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A Molecular Genetic Analysis of Thrombin Receptor Function in Mice.

**by**

Andrew J. Connolly

**DISSERTATION**

**Submitted in partial satisfaction of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY**

**in**

Biomedical Science

**in the**

**GRADUATE DIVISION**

**of the**

**UNIVERSITY OF CALIFORNIA**

**San Francisco**

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**DEDICATION:**

This dissertation is dedicated to my wife, Susan, and daughters, Sara and Diana, for their love and support during this work.

I would like to thank my colleagues in the lab for their help and good cheer. I would especially like to thank Shaun Coughlin for his patience and guidance during the past five years.

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**PREFACE:**

I would like to acknowledge the efforts of the people involved in the studies presented in Chapters 3-6. For Chapters 3 and 5, Hiroaki Ishihara was instrumental in the analysis of platelet function in *par*1 -/- mice and the cloning of PAR3. After I had produced the *par*1 -/- mice, the two of us essentially functioned as a team to investigate the two thrombin receptors in mice.

In Chapter 4, JoAnn Trejo was entirely responsible for the PAR1 signaling studies in CCL39 cells and wild type mouse fibroblasts. My contribution to the paper was to produce the *par*1 +/+ and *par*1 -/- fibroblasts and to initially characterize their thrombin-induced responses in intracellular calcium, phosphoinositide hydrolysis, and cAMP. The JBC paper was included in Chapter 4 to demonstrate the absence of responses to thrombin in *par*1 -/- fibroblasts, in contrast to the presence of responses to thrombin in *par*1 -/- platelets.

For Chapter 3, Robert Farese and his laboratory provided technical assisstance during the ES cell handling and blastocyst injection to produce the *par*1 -/- mice. For Chapter 5, Dewan Zeng performed signaling studies in COS cells to demonstrate that human PAR3 signals by a mechanism similar to that of PAR1. Yao Wu Zheng expressed PAR3 protein to demonstrate that PAR3 is cleaved by thrombin. Tracy Tram provided technical support for the Xenopus oocyte experiments. Mark Kahn and Courtney Timmons assisted in isolation of receptor genomic clones. For Chapter 6, David Suh and I performed the quantitative wound healing assays together.

The text of Chapters 3, 4, and 5 are reprints of the material as they appear in Nature and the Journal of Biological Chemistry.

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**STATEMENT BY RESEARCH ADVISOR:**

Andy's account of his contribution is factual but overly modest. He was the driving force behind the *par1* knockout, the first knockout done in our lab. Help from Robert Farese was at the level of ES cell electroporation and blastocyst injection. The resulting knockout mice provided several surprises that were reported in Nature (Chapter 3). The finding that the coagulation system apparently plays a role in embryonic development unrelated to defective blood clotting was Andy's. It has been corroborated by subsequent knockouts of tissue factor, Factor V, and prothrombin. Identification of the mechanism underlying embryonic death in the *par1* null mice is now an active area of inquiry in our lab.

The finding that par 1 null platelets still responded to thrombin while *par1* null fibroblasts did not provided definitive evidence for a second thrombin receptor in mouse platelets. Hiroaki Ishihara, D.V.M., had specialized skills in preparing and characterizing rodent platelets. Andy wisely availed himself of such a valuable resource to characterize *par1* null platelets, but he remained involved in the platelet studies both intellectually and at the bench.

Andy and Hiroaki worked together to identify PAR3. Andy took an expression cloning approach while Hiroaki used degenerate PCR. Andy contributed intellectually to all aspects of this project, even designing the degenerate PCR primers that finally worked! Both Hiroaki and Andy did a tremendous amount of work and it was difficult deciding who should be first author on the resulting Nature paper (Chapter 5). Once again this study is enabling: we expect that the *par3* null mouse will (finally) enable us to determine whether thrombin signaling is indeed critical for platelet activation.

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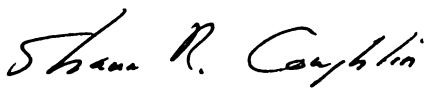
Andy's work is a major contribution to our understanding of thrombin signaling and its roles in vivo. Characterization of the *par1* knockout and the resulting dentification of PAR3 are milestones in this area and, in my opinion, constitute an outstanding thesis.



Shaun R. Coughlin
Professor, Department of Medicine
Director, Cardiovascular Research Institute
University of California, San Francisco

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**ABSTRACT:**

Thrombin is a serine protease generated at sites of vascular injury. In addition to its well-described roles in coagulation, thrombin directly activates a number of cell types including platelets, endothelial cells, vascular smooth muscle cells, fibroblasts and lymphocytes. The varied cellular activities of thrombin suggest that, in addition to its role in hemostasis, thrombin may be involved in inflammatory and reparative responses to injury. A proteolytically-activated receptor that mediates thrombin responses has been cloned. To test the necessary roles of this thrombin receptor *in vivo*, mice were created with null mutations in the thrombin receptor gene (tr -/-). One half of the tr -/- mice died *in utero* during development of the early circulatory system, revealing an unexpected role for the receptor. tr -/- mice that survived to adulthood had normal wound healing and did not have spontaneous bleeding disorders. Platelets from tr -/- mice had normal responses to thrombin while tr -/- fibroblasts lacked responses to thrombin. This provided definitive evidence for a second thrombin receptor and demonstrated that the two thrombin receptors have distinct patterns of expression. The second thrombin receptor was cloned and shown to be homologous to the first receptor. The receptor is cleaved and activated by thrombin by a mechanism similar to that of the first receptor. In mice, high-level expression of the second receptor is restricted to megakaryocytes and platelets.

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**TABLE OF CONTENTS:**


| | |
|---|---|
| i | Title Page |
| ii | Copyright Page |
| iii | Dedication |
| iv | Preface |
| v-vi | Statement by Research Advisor |
| vii | Abstract |
| viii | Table of Contents |
| ix | List of Tables |
| x | List of Figures |
| 1-6 | Chapter 1: Introduction<br><br>A) Thrombin activates many cell types *in vitro.*<br>B) The thrombin receptor is a G protein-coupled receptor.<br>C) Cellular activation by thrombin is mediated by thrombin receptor.<br>D) Rationale for a genetic approach to defining the thrombin receptor's role *in vivo*.. |
| 7-13 | Chapter 2: Methods |
| 14-18 | Chapter 3: Role of the thrombin receptor in development and evidence for a second receptor. |
| 19-25 | Chapter 4: Loss of responses in fibroblasts from receptor knockout mice. |
| 26-31 | Chapter 5: Protease-activated receptor 3 is a second human thrombin receptor. |
| 32-48 | Chapter 6: Mice lacking the thrombin receptor, PAR1, have normal skin wound healing. |
| 49-51 | Chapter 7: Summary |
| 52-57 | Bibliography |




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**LIST OF TABLES:**


| | |
|---|---|
| Chapter 3, Table 1 | Pharmacology of platelet activation. |
| Chapter 6, Table 1 | Biochemical analysis of skin wounds. |


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**LIST OF FIGURES:**


| | |
|---|---|
| Chapter 3, Figure 1 | Disruption of the thrombin receptor gene. |
| Chapter 3, Figure 2 | Hemostasis and platelet responses. |
| Chapter 3, Figure 3 | Tissue-specific roles for the cloned thrombin receptor. |
| Chapter 4, Figure 1 | Thrombin- and thrombin receptor agonist peptide-induced DNA synthesis in fibroblasts from tr +/+ and -/- mice. |
| Chapter 4, Figure 2 | Kinetics of MAP kinase activation by thrombin and thrombin receptor agonist peptide in wild-type mouse lung fibroblasts and CCL-39 cells. |
| Chapter 4, Figure 3 | Thrombin and thrombin receptor agonist peptide effects on MAP kinase activation in lung fibroblasts from tr +/+ and -/- mice. |
| Chapter 4, Figure 4 | Inhibition of thrombin-stimulated MAP kinase activity by pertussis toxin and phorbol ester pretreatment. |
| Chapter 4, Figure 5 | Inhibition of thrombin-stimulated c-raf kinase activity by pertussis toxin but not phorbol ester pretreatment. |
| Chapter 5, Figure 1 | Amino acid sequence of human PAR3. |
| Chapter 5, Figure 2 | Thrombin cleaves PAR3. |
| Chapter 5, Figure 3 | PAR3 signaling. |
| Chapter 5, Figure 4 | Expression of PAR3 mRNA. |
| Chapter 6, Figure 1 | Analysis of wound closure and tensile strength in *par*1 +/+ and -/- mice. |
| Chapter 6, Figure 2 | Histology of healing skin incisions in *par*1 +/+ and *par*1 -/- mice. |


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# Chapter 1. Introduction

**A) Thrombin activates many cell types *in vitro*.**

Thrombin is a multifunctional serine protease generated at sites of vascular injury. Active thrombin is sequestered in the fibrin clot where it is protected from neutralization by plasma proteinase inhibitors and may be released later (1-3). While thrombin's best-studied actions have been those involved in blood coagulation, thrombin is also a powerful agonist for a variety of cellular responses (4).

Thrombin is the most potent activator of platelets *in vitro* (5, 6). Thrombin causes aggregation and secretion of platelets, leading to their release of potent procoagulatant molecules and inflammatory mediators(7). Animal models (8-12) and clinical studies(13) demonstrate that thrombin itself is critical for platelet-dependent arterial thrombosis *in vivo*.

Thrombin activates vascular endothelial cells leading to increased vascular permeability (14) and increased adhesiveness for platelets and leukocytes (15). It also causes release of platelet-derived growth factor (16), platelet-activating factor (17), and monocyte chemotactic protein-1 (18) from endothelium.

Thrombin activates neutrophils (19) and is chemotactic for neutrophils (20) and monocytes (21). It is mitogenic for lymphocytes (22), fibroblasts (23), smooth muscle cells (24) and other cell types.

The apparently disparate activities of thrombin on platelets, neutrophils, monocytes, and vascular cells may be unified by viewing thrombin as an orchestrator of the tissue response to vascular injury or wounding, Perhaps thrombin mediates not only hemostatic, but also

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inflammatory and reparative responses (25). The varied cellular activities of thrombin also raise the possibility of roles in mediating the cellular events underlying arterial restenosis, atherosclerosis, vasospasm, and other pathological vascular responses (26).

