of *TNFRSF1A* (based on the genomic structure used by Fuchs et al. 1992), and we tested additional SNPs in exon 1 and in intron 5. *D12S99* and *D12S356* are telomeric flanking markers, whereas *CD4*, *D12S1695*, and *D12S77* are centromeric flanking markers. The sequence of these markers and the published genetic distances between them are as follows: *D12S99*–.02–*D12S356*–.02–*TNFRSF1A*–.01–*CD4*–.03–*D12S1695*–

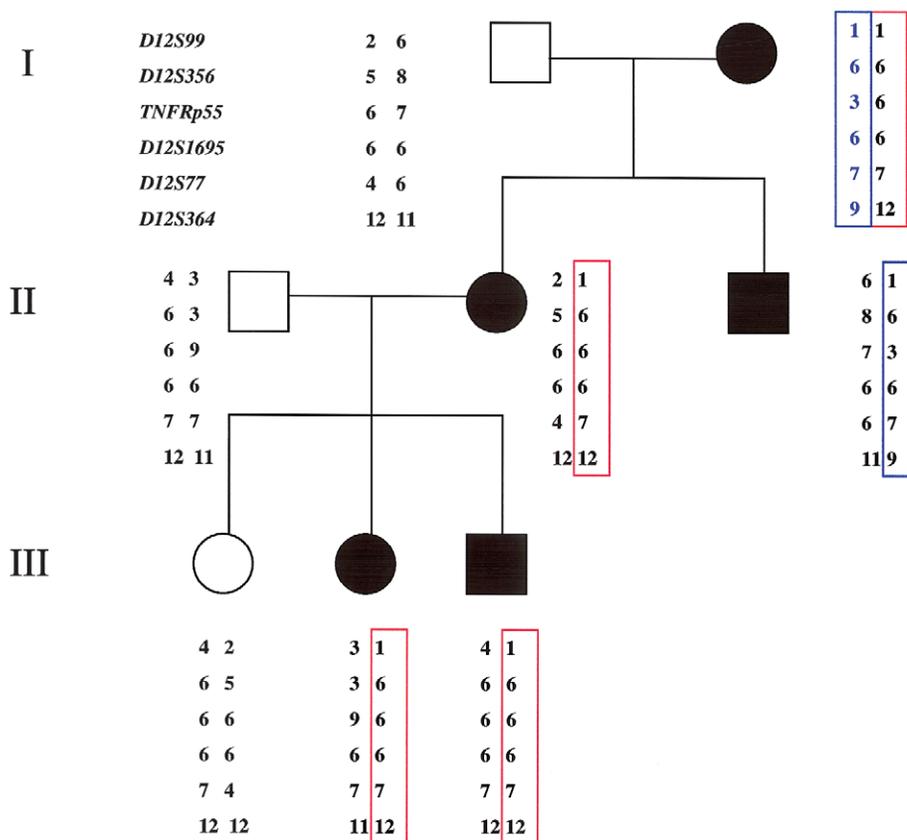


Figure 5 Pedigree of family affected with dominantly inherited periodic-fever syndrome. One affected member of the family was screened for the *TNFRSF1A* mutation, throughout the coding and promoter regions; no mutation was identified. Linkage analysis was done to rule out other mutations in *TNFRSF1A*. Genotypes for six DNA markers from chromosome 12p are shown. Affected sibs in the second generation inherited opposite haplotypes across the interval.

.03–*D12S77*. Haplotypes were deduced from the relevant pedigrees. Among five families with T50M (the recently reported Scottish American family was not studied), we identified three intragenic haplotypes: A-150 bp-T, A-152 bp-T, and G-144 bp-C (table 3). In addition, we did not find a common microsatellite founder haplotype associated with the c.193–14 G→A splicing mutation, consistent with the fact that this mutation was detected in two families of very distinct ethnicity (data not shown).

