Chromosomal Assignment of the Human Thrombin Receptor Gene: Localization to Region q13 of Chromosome 5

By Wadie F. Bahou, William C. Nierman, A. Scott Durkin, Cheri L. Potter, and Douglas J. Demetrick

A functional thrombin receptor (TR) structurally related to other members of the seven-transmembrane receptor family has been isolated from diverse cellular types intimately involved in the regulation of the thrombotic response. This receptor recapitulates many of the previously identified sequelae of thrombin-mediated cell activation phenomenon, and requires proteolytic cleavage for downstream effector-response coupling events. Using two complementary approaches, we have now completed the chromosomal assignment of the human thrombin receptor gene. Discordancy analysis of polymerase chain reaction products from a human-rodent hybrid cell mapping panel assigned the sequence to human chromosome 5 with no observed discordancies. Cytogenetic localization using fluorescence in situ hybridization on human metaphase chromosomes specifically localized the human TR gene to region q13 of chromosome 5, confirming its presence as a single-locus gene in the human genome. The chromosomal localization of the human TR gene is at or contiguous with the proximal breakpoint site identified in the majority of patients with the 5q− syndrome (dysmegakaryocytes and refractory anemia).

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THE SERINE PROTEASE α-thrombin plays a critical role in the hemostatic response. Its assorted effects include the clotting of fibrinogen and activation of factor XIII (thereby stabilizing the fibrin clot) and feedback activation of factors V, VIII, and XI, resulting in amplification of the coagulation cascade. Additionally, α-thrombin displays a number of effects on diverse cellular types. It is among the most potent of platelet-activating agents, is mitogenic for vascular smooth muscle cells and fibroblasts, is chemotactic for monocytes, and its interaction with the endothelium results in the release of von Willebrand factor, tissue factor, and endothelial cell retraction, resulting in selective permeability of the vascular wall. A functional thrombin receptor (TR) has recently been isolated from a megakaryocytic cell line. The cDNA spans 3,480 nucleotides encoding a predicted primary translation product of 425 amino acids, with structural similarity to other members of the G-protein-linked family of seven-transmembrane segment receptors. A model for TR activation has been postulated that incorporates proteolytic cleavage with subsequent activation by a newly created "tethered ligand." In platelets, peptides modeled after the new N-terminus after thrombin cleavage and proteolytically active thrombin similarly lead to accumulation of 3-phosphorylated phosphoinositides (3,4-bisphosphate and 3,4,5-trisphosphate), elevations of cytosolic calcium, and inhibition of adenyl cyclase. That the long N-terminal extension contains sites crucial for peptide ligand-mediated activation phenomenon has also been postulated.

Despite considerable investigation into the mechanism(s) of TR activation and cell signaling pathways, the molecular characterization of the human thrombin receptor gene has been incomplete. In this report, we have used two complementary approaches to complete the chromosomal assignment of the human TR gene. Discordancy analysis of polymerase chain reaction (PCR) products from a human-rodent hybrid cell mapping panel conclusively assigned the sequence to human chromosome 5. Further cytogenetic localization using fluorescence in situ hybridization (FISH) detected a unique fluorescent signal localizing the TR gene to the region 5q13, confirming that the TR is present as a single-locus copy in the human genome. The human TR gene localizes to a region on chromosome 5 that is at or contiguous to the proximal breakpoint site identified in the majority of patients with the 5q− syndrome.

MATERIALS AND METHODS

Somatic cell hybrid panels. Genomic DNA from human-rodent somatic cell hybrid lines was purchased from The Coriell Institute for Medical Research (Camden, NJ). The production and characterization of this hybrid panel has been previously described. The National Institutes of General Medical Sciences (NIGMS) panel 1 is based primarily on a mouse background retaining from 1 to 19 human chromosomes. Because of underrepresentation, two monochromosomal cell lines are included in the panel (human chromosomes 9 and 16), one of which is generated on a hamster background (chromosome 9).

PCR. The antisense oligonucleotide primer (5'-CACGAATTCTCAATGATAGACATAACAGCATG-3') corresponding to nucleotides 1106-1082 of the TR cDNA was synthesized on an Applied Biosystems, Inc (ABI) single-channel synthesizer (Foster City, CA). This primer contains a specifically engineered EcoRI site (bold) and an additional stop codon (underscored). The sense primer (5'-CACGGATCTATTATCTTTCCGCCAGTGATTGG-3') corresponding to nucleotides 705-728 was synthesized.