**B) The thrombin receptor is a G protein-coupled receptor.**

The thrombin receptor was cloned by expression cloning in Xenopus oocytes using RNA from a human megakaryocyte cell line (27). The thrombin receptor is a member of the seven transmembrane domain receptor family with a long extracellular amino terminal exodomain (27). A body of data demonstrate that the receptor is activated by a novel proteolytic mechanism (27-32). Thrombin binds to the receptor's long amino terminal exodomain via two sites. A thrombin cleavage recognition site resembling the thrombin cleavage site in protein C interacts with thrombin's active center. Carboxyl to this site is a sequence that resembles the carboxyl tail of hirudin and binds to thrombin's anion-binding exosite (27, 31-33). After binding, thrombin cleaves its receptor between $Arg^{41}$ and $Ser^{42}$ (27, 32). This cleavage event serves to unmask a new receptor amino terminus which serves as a tethered peptide ligand, binding to an as yet undefined site within the body of the receptor to effect receptor activation (27). Synthetic peptides mimicking this "agonist peptide domain" are full agonists for receptor activation (27).

The binding sites for thrombin on the amino terminus of the receptor have been studied in some detail. Structure/Activity studies suggest that the KYEPF sequence in the receptor's amino terminal exodomain binds thrombin's anion binding exosite in a manner similar to the DFEEI sequence in hirudin's carboxyl tail (31-33). X-ray crystallographic structures of co-crystals of thrombin with receptor-based peptides support this model (34). Various



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mutations in this domain were shown to produce graded shifts in the concentration response to thrombin (32). Specifically, Y52A causes an approximately 2-log rightward shift of the concentration response to thrombin; other point mutations cause lesser shifts (32).

**C) Cellular activation by thrombin is mediated by thrombin receptor.**

Many of the cellular responses to thrombin appear to be mediated by the thrombin receptor that was previously cloned and characterized (27). In various settings, this receptor has been designated "thrombin receptor (TR)," "protease-activated receptor 1 (PAR1)", and "coagulation factor 2 receptor (Cf2r)."

Platelets Activation: The cloned thrombin receptor is expressed on platelets and activation of the receptor by agonist peptides is sufficient to cause platelet aggregation and secretion (27). Moreover, antibodies to the cloned thrombin receptor inhibit human platelet activation by thrombin (28, 35). The inhibitory effects of receptor antibodies on platelets can be overcome by high concentrations of thrombin, but this also occurs in a defined system in which responses appear to be mediated solely by the cloned receptor expressed in Xenopus oocytes (28). These data suggest that the cloned receptor is the major mediator of thrombin-induced human platelet activation. However, this has not been demonstrated *in vivo*. Furthermore, there is currently no animal model to indicate the severity and scope of side effects to be expected from blocking thrombin receptor function.

Pro-inflammatory actions. Thrombin receptor agonist peptides can mimic several of thrombin's known actions on endothelial cells (27, 36, 37). The receptor is expressed by endothelial cells (26, 27) and appears to be expressed by macrophages (26). The inflammatory properties of thrombin are



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also recapitulated by thrombin receptor-activating peptides in *in vivo* models of leukocyte rolling in rat mesenteric blood vessels (38) and acute inflammation of the rat paw (39). The necessary role for thrombin receptor in mediating inflammatory events *in vivo* remains to be defined.

Proliferative actions. At least some of thrombin's mitogenic effects are accounted for by the cloned receptor in vascular smooth muscle cells (24, 29) and fibroblasts(29). Of particular interest, the thrombin receptor is selectively expressed in human atherosclerotic plaques by smooth muscle cells, poorly-defined intimal cells, and macrophages (26). In the context of thrombin's known actions on macrophages and smooth muscle cells, these data suggest a potentially causal role for thrombin receptor activation in arterial restenosis {the proliferation of intimal smooth muscle cells that often renarrows arteries after balloon dilation of an atherosclerotic plaque} and possibly in atherogenesis itself (26, 40).

Receptor Localization. As described above, thrombin receptor expression has been verified in many cell types *in vitro* (28, 29). An *in situ* hybridization survey of multiple adult tissues in the mouse revealed high expression of thrombin receptor in megakaryocytes, platelets, endothelial cells, endocardium, specific neurons, and in glomeruli (41). During development, the receptor is widely expressed, with particularly high expression noted in endothelial and endocardial cells. In situ hybridization of human atherosclerotic plaques reveals strong expression in macrophages and vascular smooth muscle cells (26). Little is known about expression in other disease states. We hypothesize that thrombin receptor may play a role in conditions in which thrombin is generated and there is a response involving cell types known to express thrombin receptor *in vitro*. These include thrombosis,

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atherosclerosis, response to arterial injury, and various forms of wound healing.

Other Thrombin Receptors? Some authors have postulated the presence of a second distinct thrombin receptor based on the ability of thrombin, unlike agonist peptide, to stimulate mitogenesis in hamster fibroblasts (42). In addition, some thrombin-induced effects are not reproduced by receptor-activating peptides in human endothelial cells (16, 43) and mouse platelets (44). However, comparisons between the agonist properties of receptor-activating peptides and thrombin are perilous, given that peptides and thrombin activate the receptor by different mechanisms, differ in potency by at least four logs (27), and differ in pharmacokinetics *in vivo* and *in vitro* (45).

**D) Rationale for a genetic approach to defining the thrombin receptor's role *in vivo*.**

Pharmaceuticals to block thrombin receptor function and define its role *in vivo* are not available. Blocking monoclonal or polyclonal antibodies to the thrombin receptor have been raised (28, 35, 46). These have been enormously useful for *in vitro* studies and structure-function analysis, but are not sufficiently avid for *in vivo* work. Similarly, analogs of the receptor's agonist peptide domain that function as weak antagonists are lacking the potency and specificity necessary for use *in vivo*. No other pharmaceutical agents targeting the thrombin receptor are available.

Genetic disorders involving the thrombin receptor gene have not been described. No candidate syndrome for a thrombin receptor genetic disease has been described. This does not, however, preclude the existence of such a



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genetic disease. Thrombin receptor is part of a receptor family that includes hundreds of different G protein-coupled receptors. The list of human diseases associated with mutations in this type of receptor has grown recently(47, 48). These include both loss of function mutations, such as in the vasopressin 2 receptor in congenital nephrogenic diabetes insipidus (49) and the ACTH receptor in isolated glucocorticoid deficiency (50, 51), and gain of function mutations, such as thyrotropin receptor in thyroid adenomas (52) and lutropin receptor in familial male precocious puberty (53). Unlike the receptors associated with these hormonal diseases, thrombin receptor has a more complex distribution. Thus, the possible phenotypes of thrombin receptor genetic diseases are more difficult to predict.

The above discussion suggests that thrombin receptor activation may be important in hemostasis/thrombosis, wound healing, and some vascular disease. The localization of receptor expression also suggests possible roles in development and neural function. Is the cloned receptor important for any of these functions *in vivo*? The only currently available and clear cut approach to alter thrombin receptor function *in vivo* and define its role in normal and pathologic states is to produce mice with targeted disruption of the thrombin receptor gene. Mice with altered thrombin receptors will then be exploited in a variety of well-defined mouse models. These include models of thrombosis(54, 55), skin wound healing (56), and atherogenesis (57, 58).

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# Chapter 2. Methods

The methods for many of the studies in this dissertation are included in published work photocopied for chapters 3-5. In this chapter, unpublished methods or elaborations of the published methods will be presented.

**A) Genomic structure and chromosomal localization.**

The cDNAs for human (27), mouse (24, 32), rat, and Xenopus thrombin receptors had been cloned before for structure/function studies. The human and mouse thrombin receptor genes were cloned and characterized in our lab (59). The receptor is encoded by two exons; the first encodes the 5'untranslated region and the signal peptide. The second encodes the balance of the receptor, i.e. the entire mature protein. These exons are separated by a 15 kb intron. The receptor's genomic structure is convenient for gene targeting because all interesting manipulations can be made within a single exon (exon 2) which encodes the receptor's agonist peptide and thrombin binding domains as well as the seven transmembrane spans. Cytogenetic localization using fluorescence in-situ hybridization specifically localized the human thrombin receptor gene to region q13 of chromosome 5 (60) and the mouse receptor gene to the syntenic 13D2 region (59). Interestingly, this places the thrombin receptor locus among a number of hematopoietic growth factors, cytokines, and receptors on the long arm of chromosome 5, including platelet derived growth factor receptor, β2-adrenergic receptor, granulocyte-macrophage colony-stimulating factor, and interleukins-3, -4, and -5.

**B) Production of tr -/- mice..**



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For gene targeting studies, the mouse thrombin receptor gene was cloned from λ phage (λ DashII, Stratagene) and P1 bacteriophage (Genome Systems) genomic libraries for the 129SV strain of mice used to produce embryonic stem cells. The clones cover over 75 kb of genomic DNA surrounding exon 2, the region to be targeted. The targeting construct for homologous recombination consists of exon 2 (1.3kb) with 2 kb of intron 1 and 5 kb of 3' flanking region [see Figure 1 in Chapter 3]. Selectable markers were derived from the pPNT plasmid constructed by Richard Mulligan (61). This plasmid contains the herpes simplex virus thymidine kinase gene (HSV-tk) and a strong neomycin resistance gene (neo), each inserted between mouse phosphoglycerate kinase-1 (PGK-1) promoter and PGK-1 polyadenylation sequences. The 2 kb neo cassette was inserted between the BspE1 and Cla1 sites in exon 2 of thrombin receptor, thus deleting transmembrane domains 1 through 7. The 3 kb HSV-tk cassette was ligated to the 3' end of the homologous region of the construct. Positive selection for integration into a chromosome was achieved by selecting with 0.25 μg/ml G418. Enrichment for clones representing homologous recombination, as opposed to random integration, was achieved by negative selection with 0.2 μM ganciclovir.

Gene targeting in D3 (62), J1 (63), AB1 (64), and RF8 (isolated by collaborator Robert Farese) ES cells was performed from published protocols (65). In brief, ES cells were grown on a feeder layer of neomycin resistant mouse embryonic fibroblasts in the presence of leukemia inhibitory factor. Linearized targeting construct (25 μg) was transfected by electroporation into 10 x $10^7$ exponentially growing ES cells and G418/ganciclovir selection was maintained for 8 days when several hundred clones became grossly apparent. Ninety-six ES clones were picked, expanded on feeder layers, and screened by Southern blot. A 1.5 kb probe was made from a genomic DNA fragment that

flanks the 3' end of the targeting construct. This detected a 28 kb EcoR1 fragment of the wild-type allele and a 8 kb fragment of the mutant allele [see Figure 1 in Chapter 3]. In addition, polymerase chain reaction (PCR) using primers from intron and neomycin resistence gene sequence confirms the presence of homologous recombination. On average, 10% of the ES clones were homologous recombinants. A total of 20 targeted ES clones were identified and isolated from D3, AB1, and RF8 cells.