However, haplotype analysis of patients with R92Q showed a high level of linkage disequilibrium within *TNFRSF1A*, where the 140-bp allele of the *TNFR1P55* microsatellite marker cosegregated strongly with the presence of the substitution at codon 92. Using this microsatellite and the two intragenic SNPs, we have identified an intragenic haplotype, defined as G-140 bp-C, associated with the R92Q mutation on all of four independent carrier chromosomes in which phase could be assigned (table 3). In all five remaining singleton cases, in which phase could not be established, genotypes

consistent with this intragenic haplotype were observed. In contrast, the frequency of this haplotype in the general northern-European population could not be >6.5%, the frequency of the 140-bp allele that we observed in 122 control chromosomes.

Genetic Heterogeneity

Among the families with a clinical history compatible with TRAPS who were referred for periodic fever, two multiplex families with dominant inheritance were identified. We did not find any mutation in *TNFRSF1A*, even after sequencing all of the exons, flanking intronic regions, and promoter regulatory sequences. One of the families was a three-generation pedigree with affected members in each generation—and, therefore, was informative for linkage analysis. Even though the first two markers were not informative in this family, from *TNFRP55* to *D12S364* opposite haplotypes were inherited in the two affected siblings in the second generation (fig. 5), thus ruling out *TNFRSF1A* mutations

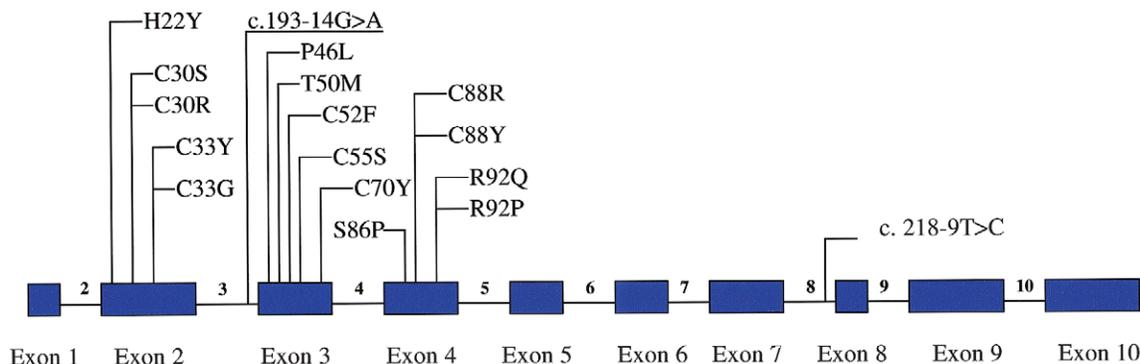


Figure 6 Genomic structure of *TNFRSF1A* (adapted from Fuchs et al. 1992). All 16 known TRAPS-causing mutations are clustered in exons 2–4, which encode the *TNFRSF1A* extracellular domain. The c.218–9 T→C polymorphism is shown in intron 8 of *TNFRSF1A*.

3' to this microsatellite locus in the intron following exon 1. When these results are considered together with our sequence analysis of exon 1 and the promoter region, it is highly unlikely that *TNFRSF1A* is the susceptibility locus. The hyperimmunoglobulinemia D with periodic fever syndrome (HIDS [MIM 260920]) and FMF were considered in the differential diagnosis, but the dominant pattern of inheritance is not consistent with either of these diagnoses. Nevertheless, the affected members were screened for a panel of 19 known FMF mutations; and they tested negative. Linkage analysis for the MWS/FCU region on distal chromosome 1q44 also identified recombinants within the family (data not shown), therefore excluding this locus as well. The second family of Irish background with parent-to-child transmission of periodic fevers was too small to be informative for linkage analysis, but no change was identified in *TNFRSF1A* when both the coding region and the promoter region were sequenced.