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Fig 1. Gel profile analysis of amplified PCR products from four representative chromosome-5-containing cell lines. In each case, DNA from NIGMS panel no. 1 individual cell lines containing human-rodent somatic cell hybrids was amplified by PCR using oligonucleotide primers and conditions described in the Materials and Methods. Samples were size-fractionated in a 6% polyacrylamide denaturing gel containing 8 mol/L urea, and data from the electrophoretic analyses were collected on an automated sequencer configured with GeneScanner software designed to quantify peak fluorescence. Size standards are delineated by open arrows (D) and are identical to those used in (A) through (C). The identical 422-bp fluorescent peak representing the amplified human thrombin receptor sequence is readily observed in all panels (solid arrow). Refer to Table 1 and text for details.

by Genosys Biotechnologies, Inc (The Woodlands, NJ) containing a fluorescein-derived 5' FAM label (ABI) and contains a specifically engineered BamHI site (underscored). Previous work in this laboratory has established that this region of the cDNA is contained within a single large exon (W.F. Bahou, unpublished data), thereby allowing for direct amplification using human genomic DNA as template. For PCR, 50 ng of hybrid cell template DNA was amplified in the presence of 40 ng of each primer in a total volume of 15 μL, essentially as previously described. Amplification conditions were as follows: 95°C for 5 minutes, followed by 25 cycles of 94°C for 1.4 minutes, 55°C for 2 minutes, 72°C for 2 minutes, and a final incubation at 72°C for 10 minutes. Two microliters of the PCR product was then pooled with 0.5 μL of fluorescently labeled internal lane size standard (GS2500; ABI) and 2.5 μL of deionized formamide. Samples were denatured in a boiling water bath for 2 minutes before loading onto a 6% polyacrylamide denaturing gel containing 8 mol/L urea. Data from the electrophoretic analyses were collected on an ABI 373A automated sequencer configured with ABI 672 GeneScanner software. For analysis, a "positive discordancy" indicates a discordancy due to the chromosome being detected when the chromosome is theoretically absent in the cell line, and a "negative discordancy" indicates discordancies due to a chromosome not being detected when the chromosome is present in a minimum of 12% of the cell line. Cell lines reported as containing a particular chromosome in fewer than 12% of metaphases were excluded from the discordancy analysis.

FISH analysis. The probe used in the hybridization is a genomic fragment of the TR isolated from an EMBL3 human genomic library (Clontech Laboratories, Inc, Palo Alto, CA). The molecular characterization of the human TR gene has been presented in preliminary form, and will be described in more detail elsewhere. The genomic probe encompasses the 3'-untranslated region, the major region of the TR coding sequence, and a region flanking the intronic sequence. FISH was performed using established methods. The 18-kb genomic fragment cloned into the Sal I site of pBluescript (Stratagene, La Jolla, CA) was directly labeled by nick translation using digoxigenin dUTP and purified over a G-50 Sephadex spin column (Pharmacia/LKB, Piscataway, NJ).

Metaphase chromosome spreads prepared from normal lymphocytes stimulated with phytohemagglutinin for 72 hours were synchronized by incubation with methotrexate (0.1 μmol/L for 16 hours), followed by thymidine rescue (10 μmol/L for 7 hours). The lymphoblasts were incubated with 0.12 μg/mL colcemid (GIBCO-BRL, Gaithersburg, MD) for 10 minutes, resuspended in methanol-acetic acid fixative, and dropped onto ethanol-cleaned glass slides; samples were subsequently air-dried and baked for 4 hours at 65°C. Before hybridization, chromosome preparations were denatured for 4 minutes at 85°C in 70% formamide/2× SSC (1× SSC is 150 mmol/L NaCl, 15 mmol/L Na citrate), quenched immediately in ice-cold 70% ethanol, and dehydrated in room temperature 95% and 100% ethanol. Hybridization occurred overnight in a humidified chamber at 37°C with 1 μL of probe in a solution containing 50% formamide, 10% dextran sulphate, 2× SSC, and 1 mg/mL human DNA in a total volume of 10 μL. After hybridization, the slides were washed for 5 minutes each in 50% formamide/2× SSC (3×) and 2× SSC (3×) at 44°C, followed by a room temperature rinse in phosphate-buffered saline (PBS), and then stained with...
FITC/Texas Red/DAPI; Chroma Technology, Inc, Brattleboro, described in the Materials and Methods amplified a single slide film (Eastman Kodak Co, Rochester, NY).