ES cells in which a single thrombin receptor allele had been targeted were injected into the blastocoele cavity of C57BL/6 blastocysts and implanted into pseudopregnant females, with the help of Dr. Robert Farese at the Gladstone Institute of UCSF. Resulting chimeras were identified by the agouti coat color. The third and fourth ES clones injected into blastocysts were from RF8 cells (clones 12 and 18) and yielded numerous chimeras with >90% agouti coat color. The mature male chimeras were mated with C57BL/6 and 129sv females. Germline transmission of the null mutation in thrombin receptor was verified by PCR and Southern blots.

**C) Platelet studies.**

Platelet Preparation Protocol:

1. Anesthetize mice with pentobarbital 100 mg/kg or Avertin intraperitoneally. Cut down to the inferior vena cava. Collect 4-5ml whole blood from 5-6 mouse with 1/10(v/v) 3.15% citrate (pH7.4). There is the least chance of activating platelets by this approach.
2. Spin 200g* for 7 min at room temperature with half brake to get 1-2ml platelet rich plasma (PRP). This g* is very critical to get good yield of PRP, so you have to determine the exact rpm with your centrifuge.



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3. Add 10mM EDTA and 1uM PGI2 or PGE1 (both final concentrations) to PRP.

4. Spin at 500g for 10min at room temperature to get first platelet pellet.

Washed Platelet Lumi-Aggregation Protocol:

1. Resuspend the first platelet pellet in platelet buffer and add 1mM EDTA and 1uM PGI2 or PGE1. (Platelet buffer: 20mM Tris-HCl (pH 7.4), 140mM NaCl, 2.5mM KCl, 1mM MgCl2, 1mg/ml glucose, 0.2% BSA)

2. Spin 500g for 10min at room temperature to get platelet pellet.

3. Resuspend in about 4ml of buffer. This gives an OD500 of about 1.0. This can be diluted even further with good results.

4. The platelet assays are done on a Chronolog lumi-aggregometer using the foam booster pads at the bottom of the tubes to decrease the volumes needed.

5. Add 2mM CaCl2 just before each trace. Add appropriate luciferase before each trace if doing secretion assays.

Washed Platelet Fura-2 Assay Protocol:

1. Resuspend the first platelet pellet in platelet buffer with 10 mM EDTA and spin at 500g for 10 min at room temperature.

2. Resuspend this washed pellet in 3 mL of platelet buffer with 1mM EDTA and 4ug/mL Fura-2AM.

3. Incubate in the dark at 37 C for 30 minutes. Mix gently every 10 minutes.

4. Spin 500g for 10min at room temperature to get platelet pellet.

5. Resuspend in about 2 ml of buffer. This should give you OD500 about 2.0. If necessary you can dilute this even further and still get good scans.



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6. Intracellular calcium measurements are performed on a Hitachi F2000 fluorimeter with thick-walled quartz cuvettes that take 0.5 mL of cell suspension per run.

7. Add 0.1 mM EGTA at time zero in each scan. This will block aggregation.

8. Add agonists at various times.

9. Add 2 mM CaCl2 immediately before calibration with Triton then EGTA.

**D) Fibroblast studies.**

Fibroblast isolation and culture:

Each primary culture was prepared from four adult mouse lungs or from single mouse embryos at E13.5. Lung tissues were minced, washed with phosphate-buffered saline (PBS), and incubated with 0.05% trypsin/EDTA ( 0.2 g/L) for 15 min at 37 °C. Embryos were rinsed with PBS, placed in 5 mL of trypsin/EDTA solution, passed once through the orifice of a 30 mL syringe, and incubated for 15 min. at 37C. After inactivating trypsin with serum, the preparation was triturated and cells were plated on 100-mm plates using Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS).

To establish fibroblast cell lines, cells were replated on every third day at approximately 25% confluency to minimize cell contact. By the 20-25th passage, permanent monomorphic, contact-inhibited fibroblast cell lines were derived. The presence or absence of wild type and disrupted thrombin receptor genes was confirmed by Southern Blot and Polymerase Chain Reaction (PCR).

Cell lines were maintained in DMEM H-16 3 g/L glucose, 0.584 g/L L-glutamine, 0.11 g/L sodium pyruvate, 3.7 g/L $NaHCO_3$ supplemented with



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5% FBS and 1% streptomycin (100 µg/ml) and penicillin G (100 units/ml). Cells were passaged using 0.05% trypsin/EDTA (0.2g/L) and maintained in a 5% $CO_2$ incubator at 37 °C.

Fibroblast Fura-2AM Assay Protocol:

For attached cells, such as fibroblasts, that cannot be exposed to trypsin before the fura2-AM assay, a commercially available enzyme-free cell dissociation buffer (Gibco, Cat#13150-016) is used.

1) Grow cells to 75% confluence in 3 or more T185 flasks.

2) Wash each flask 1x with 10 mL Cell Dissociation Buffer (CDB)

3) Incubate in 10 mL CDB at 37C for 20 min. (The cells should begin lifting off)

4) Add 10 mL RPMI to each flask and triturate.

5) Spin down cells at 750 - 1000 g for 3 min.

6) Resuspend in 3 mL of RPMI + BSA(1mg/mL) + HEPES (25 mM) + 5% CDB.

7) Add Fura2-AM (4 ug/mL) and incubate in the dark at 37C for 30 min. Gently mix the cells about every 10 minutes.

8) Spin. Wash 1x with RPMI/BSA/HEPES + %5 CDB.

9) Finally resuspend in 6 - 10 mL of RPMI/BSA/HEPES without CDB.

10) Measure calcium by fluorimetry immediately.

**E) Animal model studies.**

Mice were raised in microisolator cages in the Transgenic Core Facility at the Parnassus campus of the University of California, San Francisco. The strains used are as noted in Chapters 3-6. The inbred 129sVter mice were obtained by crossing the original male chimera "12-1" with 129sVter females from the colony of Robert Farese. This is the same colony from which the RF8 ES cells were derived.



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The bulk of the mice for these studies were 50% C57BL/6J and 50% 129sVter, These arose from interbreeding of heterozygotes from the original crosses of the "12-1" chimera with C57BL/6J mice obtained from Jackson Laboratories. Mice of higher C57BL/6 strain were obtained by backcrossing these 50%/50% mice with C57BL/6J mice 5 times.

For possible future atherosclerosis studies, Par1 -/- mice of 50%/50% mixed strain were also crossed with Apo E -/- mice that were received from the Induced Mutant Resource of Jackson Laboratories. By report these mice were produced by Dr. Maeda at the University of North Carolina, Chapel Hill and are of nearly 100% C57BL/6 (backcross N=10). The double heterozygote offspring were intercrossed and *par*1 +/+ and *par*1 -/- mice were generated in an apoE -/- background of 75% C57BL/6, 25% 139sVter strain.

# Chapter 3

Photocopied from:

Connolly A.J., Ishihara H., Kahn M., Farese R.V. and Coughlin S.R. Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**: 516-519 (1996).

# Role of the thrombin receptor in development and evidence for a second receptor

**Andrew J. Connolly*†‡, Hiroaki Ishihara*†, Mark L. Kahn*†§, Robert V. Farese Jr||§ & Shaun R. Coughlin*†§**

* Cardiovascular Research Institute, † Daiichi Research Center, Departments of ‡ Pathology and § Medicine, and || Gladstone Institute for Cardiovascular Disease, University of California, San Francisco, HSW-831, 505 Parnassus Avenue, San Francisco, California 94143-0524, USA

**THROMBIN, a coagulation protease generated at sites of vascular injury, activates platelets, endothelial cells, leukocytes and mesenchymal cells[1,2]. A G-protein-coupled receptor that is proteolytically activated by thrombin[3] is a target for drug development aimed at blocking thrombosis, inflammation and proliferation. Here we show that although disruption of the thrombin-receptor (*tr*) gene in mice causes about half of the $tr^{-/-}$ embryos to die at embryonic day 9–10, half survive to become grossly normal adult mice with no bleeding diathesis. Strikingly, $tr^{-/-}$ platelets respond strongly to thrombin, whereas $tr^{-/-}$ fibroblasts lose their ability to respond to thrombin. We conclude that the thrombin receptor plays an unexpected role in embryonic development, suggesting a possible new function for the 'coagulation' proteases themselves. Moreover, a second platelet thrombin receptor exists, and different thrombin receptors have tissue-specific roles. This may allow development of therapeutics that will selectively block thrombin's different cellular actions.**

To define the roles of the cloned thrombin receptor *in vivo* and to answer definitively whether other receptors mediate some cellular responses to thrombin, we disrupted the cloned thrombin receptor (*tr*) gene in mice (Fig. 1). Surprisingly, $tr^{-/-}$ progeny were born at about one-half of the expected rates. Litters from $tr^{+/-}$ matings yielded 41 $tr^{+/+}$, 110 $tr^{+/-}$, and only 24 $tr^{-/-}$ offspring ($P < 0.01$, $\chi^2$ test). $tr^{-/-}$ male X $tr^{+/-}$ female matings yielded 98 $tr^{+/-}$ and only 33 $tr^{-/-}$ mice ($P < 0.01$, $\chi^2$ test). At embryonic day 8.5 (E8.5), $tr^{+/-}$ and $tr^{-/-}$ embryos were morphologically indistinguishable and had beating hearts and a functional vitelline circulation (not shown). At E9.0, however, $tr^{-/-}$ embryos were uniformly smaller than $tr^{+/-}$ embryos (Fig. 1). By E9.5 this size