Moreover, in our mutational survey of 150 patients with periodic fever and clinical histories suggestive of TRAPS, 120 had no mutations in exons 2–5. Ninety patients were further analyzed for mutations in the remainder of *TNFRSF1A* (exons 1 and 6–10), by a combination of DHPLC and sequencing; no mutation was identified, except a new polymorphism (i.e., SNP) in intron 8, at position c.218–9 T→C (fig. 6). This sequence change was evaluated in 88 control chromosomes (CEPH and Irish), and allele frequencies were .78 (T) and .22 (C). In 80 chromosomes from patients, the frequencies were the same—that is, .78 (T) and .22 (C).

Discussion

In the present article, we have reported six novel mutations, in *TNFRSF1A*, as a cause of the recently described dominantly inherited periodic fever syndrome, TRAPS. These novel mutations confirm our initial report

demonstrating a role for the p55 molecule in the pathogenesis of autoinflammatory disease (McDermott et al. 1999). The total number of *TNFRSF1A* mutations that have been described thus far is 16, including 4 mutations (C30S, R92P, C55S, and C70Y) that recently have been reported elsewhere (Dodé et al. 2000; Aganna et al. 2001; Jadoul et al. 2001; Simon et al. 2001). In contrast to our previous study, in which five of six *TNFRSF1A* mutations involved cysteine residues, in the present study five of six novel TRAPS mutations were identified at noncysteine residues. As a group, the noncysteine mutations are less frequently associated with amyloi-

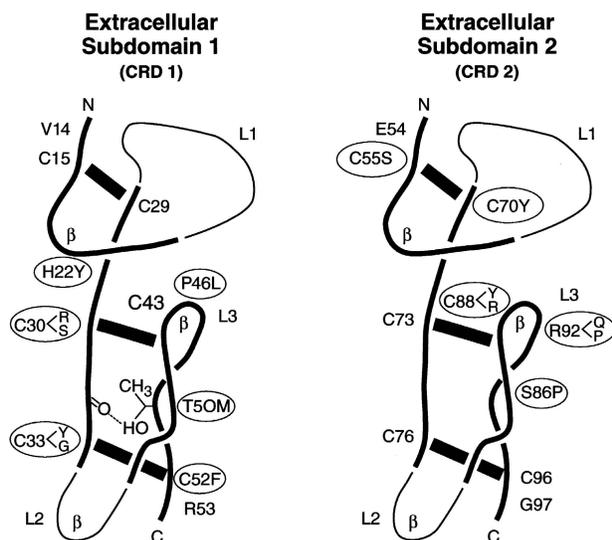


Figure 7 Crystallographically determined structure of the *TNFRSF1A* extracellular domains 1 and 2 (Banner et al. 1993). TRAPS mutations are shown as circled amino acids. The three disulfide bonds of CRD1 and CRD2 are depicted by thick black bars. Structurally conserved regions of the CRDs are represented by thicker lines. The β -turn positions are indicated by “ β .” Loop domains are denoted as “L1”–“L3.”

dosis—and perhaps are less penetrant—than the cysteine substitutions.

Our new data are consistent with our previous observation that dominantly inherited periodic fevers can be caused by structural changes in the extracellular domain of *TNFRSF1A*, but we now have a broader view of the mutations that can cause this phenotype. Of the 16 TRAPS mutations described thus far, 15 are missense, and all 16 are clustered within the first two cysteine-rich extracellular subdomains (i.e., CRD1 and CRD2) of the protein, spanning exons 2–4 of the genomic sequence (fig. 6). If one of the mechanisms by which TRAPS mutations cause an autoinflammatory state is interference with activation-induced receptor cleavage, then it may well be that more-drastic nonsense or frameshift mutations, resulting in a truncated or non-expressed protein, would not have the same phenotype. Extensive analysis of coding and exon-flanking sequences of 90 patients referred for TRAPS did not identify any disease-causing mutation, either in the transmembrane or in intracellular domains of *TNFRSF1A*.

The nine cysteine substitutions thus far identified all disrupt conserved extracellular disulfide bonds (fig. 7): C30R, C30S, C33Y, C33G, and C52F would disrupt the second or third disulfide bond in the first extracellular subdomain (i.e., CRD1), whereas C55S, C70Y, C88R, and C88Y would abolish the first or second intrachain disulfide bond in the second extracellular subdomain (i.e., CRD2). The observation of multiple mutations at residues C30, C33, and C88 suggests a critical role for these sites that is not immediately apparent from the p55 crystal structure (Banner et al. 1993; Naismith et al. 1996).