15 µg/mL of sheep antidigoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) for 45 minutes at 25°C. After washing, slides were then incubated with a 1/40 dilution of Cy3-conjugated sheep antiserum (Jackson Immunoresearch, West Grove, PA) for 45 minutes at 25°C, washed as above, and then stained for 20 minutes in 4,6 diamidin-2-phenylindole dihydrochloride (DAPI; Fluka Chemika-Biochemika, Ronkonkoma, NY) (0.25 µg/mL). After a brief rinse in PBS, the slides were stained with actinomycin D (0.25 mg/mL) in 10 mmol/L sodium phosphate/1 mmol/L EDTA for 20 minutes,17 rinsed twice with PBS, and then mounted in antifade medium18 and visualized using a Microphot FXA microscope (Nikon, Garden City, NY) and triple bandpass (FITC/Texas Red/DAPI; Chroma Technology, Inc, Brattleboro, VT) filter. Photographs were prepared utilizing Kodachrome 200 slide film (Eastman Kodak Co, Rochester, NY).

Table 1. Assignment of Human TR to Chromosome 5

<table>
<thead>
<tr>
<th>Human Chromosomes</th>
<th>No. of Hybrid Clones* (human TR/chromosome present)†</th>
<th>% Discordant</th>
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<tr>
<td>1</td>
<td>2 9 6 2</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>3 8 5 3</td>
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<td>8</td>
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<td>3 10 4 0</td>
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The human TR sequences were amplified from the rodent-human somatic cell hybrid panel using the oligonucleotide primers and conditions described in the Materials and Methods (also refer to Fig 1). The human TR gene localizes to human chromosome 5 with no observed discordancies in 17 cell lines. * Use of a hybrid in the discordancy analysis requires that the cell line possess the chromosome under evaluation in more than 12% of the metaphase spreads used to characterize the cell line. † The numerator indicates the presence (+) or absence (−) of the human TR PCR product. The denominator indicates the presence (+) or absence (−) of the specific human chromosome. ‡ The percent discordant is calculated by summing the number of discordant hybrids (+/− and −/+) and then dividing the sum by the total number of hybrids analyzed.

RESULTS

The PCR product using the oligonucleotide primer pair described in the Materials and Methods amplified a single product of the appropriate size (422 bp) from human genomic DNA, as judged by polyacrylamide gel electrophoresis (not shown). The identical primer pair failed to amplify endogenous rodent TR sequence using murine or hamster total genomic DNA as template, either suggesting DNA sequence differences in the portions of the rodent genes contained within the primer sequence or a different genomic organization with an associated large intron. Although the sequence of the murine TR homolog is not available, the cDNAs for both rat19 and hamster TRs20 allow for comparative analysis. Within the regions of the oligonucleotides, limited homologies of 75% to 83% are observed between human and rat/hamster sequences (the sense primer alone was 96% homologous to hamster sequence), suggesting that the nucleotide discrepancies cross-species are primarily responsible for the lack of amplification of nonhuman TR sequences.

The chromosomal assignment of the TR gene was then established using the NIGMS panel of somatic cell hybrids and the same oligonucleotide primer pair. The gel profile of the analysis of the PCR product of four of the eight cell lines shown to contain human TR sequences is displayed in Fig 1. In all situations, a single discrete amplifiable fragment of the identical size was identifiable. No amplifiable product was detected in the remaining cell lines. The pattern of amplification (Fig 1 and Table 1) unambiguously places the thrombin receptor gene on chromosome 5, with no discordances from 17 evaluable cell lines. The chromosome with the next fewest discordancies was chromosome 21, which displayed 3 discordant cell lines, thereby conclusively supporting the chromosome 5 assignment.