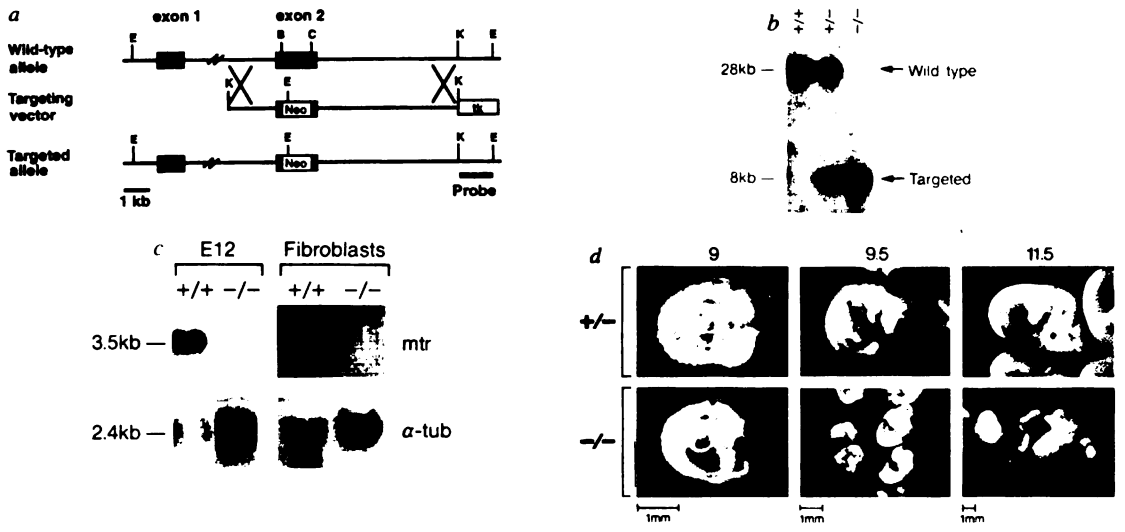



FIG. 1 Disruption of the thrombin-receptor gene in mice. *a*, Gene-targeting strategy: structure and partial restriction maps of *tr* gene, targeting construct and targeted allele, and location of the probe used for Southern analysis. Restriction endonuclease sites: E, *Eco*RI; B, *Bsp*EI; C, *Cla*I; K, *Kpn*I. *b*, Southern blot analysis of tail DNA from $tr^{+/+}$, $tr^{+/-}$, and $tr^{-/-}$ mice. The 28-kb wild-type and 8-kb targeted *Eco*RI fragments were identified using the 1.5-kb *Kpn*I–*Eco*RI probe shown in *a*. *c*, Northern blot analysis of *tr* mRNA expression in $tr^{+/+}$ and $tr^{-/-}$ E12 embryos, and embryonic fibroblasts. *tr* mRNA was identified using the *Bsp*EI–*Cla*I probe (upper panel); hybridization for α-tubulin was a control for lane loading (lower panel). *tr* mRNA was also absent from tissues of adult $tr^{-/-}$ mice (not shown). Sequencing of the product of reverse transcription and polymerase chain reaction (PCR) derived from $tr^{-/-}$ embryonic fibroblast RNA confirmed targeted disruption of the *tr* gene (not shown). *d*, Global growth retardation followed by death or recovery of $tr^{-/-}$ embryos. $tr^{+/+}$ or $tr^{+/-}$ embryos at E9.0, 9.5 or 11.5 are shown. Magnifications are the same for $tr^{-/-}$ and $tr^{+/-}$ embryos at each gestational age; 1 mm size standard is shown. No differences between $tr^{+/+}$ and $tr^{+/-}$ embryos were detected (not shown). Results are representative of 4 litters examined at each time point.

METHODS. The mouse *tr* gene's exon 1 encodes the 5′ untranslated sequence and signal peptide. Exon 2 encodes the entire mature receptor protein and 3′ untranslated region[12]. The targeting vector was constructed using a 7.5-kb *Kpn*I genomic fragment and the PGK-Neo and PGK-tk cassettes from pPNT[25]. The PGK-Neo cassette was inserted between *Bsp*EI and *Cla*I sites in exon 2, thus replacing the gene segment encoding transmembrane domains 1–7. Culture and electroporation of RF8 ES cells (generated by R. Farese) and production of chimaeric mice was as described[26]. Homologous recombination and germline transmission were confirmed by Southern blot and PCR. For PCR, primers to the inserted PGK-Neo cassette and to genomic sequence outside the 2.0-kb 5′ flanking sequence in the targeting vector were used. $tr^{+/+}$ or $tr^{+/-}$ embryos were generated by mating $tr^{+/+}$ or $tr^{+/-}$ males to $tr^{+/-}$ females. Unless otherwise indicated, all results were obtained using mice that were 50% C57BL/6 and 50% 129/Sv strain.

difference was marked, and about half of the $tr^{-/-}$ embryos had no heart beat (Fig. 1). A delay in placental development paralleled the delay seen in the $tr^{-/-}$ embryo proper at these time points. $tr^{-/-}$ embryos that survived the critical period from E9–10 had 'caught up' with their $tr^{+/-}$ counterparts by E11.5 (Fig. 1) and developed normally through to birth.

The cause of this embryonic death is unknown. Its partial nature is probably stochastic rather than genetic because it occurred even in an inbred 129/Sv background strain of mice (not shown). Failed haemostasis was not observed and would not be expected, given the platelet phenotype described below. Moreover, embryos lacking platelets[4] or fibrinogen to support platelet aggregation[5] survived to birth. At E9.0–9.5, *tr* messenger RNA was widely expressed in tissues critical for development at this time, including heart and blood vessels, haematopoietic cells, placenta, as well as brain and mesenchymal cells (ref. 6 and data not shown). Which of these tissues is responsible for the $tr^{-/-}$ embryonic lethality remains to be discovered. Regardless, our results reveal an unexpected developmental role for this receptor and raise new questions. Could thrombin produced upon invasion of maternal blood vessels be a signal for placental development, or might thrombin produced in the embryo have an unknown function? Alternatively, does another protease or even a peptide agonist activate the thrombin receptor during development?

Despite this partial embryonic loss, one-half of the $tr^{-/-}$ mice survived to birth, grew to maturity and were grossly normal. To our surprise, spontaneous bleeding was not seen in $tr^{-/-}$ mice and tail bleeding times, a platelet and thrombin-dependent *in vivo* measure of haemostasis[7], were indistinguishable from those of wild-type mice (Fig. 2*a*). Moreover, thrombin activated platelets from $tr^{-/-}$ or $tr^{+/-}$ mice equally (Fig. 2*b*). Strong secretion responses to thrombin were noted in platelets from $tr^{-/-}$ mice even in the absence of aggregation, so the secretion response was not aggregation-induced. Prostacyclin ($PGI_2$) blocked thrombin-induced secretion and aggregation in $tr^{-/-}$ platelets (Fig. 2*c*). Last, thrombin triggered rapid increases in cytoplasmic calcium in $tr^{-/-}$ platelets in the absence of extracellular calcium and aggregation (Fig. 3). These increases presumably reflect phosphoinositide hydrolysis and mobilization of calcium from intracellular stores. Thus thrombin can cause second messenger responses and functional activation in mouse platelets via a cell-surface receptor that is distinct from the cloned thrombin receptor.

What is the nature of this second receptor? The pharmacology of responses in $tr^{+/+}$ and $tr^{+/-}$ mouse platelets, human platelets, and *Xenopus* oocytes expressing cloned thrombin receptors was similar in many respects (Table 1). The protease inhibitor PPACK[8] ablated thrombin's ability to activate $tr^{-/-}$ mouse platelets. γ-Thrombin, which is defective in its fibrinogen-binding exosite[9], was two logs less potent than α-thrombin in activating $tr^{-/-}$ mouse platelets, and blockade of thrombin's fibrinogen-binding exosite with hirugen[10] markedly right-shifted the dose response to thrombin. Thus thrombin's protease activity and its fibrinogen-binding exosite are important for activation of both the cloned and the second thrombin receptor. The cloned thrombin receptor and protease-activated receptor 2 (PAR2), which signals to trypsin but not thrombin, are clearly the result of gene

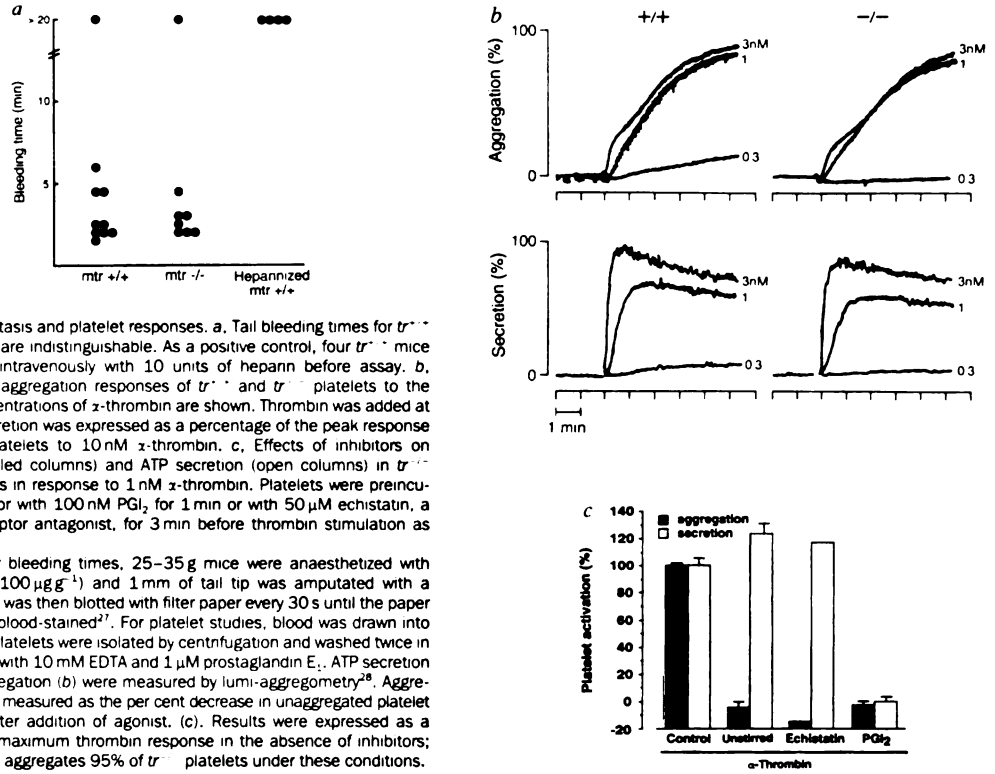



FIG. 2 Haemostasis and platelet responses. *a*, Tail bleeding times for $tr^{+/+}$ and $tr^{-/-}$ mice are indistinguishable. As a positive control, four $tr^{+/+}$ mice were injected intravenously with 10 units of heparin before assay. *b*, Secretion and aggregation responses of $tr^{+/+}$ and $tr^{-/-}$ platelets to the indicated concentrations of α-thrombin are shown. Thrombin was added at 2 min. ATP secretion was expressed as a percentage of the peak response of wild-type platelets to 10 nM α-thrombin. *c*, Effects of inhibitors on aggregation (filled columns) and ATP secretion (open columns) in $tr^{-/-}$ mouse platelets in response to 1 nM α-thrombin. Platelets were preincubated without or with 100 nM $PGI_2$ for 1 min or with 50 μM echistatin, a fibrinogen-receptor antagonist, for 3 min before thrombin stimulation as indicated.