Among the six noncysteine missense substitutions, T50M most likely prevents the formation of a highly conserved intrachain hydrogen bond (fig. 7). Three other sequence variants—P46L, S86P, and R92P—involve proline residues that would be predicted to introduce a bend into the protein secondary structure, and therefore such mutations might have an important effect on the folding of the extracellular domain. The ligand-binding domain of the p55 molecule is between amino acid 77 and amino acid 114 (Banner et al. 1993), and thus the mutations at residues 86 and 92 may affect TNF binding. H22Y, P46L, and R92Q fall within the β turns of loop 3 of CRD1 and CRD2 (fig. 7), and therefore these replacements may have a significant impact on overall loop structure.

This article is also the first report of a *TNFRSF1A* splice mutation associated with periodic fever. We have provided strong evidence, both from the cDNA sequence in patients and from the analysis of minigene constructs, that the c.193–14 G→A mutation results in the addition of four amino acids to CRD1. This and other mutations within CRD1 (amino acids 1–54) may

affect the ligand-independent assembly of *TNFRSF1A* trimers on the cell membrane (Chan et al. 2000).

We have previously provided evidence that the C52F mutation is a novel type of dominant negative, in which “negative” refers not to a failure of signaling but, rather, to a failure in cytokine-receptor clearance (McDermott et al. 1999). Receptor cleavage may have a homeostatic effect both by reducing the number of available membrane sites for repeated stimulation and by producing a pool of potentially antagonistic soluble receptors, and thus a defect in cleavage could lead to a hyperinflammatory state. In the present article, we have extended the finding of impaired activation-induced shedding to other mutations. However, we were unable to demonstrate a defect in shedding *in vitro* for monocytes from patients with R92Q and the splicing mutation, despite the facts that (a) serum p55 levels did not increase with attacks for R92Q and (b) the splicing mutation was seen only in families with TRAPS symptoms. These data suggest that additional pathogenetic mechanisms may be operative in TRAPS.

The present study also provides important insights into genotype-phenotype correlations and related issues of penetrance. This article presents the first comprehensive analysis of *TNFRSF1A* genotypes seen in association with systemic amyloidosis, the most serious and life-threatening complication of TRAPS. Overall, we found that ~14% of patients thus far reported with TRAPS have developed amyloidosis, with the risk skewed strongly toward individuals with cysteine substitutions. The prevalence of amyloidosis among patients with TRAPS is significantly higher than previously recognized. This is most likely due to the fact that, prior to the identification of *TNFRSF1A* as the underlying gene, it was unclear whether families with periodic fever and amyloidosis (Gertz et al. 1987; Zaks and Kastner 1997) should be grouped with the index family for FHF, in which amyloidosis was uncommon. Molecular analysis has enabled proper diagnosis and the accurate assessment of the true prevalence of this complication. We also found that, relative to individuals with cysteine substitutions, individuals with noncysteine mutations have a tendency toward lower overall penetrance, although it should be emphasized that, even for cysteine substitutions, penetrance is not 100%. At present, we have insufficient clinical data to determine whether, aside from amyloidosis, patients with cysteine substitutions have more-severe disease.

Of the *TNFRSF1A* substitutions reported here, P46L and R92Q are likely to have the lowest penetrance for TRAPS, with each having been found in ~1% of control chromosomes. Despite these high frequencies in controls, we believe that both substitutions are either low-penetrance mutations or functional variants, rather than benign polymorphisms. As noted above, P46L would

be predicted to have a significant effect on the loop structure of p55 molecules, and the one patient with this substitution whom we have seen has symptoms very typical of TRAPS, with a favorable response to anti-TNF therapy. Moreover, we were able to demonstrate a defect in activation-induced TNFRSF1A shedding in monocytes from a patient with P46L. In the case of R92Q, the clinical picture is somewhat more heterogeneous, but the frequency of this substitution in our cohort with periodic fever was significantly higher than that in the control population. Although we did not observe a defect in R92Q TNFRSF1A shedding in vitro, soluble p55 serum levels in vivo did not increase with attacks.