To more precisely map the human TR gene, FISH analysis was completed using chromosome spreads from lymphocyte cultures of a normal volunteer. Figure 2A illustrates a single mitosis showing the presence of dual-chromatid staining of both copies of chromosome 5. No other sites of staining were present, confirming that the TR gene exists at a single locus on chromosome 5. Higher resolution of a single chromosome 5 (Fig 2B) shows the fine localization of the gene to band q13, a pattern that was evident in most metaphase preparations. The human TR gene localizes to a region on chromosome 5 that contains the genes for a number of hematopoietic growth factors, cytokines, and receptors, and is at or contiguous to the proximal breakpoint site identified in the majority of patients with interstitial deletions of the long arm of chromosome 5 (5q− syndrome).21

DISCUSSION

The identification of a functional TR on a number of responsive cell lines has enhanced our appreciation of the events involved in thrombin-mediated cell signaling and activation. The cDNAs for identical receptors have now been isolated from platelets,2 human umbilical vein endothelial cells,1 rat vascular smooth muscle cells,1 and hamster fibroblasts.20 This functional TR recapitulates many of the previously identified sequelae of thrombin activation, including phosphoinositide hydrolysis, elevations of cytosolic calcium transients, inhibition of adenyl cyclase, and tyrosine phosphorylation of multiple platelet proteins.8,10,22,25 Although
its role in platelet activation is well-characterized, its precise role in α-thrombin–induced mitogenesis remains inconclusive. In fibroblasts and vascular smooth cells, α-thrombin–induced mitogenic pathway(s) appears to be distinct from those associated with elevations of [Ca$^{2+}$], transients. Indeed, responses to α-thrombin in diverse cellular types suggest the presence of dual activation pathways and/or coupling mechanisms.

Preliminary work in this and other laboratories has suggested that the TR gene is of limited size and complexity. Based on the genomic organization of the human TR gene, we have used specifically designed oligonucleotide primers and PCR analysis of a rodent somatic cell hybrid panel for initial assignment of the human TR gene to chromosome 5. The presence of a unique signal on region q13 as detected by FISH analysis more precisely localizes the TR gene, and confirms previous observations that the gene is present as a single copy in humans and rats. The long arm of human chromosome 5 contains the genes for a number of hematopoietic growth factors, cytokines, and receptors. To date, the granulocyte-macrophage colony-stimulating factor gene, the cytokine gene cluster (interleukins-3, -4, and -5), the colony-stimulating factor-1 gene, and the colony-stimulating factor-1 receptor gene have all been localized to a defined region of 5q. Of note, another seven-transmembrane receptor, the β$_2$-adrenergic receptor, has also been localized to this portion of chromosome 5.

Loss of a portion of the long arm of chromosome 5 has been observed in distinct hematologic conditions, including chemotherapy-associated myelodysplasia, acute leukemia, and refractory anemia with dysmegakaryocytosis (5q– syndrome). The association of an acquired interstitial deletion of chromosome 5 with malignant or dysplastic hematopoiesis raises presumptive evidence that the deleted growth factor and receptor gene(s) may be involved in the process of transformation and clonal cell growth. However, the mechanism(s) whereby a hemizygous deletion within this gene cluster could result in the altered maturation and growth of hematopoietic cells remains enigmatic. In this regard, the recent observation of homozygous gene deletions of both CSF1R alleles in a subset of patients with myelodysplasia is especially noteworthy.

Although some variability exists in the regions on chromosome 5 involved in the breakpoint, the interstitial deletion generally shows a proximal breakpoint in the region q13-q15 and a distal breakpoint at q31-q33. A recent study of 43 consecutive patients with the 5q– syndrome documented that the proximal breakpoint site in 70% of the patients was located within the region q13. This site is at or contiguous with our chromosomal localization data for the human TR gene. It is noteworthy that a characteristic morphologic manifestation of the 5q– syndrome is dysmegakaryocytosis with poorly lobulated nuclei. To date, the isolation and characterization of cytokines specifically
regulating the growth and differentiation of megakaryocytes is incomplete, although a thrombopoietin expressly involved in “megakaryocytic polyploidyization” has been postulated. Interestingly, α-thrombin has been shown to stimulate megakaryocytic differentiation in a human megakaryoblastic leukemia cell line, although this effect is not conclusively mediated by the functional TR. Whether the TR gene is deleted in patients with the 5q− syndrome, and if so, how altered expression of the TR could be pathogenically involved in the clinical and molecular features of this syndrome is currently under investigation.

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