METHODS. For bleeding times, 25–35 g mice were anaesthetized with pentobarbital (100 $\mu g g^{-1}$) and 1 mm of tail tip was amputated with a scalpel; the tail was then blotted with filter paper every 30 s until the paper was no longer blood-stained[27]. For platelet studies, blood was drawn into citrate buffer. Platelets were isolated by centrifugation and washed twice in Tyrode's buffer with 10 mM EDTA and 1 μM prostaglandin $E_1$. ATP secretion (*b*, *c*) and aggregation (*b*) were measured by lumi-aggregometry[28]. Aggregation was also measured as the per cent decrease in unaggregated platelet count 4 min after addition of agonist. (*c*). Results were expressed as a percentage of maximum thrombin response in the absence of inhibitors; thrombin alone aggregates 95% of $tr^{-/-}$ platelets under these conditions.

duplication[11,12]. Whether the second thrombin receptor is another protease-activated receptor derived by gene duplication or something different remains to be determined.

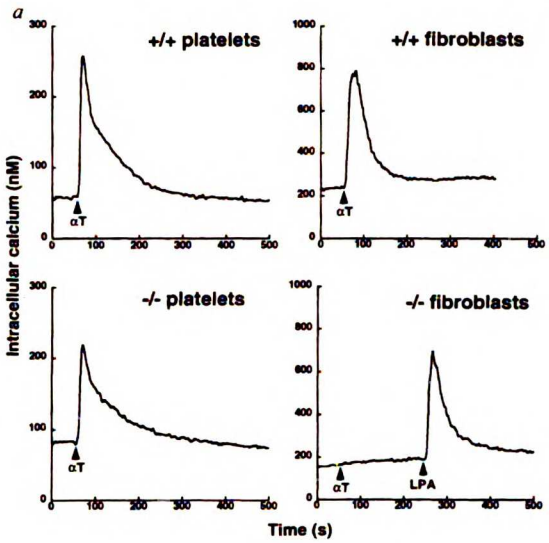
What is the relative importance of the second thrombin receptor in human as opposed to mouse platelets? Synthetic peptides that mimic the new amino terminus that is unmasked when thrombin cleaves the cloned thrombin receptor are agonists for that receptor[3]. Such peptides fail to activate wild-type and $tr^{-/-}$ mouse platelets but readily activate human platelets (Table 1 and ref. 3). Similar species differences in agonist peptide potency have been reported but their basis is uncertain[13,14]. The difference between mouse and human platelets was not accounted for by unresponsiveness of the cloned mouse thrombin receptor to agonist peptide; the mouse and human homologues responded similarly to agonist peptides or thrombin (Table 1). In addition, the failure of mouse platelets to respond to agonist peptides was not due to an inhibitor unique to mouse platelet preparations, because mixing of mouse and human platelets did not inhibit peptide-induced activation of human platelets (not shown). In the context of the observation that wild-type and $tr^{-/-}$ platelets respond identically to thrombin (Fig. 2), these results suggest that the cloned thrombin receptor plays little part in mouse platelet function. By contrast, the cloned thrombin receptor does appear to be important for human platelets; thrombin-receptor-activating peptides cause a strong response in human platelets and thrombin-receptor antibodies attenuate their response to thrombin[15–17]. However, thrombin is more effective than agonist peptide in eliciting certain responses in human platelet responses[18–21], so a second receptor may also operate in these cells.

In contrast to platelets, thrombin responses were lost in fibroblasts from $tr^{-/-}$ mice. Unlike their wild-type counterparts, fibroblasts derived from $tr^{-/-}$ embryos or adult lung failed to show

TABLE 1 Pharmacology of platelet activation versus cloned thrombin receptor activation

| | $EC_{50}$ | | | | |
|---|---|---|---|---|---|
| | Platelet ATP secretion | | | Cloned receptor activation | |
| | $^{-/-}$ Mouse | $^{+/+}$ Mouse | Human | Mouse | Human |
| α-Thrombin (nM) | 0.7 | 0.8 | 0.9 | 0.03 | 0.05 |
| PPACK-α-thrombin (nM) | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| γ-Thrombin (nM) | 60 | ND | 60 | 2 | 5 |
| α-Thrombin (nM) + hirugen | 40 | ND | 30 | 25 | 40 |
| SFLLRN (human) (μM) | >200 | >200 | 6 | 0.7 | 0.2 |
| SFFLRNPSE (mouse) (μM) | >200 | >200 | 180 | 3 | 3 |

Agonist-induced platelet ATP secretion was measured as in Fig. 1. Values of the half-maximal effective concentration ($EC_{50}$) were estimated from concentration–response curves using duplicate determinations at 4 different agonist concentrations. The fibrinogen-binding exosite inhibitor hirugen was incubated at 100 $\mu g ml^{-1}$ with α-thrombin before addition to cells. To assess responses mediated by the cloned mouse and human receptors, cRNAs encoding these molecules were transcribed and injected into *Xenopus* oocytes. After 24 h in culture, oocytes were loaded with $^{45}Ca$ and agonist-induced release of $^{45}Ca$ was measured as an index of intracellular calcium flux[3]; $EC_{50}$ values were estimated as described[23]. $EC_{50}$ values in the *Xenopus* system are uniformly lower than those obtained with platelets because of the high level of receptor expression achieved in the former[24]. Note, however, that the relative potencies of the various agonists compared to α-thrombin is similar in the two systems.



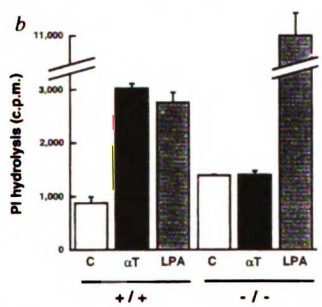




FIG. 3 Tissue-specific roles for the cloned thrombin receptor. *a*, Intracellular calcium measurements in $tr^{+/+}$ and $tr^{-/-}$ platelets and fibroblasts. Increases in cytosolic calcium in response to thrombin (αT; 10 nM) or lysophosphatidic acid (LPA; 1 μM) were determined using Fura-2. Agonists were added at the indicated times (arrow). *b*, Agonist-induced phosphoinositide hydrolysis in $tr^{+/+}$ and $tr^{-/-}$ fibroblasts in response to thrombin (αT; 10 nM) or LPA (1 μM).
METHODS. Platelets or fibroblasts were loaded with Fura-2AM dye at 37 °C for 30 min, suspended in calcium-free Tyrode's buffer containing 0.1 mM EGTA, and assayed using a Hitachi F2000 fluorometer[29]. Absence of platelet aggregation during the calcium assay was confirmed by counting unaggregated platelets in a haemocytometer before and 4 min after addition of thrombin. Fibroblasts were cultured from wild-type or $tr^{-/-}$ E12.0 embryos or from the lungs of adult wild-type or $tr^{-/-}$ mice. Thrombin- or LPA-induced phosphoinositide hydrolysis was assessed as described[30], except that agonist stimulation lasted for 60 min. Data shown were obtained with lung fibroblasts at passages 20–24. Results were similar with fibroblasts from primary cultures and with passaged fibroblasts from two independent isolates. The magnitude of the LPA response differed for each fibroblast line tested.

thrombin-induced phosphoinositide hydrolysis and calcium mobilization (Fig. 3). Thrombin-induced mitogenic responses were also ablated in these cells (J. Trejo, A.J.C., and S.R.C., unpublished observations). The cloned thrombin receptor is thus critical for triggering classical thrombin-induced events in some mouse mesenchymal cells, a role that may contribute to the observed developmental phenotype.

In summary, the thrombin-receptor knockout mouse reveals an unexpected role for the cloned thrombin receptor in embryonic development and that the cloned receptor is critical in fibroblast signalling. The persistence of thrombin-induced responses in $tr^{-/-}$ platelets and its absence in $tr^{-/-}$ fibroblasts uncovers a second platelet thrombin receptor and shows that different thrombin receptors have tissue-specific functions. Thrombin stimulation of mesenchymal cells may be important in normal wound healing and in pathological proliferative responses such as atherosclerosis and restenosis[5,22]. With intact haemostatic function but absent fibroblast responses to thrombin, $tr^{-/-}$ mice provide an opportunity to test thrombin's role in *in vivo* models of these processes. Last, to the extent that multiple thrombin receptors probably also operate in humans, these studies suggest the possibility of developing pharmaceuticals that selectively block proliferative or haemostatic responses to thrombin. □

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CORRESPONDENCE and requests for materials should be addressed to S.R.C. (e-mail: shaun_coughlin.src@quickmail.ucsf.edu).

# Chapter 4

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# The Cloned Thrombin Receptor Is Necessary and Sufficient for Activation of Mitogen-activated Protein Kinase and Mitogenesis in Mouse Lung Fibroblasts

LOSS OF RESPONSES IN FIBROBLASTS FROM RECEPTOR KNOCKOUT MICE*

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**JoAnn Trejo‡§, Andrew J. Connolly‡¶‖, and Shaun R. Coughlin‡¶**‡‡**

*From the ‡Cardiovascular Research Institute, the ¶Daiichi Research Center, and the Departments of **Medicine and ‖Pathology, University of California, San Francisco, California 94143-0524*

**The mitogenic activity of thrombin on fibroblasts and smooth muscle cells may contribute to embryonic development and normal wound healing, and it may also play a role in pathological responses to vascular injury. To examine the importance of thrombin signaling *in vivo* and to define the cloned thrombin receptor's role, we disrupted the thrombin receptor gene (*tr*) in mice. Platelets from $tr^{-/-}$ mice responded normally to thrombin, but $tr^{-/-}$ fibroblasts showed no thrombin-induced calcium mobilization or phosphoinositide hydrolysis. Thus distinct thrombin receptors act in different tissues. This study focuses on the role of the thrombin receptor in thrombin-induced mitogenesis and mitogen-activated protein (MAP) kinase activation in mesenchymal cells. Thrombin and thrombin receptor agonist peptide both stimulated DNA synthesis and MAP kinase activation in fibroblasts derived from wild-type mice. These responses were selectively lost in fibroblasts from $tr^{-/-}$ mice. Activation of the cloned thrombin receptor is therefore necessary and sufficient for thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. The $tr^{-/-}$ mouse thus provides a valuable model for defining the role of thrombin-induced proliferative events *in vivo*. Because thrombin-induced MAP kinase activation was attributable to a single receptor expressed at natural levels, mouse lung fibroblasts presented an opportunity to define the pathways that normally mediate activation of MAP kinase by the thrombin receptor. Elimination of phorbol-sensitive protein kinase C by prolonged exposure to phorbol ester only partially inhibited MAP kinase activation by thrombin but completely blocked c-Raf kinase activation. Pertussis toxin partially inhibited MAP kinase activation by thrombin but had no significant effect on c-Raf kinase activation. Thus in mouse lung fibroblasts, one thrombin receptor utilizes two pathways for MAP kinase activation: one is protein kinase C- and c-Raf dependent, and a second is $G_i$-dependent and c-Raf-independent.**

The ability of thrombin to stimulate proliferation of fibroblasts and smooth muscle cells has led to the hypothesis that thrombin, when generated at sites of vascular injury, may contribute to both normal wound healing and pathological proliferative responses such as restenosis and atherosclerosis. Understanding how thrombin activates mesenchymal cells is, therefore, potentially important for therapeutic development. A recently cloned receptor mediates thrombin signaling in a variety of cell types (1). This receptor is a member of the G protein-coupled receptor family but is activated by a novel proteolytic mechanism. Thrombin cleaves the amino-terminal exodomain of its receptor to unmask a new amino terminus that then functions as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect activation. A soluble peptide mimicking the first six residues after the cleavage site is an agonist for the receptor. Such "agonist peptides" grossly mimic the actions of thrombin on a variety of cell types including fibroblasts (2–7). However, thrombin is more effective than agonist peptide in activating MAP[1] kinase and stimulating proliferation of CCL-39 hamster lung fibroblasts (8, 9). *A priori*, such discrepancies might be caused by differences in signaling by the cloned receptor when activated by thrombin *versus* agonist peptide (*i.e.* ligation by tethered *versus* free agonist might yield different kinetics of ligation or desensitization or different active receptor conformations). Alternatively, a distinct thrombin receptor that cannot be activated by agonist peptide might mediate some of the actions of thrombin.