On the basis of the hypothesis that R92Q might have a broader influence on susceptibility to inflammation, we have examined the frequency of this substitution in a cohort of 135 patients with early arthritis; we found 7 patients to be positive ($P = .06$, Fisher exact test), and a larger study is in progress. It is possible that the phenotypic manifestations of R92Q depend on other linked or unlinked modifying genes and/or on modifying environmental factors (Dipple and McCabe 2000). These results are particularly intriguing in light of the recently reported possible susceptibility locus for rheumatoid arthritis, in the *TNFRSF1A* region of chromosome 12 (Jawaheer et al. 2001).

Haplotype analysis of TRAPS chromosomes bearing R92Q revealed a common intragenic haplotype (G-140 bp-C) associated with this substitution. Since this haplotype comprises only intragenic markers (and does not extend to flanking microsatellites) and is seen in a number of different populations, the data suggest a very ancient common founder. In light of the current theories of the genetics of complex diseases (Lander 1996; Risch and Merikangas 1996; Collins et al. 1997), it will be interesting to determine whether R92Q carriers with non-TRAPS early arthritis also carry this haplotype. It is also noteworthy that we did not identify a common founder haplotype associated with T50M, a mutation detected in several families of Scottish or Irish descent; our data suggest instead that T50M is a recurrent mutation arising at a CpG hotspot.

The diversity of T50M carrier chromosomes in the Scottish/Irish families is but a part of the rather unexpected genetic heterogeneity of TRAPS seen in these populations. Although TRAPS has been seen in several ethnic groups, a disproportionately large percentage of families are of Irish or Scottish descent. Altogether, we have observed 10 of 16 currently known mutations in families of Irish and/or Scottish ancestry. Although ascertainment bias remains a possibility that is difficult to exclude, it is also possible that there is a high frequency of permissive alleles at one or more interacting loci in

the Irish and Scottish populations, thereby increasing the penetrance of *TNFRSF1A* mutations. A similar hypothesis has been proposed to explain the high frequency of limb-girdle type 2A muscular dystrophy on Reunion Island (the “Reunion Paradox”), as well as Hurler syndrome and metachromatic leukodystrophy in the lower Galilee in Israel, in the absence of high-frequency founder mutations (Beckmann 1996).

The last 5 years have witnessed striking advances in our understanding of the molecular pathogenesis of the hereditary periodic fevers (Centola et al. 1998; Drenth and van der Meer 2000). The genes underlying FMF (French FMF Consortium 1997; International FMF Consortium 1997), HIDS (Drenth et al. 1999; Houten et al. 1999), and TRAPS have all been identified, and susceptibility loci for MWS and FCU have been mapped to chromosome 1q44 (Cuisset et al. 1999; Hoffman et al. 2000; McDermott et al. 2000). Data presented in the present article suggest that yet other loci play a role in the pathogenesis of periodic fever and inflammation in man. Whether these loci encode new members of the pyrin, TNF, or mevalonate pathways or encode proteins in yet other pathways remains to be established. As is the case for the p55 TNF receptor, it is likely that these other genes will eventually be found to play an important role in a number of human inflammatory diseases.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Centers for Disease Control and Prevention, <http://www.cdc.gov/epiinfo/ei6.htm> (for Epi Info 6 statistical software packages)
 HGSDBASE, <http://hgbase.cgr.ki.se> (for allele frequencies of SNP in exon 1 [accession number SNP000005194] and SNP in intron 5 [accession number SNP000005195])
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for FCU [MIM 120100], FHF [MIM 142680], FMF [MIM 249100], HIDS [MIM 260920], and MWS [MIM 191900])

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