To define the roles of the cloned thrombin receptor *in vivo* and to answer definitively whether other receptors mediate some cellular responses to thrombin, we disrupted the cloned thrombin receptor gene in mice (10). Platelets from mice homozygous for the thrombin receptor gene deletion ($tr^{-/-}$) responded normally to thrombin, but $tr^{-/-}$ fibroblasts showed no thrombin-induced calcium mobilization or phosphoinositide hydrolysis. Thus a second thrombin receptor does exist, and distinct thrombin receptors mediate thrombin signaling in different tissues. Defining which thrombin receptors mediate various cellular responses therefore becomes an important biological question and one that is central to therapeutic development. This report focuses on the role of the cloned receptor in thrombin-induced mitogenesis and MAP kinase/extracellular-regulated protein kinase (ERK) activation in mesenchymal

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‡‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Box 0524, HSW-831, 505 Parnassus Ave., University of California, San Francisco, CA 94143-0524. Tel.: 415-476-6174; Fax: 415-476-8173; E-mail: shaun_coughlin.src@quickmail.ucsf.edu.

[1] The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular-regulated protein kinase; MEK, MAP/ERK kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; MBP, myelin basic protein; PIPES, 1,4-piperazinediethanesulfonic acid; WT, wild type.

cells. The MAP kinase pathway is an important route through which a variety of mitogens signal from cell surface receptors to the nucleus to effect transcriptional regulation (11, 12). We report that activation of the cloned thrombin receptor is necessary and sufficient for both thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. These cells therefore provided a valuable tool for identifying signaling pathways activated by a single G protein-coupled receptor in an untransfected cell line. In these cells, the thrombin receptor utilizes two pathways to mediate MAP kinase activation: one that is protein kinase C- and c-Raf-dependent, and a second is $G_i$-dependent and c-Raf-independent.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—The thrombin receptor knockout mouse was generated as described (10). Lung fibroblast cell lines were derived from wild-type and *tr* mice by published methods (13). Each primary culture was prepared from four adult mouse lungs. Briefly, tissues were minced, washed with phosphate-buffered saline (PBS), and incubated with 0.05% trypsin/EDTA (0.2 g/liter) for 15 min at 37 °C. After inactivating trypsin with serum, the preparation was triturated, and cells were plated on 100-mm plates using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). On every 3rd day, cells were replated at approximately 25% confluency to minimize cell contact. By the 20–25th passage, permanent monomorphic, contact-inhibited fibroblast cell lines were derived. The presence or absence of wild-type and disrupted thrombin receptor genes was confirmed by Southern blot and polymerase chain reaction (10). CCL-39 hamster lung fibroblast cells were obtained from American Type Culture Collection. Cell lines were maintained in DMEM H-16, 3 g/liter glucose, 0.584 g/liter L-glutamine, 0.11 g/liter sodium pyruvate, 3.7 g/liter $NaHCO_3$ supplemented with 5% FBS and 1% streptomycin (100 μg/ml), and penicillin G (100 units/ml). Cells were passaged using 0.05% trypsin/EDTA (0.2 g/liter) and maintained in a 5% $CO_2$ incubator at 37 °C.

*[$^3$H]Thymidine Incorporation*—Wild-type and *tr* mouse lung fibroblasts were plated in 12-well plates at $2.5 \times 10^4$ cells/well in DMEM with 5% FBS. After 24 h, the medium was changed to DMEM H-16 medium containing 1 μg/ml insulin, 5 μg/ml transferrin, and 0.5 mg/ml bovine serum albumin with or without agonists. Following 24 h of incubation with agonists, [$^3$H]thymidine (1 μCi/well; 3 μM thymidine) was added, and the incubation was continued for an additional 24 h. Cells were then washed with cold PBS and [$^3$H]thymidine incorporation was determined as trichloroacetic acid-insoluble radioactivity by liquid scintillation counting as described previously (14).

α-Thrombin was generously provided by J. Fenton II, Albany Medical College, Albany, NY. Mouse thrombin receptor agonist peptide SFFLRNPSE was synthesized as the carboxyl amide and high performance liquid chromatography purified. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were obtained from Boehringer Mannheim. Pertussis toxin was from List Biological Chemicals, and phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) were from LC Services Corp.

*MAP Kinase Assay*—MAP kinase activity was assayed by a modification of published methods (15). Mouse lung fibroblasts and CCL-39 cells were seeded in 6-well plates at $1 \times 10^5$ cells/well and maintained overnight in DMEM H-16 containing 5% FBS. Cell monolayers were rinsed twice and maintained in serum-free DMEM H-16 for 48 h. Agonists were then added in DMEM H-16 containing 0.1% bovine serum albumin for the indicated times at 37 °C. In some experiments, 0.1% dimethyl sulfoxide ($Me_2SO$) was added to control for $Me_2SO$ in the phorbol solutions. This concentration of $Me_2SO$ had no effect on MAP kinase activity. Following agonist exposure, cells were rinsed twice in cold PBS and lysed in 0.5 ml of lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10 mM sodium pyrophosphate, 2 μg/ml aprotinin, 1.0 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, and 0.5 mM $Na_3VO_4$). Insoluble material was removed from cell lysates by centrifugation at $14,000 \times g$ for 10 min at 4 °C. The supernatant was precleared by incubation with 25 μl of Protein A-agarose beads (50% (v/v) in PBS; Oncogene Science Inc.) for 30 min at 4 °C. Protein A-agarose beads were removed by centrifugation ($4,000 \times g$) for 2 min. Rabbit polyclonal antiserum (0.2 μg), which recognized ERK2 (p42) and ERK1 (p44) (Santa Cruz Biotechnology Inc.), was added to the precleared lysate and incubated along with 25 μl of 50% Protein A-agarose beads for 2 h at 4 °C. The immunoprecipitate was recovered by centrifugation, washed once with lysis buffer, and twice with 40 mM HEPES, pH 8, 2 mM dithiothreitol, 1 mM EGTA, 5 mM $MgCl_2$, and 200 μM $Na_3VO_4$ kinase wash buffer. After the final wash, immunoprecipitates were resuspended in 50 μl of 40 mM HEPES, pH 8, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA, and 200 μM $Na_3VO_4$ containing 12.5 μg of myelin basic protein (MBP) (Life Technologies, Inc.) and 5 μCi of [$\gamma$-$^{32}$P]ATP (DuPont NEN). *In vitro* phosphorylation reactions were allowed to proceed for 10 min at 30 °C. Reactions were terminated with 5 × Laemmli sample buffer, and the products were analyzed by 15% SDS-polyacrylamide gel electrophoresis autoradiography. Phosphorylated MBP was quantitated using a PhosphorImager (Molecular Dynamics Inc.).

*c-Raf Kinase Assay*—c-Raf kinase activity was determined by an *in vitro* immune complex assay using the kinase substrate histone H1 (16) in a procedure modified from that previously described (17). Briefly, mouse lung fibroblasts were plated at $0.5 \times 10^6$ cells/100-mm plate and grown overnight in DMEM H-16 containing 5% FBS. Cells were washed and maintained in serum-free DMEM H-16 for 48 h. Cells were then stimulated with agonists and lysed as described above. c-Raf kinase was immunoprecipitated from precleared lysates using 3 μl of rabbit polyclonal antibody generated against a c-Raf kinase carboxyl-terminal peptide (kindly provided by Randall C. Shatzman, Roche Bioscience) together with 25 μl of Protein A-agarose beads. Mixtures were incubated for 2 h at 4 °C. Immunoprecipitates were collected by centrifugation at $4,000 \times g$ for 2 min and were washed once in lysis buffer and twice with 10 mM PIPES, pH 8, 100 mM NaCl, and 0.4 μg/ml aprotinin. Immunoprecipitates were resuspended in 50 μl of 20 mM PIPES pH 8, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 4.5 μg of histone H1 (Life Technologies, Inc.), and 5 μCi of [$\gamma$-$^{32}$P]ATP. *In vitro* phosphorylation reactions were carried out at room temperature for 30 min and terminated with 5 × Laemmli sample buffer. Samples were separated on 12% SDS-polyacrylamide gel electrophoresis, and gels were vacuum dried. Polyacrylamide gels were exposed to autoradiography film or quantitated using a PhosphorImager.

## RESULTS AND DISCUSSION

To define the role of the cloned thrombin receptor in mediating thrombin-induced mesenchymal cell proliferation, we examined the action of thrombin on fibroblasts cultured from $tr^{-/-}$ mice. Thrombin and thrombin receptor agonist peptide stimulated DNA synthesis in fibroblasts derived from wild-type mice. By contrast, neither agonist caused DNA synthesis in $tr^{-/-}$ lung fibroblasts (Fig. 1*A*). Wild-type and $tr^{-/-}$ fibroblasts both showed comparable serum- and growth factor-induced increases in DNA synthesis; thus the failure of the $tr^{-/-}$ cells to respond to thrombin was selective. Similar results were obtained with other wild-type and $tr^{-/-}$ fibroblast lines derived from independent primary cultures (data not shown). The ability of both thrombin and thrombin receptor agonist peptide to stimulate DNA synthesis in wild-type fibroblasts and the selective loss of these responses in receptor-deficient cells provide strong evidence that the cloned receptor is both necessary and sufficient for thrombin-induced mitogenesis.

bFGF has been reported to cooperate with thrombin to stimulate DNA synthesis in hamster lung fibroblasts (18). In wild-type mouse lung fibroblast cultures, thrombin and the *bona fide* growth factor bFGF each increased DNA synthesis to a similar extent (Fig. 1*B*). When used in combination, the effects of thrombin and bFGF were approximately additive. In $tr^{-/-}$ fibroblast cultures, bFGF stimulated DNA synthesis to a degree comparable with that seen in wild-type fibroblasts, but thrombin plus bFGF did not stimulate DNA synthesis more than bFGF alone. Thus in mouse lung fibroblasts, the cloned thrombin receptor is required for thrombin-induced DNA synthesis even in the presence of bFGF.

We next examined the role of the cloned thrombin receptor in activating MAP kinase, an important mitogenic signaling pathway. Thrombin caused robust activation of MAP kinase in lung fibroblasts derived from wild-type mice (Fig. 2, *A* and *B*). The response followed a time course similar to that previously seen with CCL-39 hamster lung fibroblasts (19), with a peak at 5 min followed by a plateau that lasted >120 min (Fig. 2). Mouse thrombin receptor agonist peptide also rapidly activated MAP

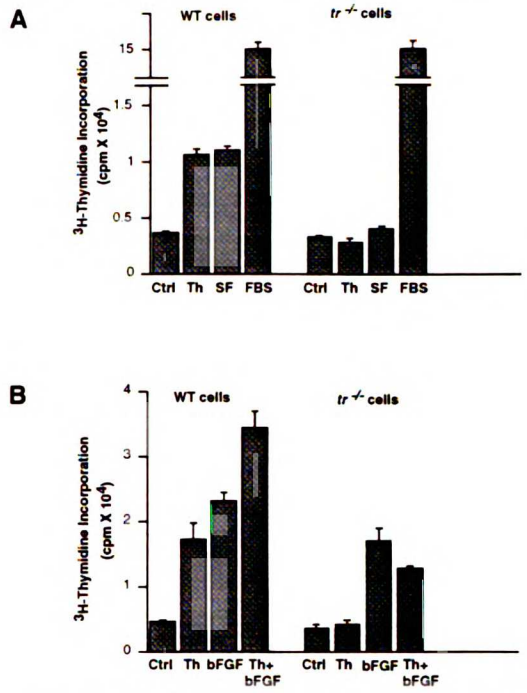


FIG. 1. **Thrombin- and thrombin receptor agonist peptide-induced DNA synthesis in fibroblasts from wild type and $tr^{-/-}$ mice.** *A*, agonist-induced [$^3$H]thymidine uptake in mouse lung fibroblasts. WT and $tr^{-/-}$ cells were incubated with DMEM alone (*Ctrl*) or DMEM containing 10 nM α-thrombin (*Th*), 300 μM SFFLRNPSE (*SF*) or 10% FBS. [$^3$H]Thymidine incorporation was measured as described under "Experimental Procedures." Data shown are the mean ± S.E. of three replicates in a single experiment. Thrombin and SFFLRNPSE responses were different in WT *versus* $tr^{-/-}$ cells when assessed by two-way analysis of variance, followed by Bonferroni's *t* test ($p < 0.05$) (35). Similar results were obtained in two other independent experiments. *B*, thrombin-stimulated [$^3$H]thymidine incorporation in the presence of bFGF. WT and $tr^{-/-}$ cells were incubated in DMEM without (*Ctrl*) or with 10 nM α-thrombin (*Th*), 100 ng/ml bFGF (*bFGF*) or both (*Th + bFGF*). [$^3$H]Thymidine incorporation was determined. The data shown are the mean ± S.E. of triplicate determinations from one experiment. The effect of thrombin was again different in WT *versus* $tr^{-/-}$ cells when assessed by two-way analysis of variance, followed by Bonferroni's *t* test ($p < 0.05$). Two other separate experiments yielded similar results. Fibroblasts for these studies were obtained as described under "Experimental Procedures." Similar results were obtained with other independently derived wild-type and $tr^{-/-}$ mouse lung fibroblast cell lines.

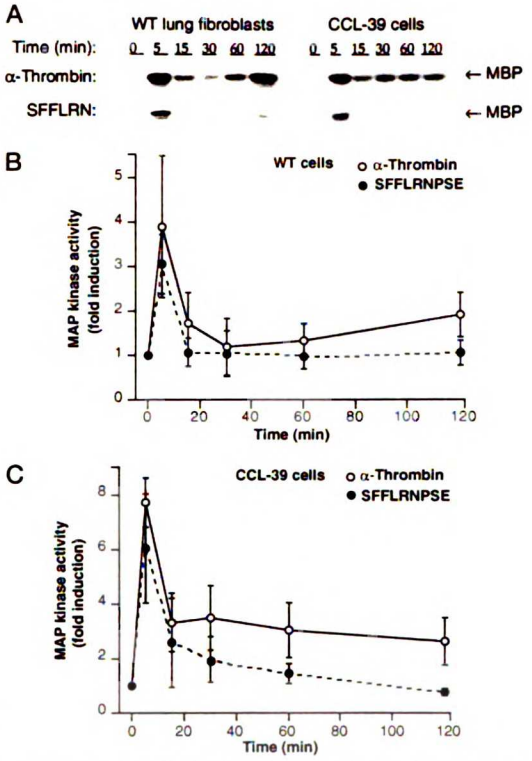


FIG. 2. **Kinetics of MAP kinase activation by thrombin and thrombin receptor agonist peptide in wild-type mouse lung fibroblasts and CCL-39 cells.** *A*, MAP kinase activation by α-thrombin and SFFLRNPSE. WT mouse lung fibroblasts and CCL-39 cells were exposed to α-thrombin (10 nM) or SFFLRNPSE (300 μM) for various times. MAP kinase activity was determined using myelin basic protein (MBP) in an *in vitro* phosphorylation assay as described under "Experimental Procedures." The time course of agonist-stimulated phosphorylation of MBP from a representative experiment is shown. *B*, time course of MAP kinase activation in WT lung fibroblasts. WT cells were exposed to 10 nM α-thrombin or 300 μM SFFLRNPSE for the indicated times. MAP kinase activity was measured as described above. Data are expressed as the fold increase over basal and represent the mean ± S.E. of three experiments. *C*, time course of MAP kinase activation in CCL-39 cells. Cells were stimulated with 10 nM α-thrombin or 100 μM SFFLRNPSE for the times indicated. The activity of MAP kinase was measured as described above. Results are expressed as the fold increase over basal and represent the mean ± S.E. of three experiments. For both *B* and *C*, the responses to α-thrombin *versus* SFFLRNPSE were different when assessed by two-way analysis of variance ($p < 0.05$), and the responses to α-thrombin *versus* SFFLRNPSE at late times (120 min) were different ($p < 0.05$) when assessed using Bonferroni's *t* test.

kinase in wild-type mouse fibroblasts, but, as in CCL-39 cells (9), the agonist peptide was less effective than thrombin at eliciting *prolonged* activation of MAP kinase (Fig. 2, *A* and *C*). In contrast to the wild type fibroblasts, cells derived from $tr^{-/-}$ mice failed to show either early or late MAP kinase activation in response to thrombin (Fig. 3, *A* and *B*). Thrombin-induced calcium mobilization and phosphoinositide turnover were also absent in the $tr^{-/-}$ fibroblasts (10), while activation of MAP kinase by other agents such as the phorbol ester PMA was preserved (data not shown). Similar results were obtained in other independently derived wild-type and $tr^{-/-}$ mouse lung fibroblast cell lines. These results indicate that the cloned thrombin receptor is necessary for activation of MAP kinase by thrombin in mouse lung fibroblasts.

MAP kinase activation by thrombin receptor agonist peptide was also lost in $tr^{-/-}$ fibroblasts (Fig. 3, *A* and *C*); thus the response to agonist peptide seen in the wild-type cells was mediated by the cloned receptor and not by a second protease-activated receptor. This possibility was not just a formal one because thrombin receptor agonist peptide does activate protease-activated receptor 2 (20). These findings suggest that, despite their differing tempos, MAP kinase activations by thrombin and agonist peptide are both mediated by the cloned thrombin receptor in mouse lung fibroblasts. How then are the differing tempos caused? Degradation of the agonist peptide might seem to be a plausible explanation. However, the bioassay of conditioned medium using agonist-induced $^{45}Ca^{2+}$ release in *Xenopus* oocytes expressing only the cloned thrombin receptor suggested that only approximately half of agonist pep-

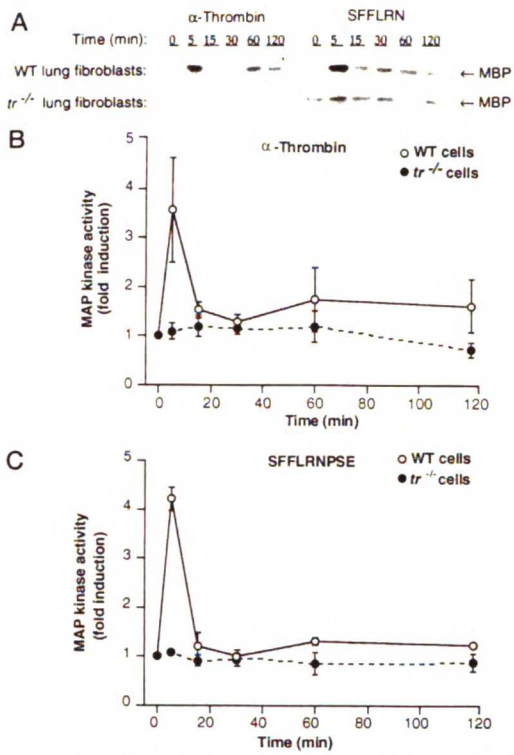


FIG. 3. **Thrombin and thrombin receptor agonist peptide effects on MAP kinase activation in lung fibroblasts from wild-type and $tr^{-/-}$ mice.** *A*, MAP kinase activation by α-thrombin and SFFLRNPSE in wild-type (*WT*) and $tr^{-/-}$ cells. Cells were treated with either α-thrombin (10 nM) or SFFLRNPSE (300 μM) for the specified times. MAP kinase activity was determined using MBP in an *in vitro* phosphorylation assay (see "Experimental Procedures"). Data show the time course of agonist-stimulated phosphorylation of MBP from a single experiment. *B*, comparison of MAP kinase activation by α-thrombin in WT and $tr^{-/-}$ mouse lung fibroblasts. WT and $tr^{-/-}$ cells were exposed to 10 nM α-thrombin for the specified times. MAP kinase activity was measured as described under "Experimental Procedures." Data are expressed as the fold increase relative to basal and represent the mean ± S.E. of five experiments. *C*, comparison of MAP kinase activation by SFFLRNPSE in WT and $tr^{-/-}$ lung fibroblasts. Cells were treated with 300 μM SFFLRNPSE for the indicated times. Data are expressed as fold increase in MAP kinase activity over basal and represent the mean ± S.E. of duplicate experiments. In *B* and *C*, the responses of WT *versus* $tr^{-/-}$ fibroblasts to either α-thrombin or SFFLRNPSE were different ($p < 0.05$) when assessed by two-way analysis of variance. These results were also replicated in independently derived lung fibroblasts from wild-type and $tr^{-/-}$ mice.

tide activity was lost during the 2-h incubation shown in Fig. 2, *A* and *B*. Moreover, the addition of 300 μM fresh agonist peptide to wild-type lung fibroblasts after a 2-h incubation in the presence of agonist peptide failed to cause a second increase in MAP kinase activity (data not shown), suggesting that absence of agonist peptide does not account for its failure to cause prolonged MAP kinase activation. The possibility that agonist peptide activates both the cloned thrombin receptor that stimulates MAP kinase and an unknown receptor that inhibits late MAP kinase activation was also tested. The addition of peptide with thrombin did not prevent prolonged MAP kinase activation. It also remains formally possible that thrombin activates a second receptor that is insensitive to agonist peptide and incapable of activating MAP kinase (as well as phosphoinositide hydrolysis and calcium mobilization) on its own but is capable of prolonging MAP kinase activation when activated in concert with the cloned receptor. This explanation lacks parsimony. Differences in the kinetics of receptor activation or desensitization or perhaps differences in signaling by thrombin-*versus* free peptide-activated thrombin receptors may account for the distinct temporal patterns of MAP kinase activation caused by the two agonists.

Taken together, the observations presented above strongly suggest that the cloned thrombin receptor fully accounts for thrombin-induced MAP kinase activation in mouse lung fibroblasts. The wild-type mouse lung fibroblasts thus provided a unique opportunity to dissect the pathway from receptor to MAP kinase activation in an untransfected cell in which responses can be attributed to a single cloned receptor expressed at natural levels. The pathway from thrombin receptor to MAP kinase activation is potentially complex. The thrombin receptor shows robust coupling to G proteins $G_i$ and $G_q$ (21–23). Downstream signaling molecules that might mediate the actions of these G proteins on MAP kinase include *ras*, protein kinase C, and c-Raf kinase as well as other MEK (MAP/ERK kinase) kinases (24). c-Raf and other MEK kinases converge on MEK; phosphorylated and therefore activated MEK in turn phosphorylates and activates MAP kinase (see Ref. 25). Toward defining the pathways from thrombin receptor to MAP kinase activation in mouse fibroblasts, we examined the importance of $G_i$-like G proteins and protein kinase C in c-Raf and MAP kinase activation.

Elimination of $G_i$ function by treatment of cells with pertussis toxin partially inhibited MAP kinase activation but had no significant effect on c-Raf kinase activation by thrombin (Figs. 4*A* and 5*A*). Pertussis toxin did not inhibit either thrombin-induced phosphoinositide hydrolysis (data not shown) or phorbol ester and EGF stimulation of MAP kinase in these cells (Fig. 4*A*; data not shown). These data are consistent with pertussis toxin selectively ablating $G_i$ function without nonspecifically inhibiting either thrombin signaling or MAP kinase activation in general. One can also conclude that the thrombin receptor activates phosphoinositide hydrolysis mainly through a pertussis-insensitive, presumably $G_q$-like G protein (26, 27) in these cells (Fig. 6). Pertussis toxin treatment ADP-ribosylated more than 95% of available substrate in these cells (data not shown) (6); therefore the partial nature of pertussis inhibition of thrombin-induced MAP kinase activation was not due to incomplete action of the toxin. These results show that the cloned thrombin receptor utilizes both pertussis-sensitive and -insensitive G proteins to activate MAP kinase (Fig. 6). Given the receptor's ability to couple to each (21, 22), $G_i$ and $G_q$ are excellent candidates for mediating such pathways.

Elimination of phorbol-sensitive protein kinase C activation by prolonged PDBu pretreatment of wild-type fibroblasts partially inhibited MAP kinase activation by thrombin but completely blocked activation by phorbol ester (Fig. 4*B*). PDBu pretreatment had little effect on MAP kinase activation by EGF and no effect on thrombin-induced phosphoinositide hydrolysis (Fig. 4*B*; data not shown). These data suggest that the thrombin receptor utilizes both protein kinase C-dependent and -independent pathways to couple to MAP kinase.

In contrast to MAP kinase activation, prolonged PDBu pretreatment completely blocked c-Raf kinase activation by thrombin (Fig. 5*B*). Protein kinase C activation is therefore critical for c-Raf activation by the thrombin receptor in mouse lung fibroblasts. The persistence of thrombin-induced MAP kinase activation in the absence of c-Raf activation in PDBu-pretreated mouse fibroblasts demonstrates a c-Raf independent pathway to MAP kinase activation in these cells.

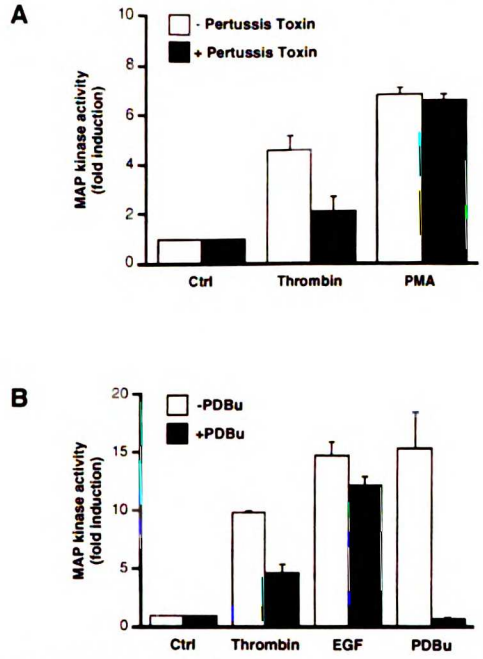


FIG. 4. **Inhibition of thrombin-stimulated MAP kinase activity by pertussis toxin and phorbol ester pretreatment.** *A*, effect of pertussis toxin on thrombin-induced MAP kinase activity. Serum-starved WT lung fibroblasts were incubated in the presence or absence of 100 ng/ml pertussis toxin for 5 h at 37 °C. Cells were then stimulated with DMEM alone (*Ctrl*) or with DMEM containing 10 nM α-thrombin or 100 ng/ml PMA for 5 min at 37 °C. MAP kinase activity was measured and expressed as "fold induction" relative to controls. Data shown are the mean ± S.E. of an individual experiment performed in duplicate. The basal activity of MAP kinase was similar in pertussis toxin-treated and untreated cells. Similar findings were observed in three separate experiments, each done in duplicate. *B*, effect of PDBu treatment on thrombin-stimulated MAP kinase activity. Serum-deprived WT cells were incubated with 0.1% $Me_2SO$ (vehicle) or 1 μg/ml PDBu for 18 h and then exposed to DMEM alone (*Ctrl*) or to DMEM with 10 nM α-thrombin, 100 ng/ml EGF, or 1 μg/ml PDBu for 5 min at 37 °C. Increases in MAP kinase activity were determined and expressed as fold induction over basal activity. The basal activity was similar in control and PDBu-pretreated cells. Two separate experiments performed in duplicate revealed similar results.

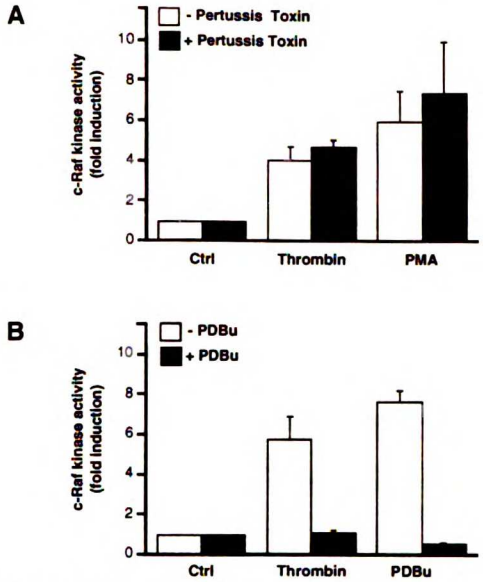


FIG. 5. **Inhibition of thrombin-stimulated c-Raf kinase activity by pertussis toxin but not phorbol ester pretreatment.** *A*, effect of pertussis toxin on thrombin-induced c-Raf kinase activation. WT lung fibroblasts were serum-deprived and treated for 5 h at 37 °C with 100 ng/ml pertussis toxin. Cells were then incubated with DMEM alone (*Ctrl*) or DMEM containing 10 nM α-thrombin or 100 ng/ml PMA for 5 min at 37 °C. c-Raf was immunoprecipitated, and kinase activity was determined using histone H1 in an *in vitro* kinase reaction (see "Experimental Procedures"). Data are presented as fold induction relative to controls (mean ± S.E. ($n$ = 2)). Basal c-Raf kinase activity was similar in control and pertussis-pretreated cells. These results were replicated in two independent experiments performed in duplicate. *B*, effect of PDBu pretreatment on c-Raf kinase activation stimulated by thrombin. Serum-starved WT cells were exposed to 0.1% $Me_2SO$ (vehicle) or to 1 μg/ml PDBu for 18 h. Cells were then exposed to DMEM with 10 nM α-thrombin, 1 μg/ml PDBu, or to DMEM alone (Ctrl) for 5 min at 37 °C. c-Raf kinase activity was determined as above. Each experiment was performed in duplicate and results expressed as the mean ± S.E. PDBu pretreatment did not alter basal c-Raf kinase activity. These results were replicated in an independent experiment performed in duplicate.

G protein-coupled receptors have been shown to activate MAP kinase via $G_q$ or $G_i$ in both transiently transfected cell systems (28–30) and in stably transfected cell lines (31–34). $G_q$-mediated MAP kinase activation involves $\alpha_q$ coupling to phospholipase C and protein kinase C and in some cell types involves a c-Raf kinase-dependent pathway (30). In contrast, $G_i$ mediates MAP kinase activation through an incompletely characterized pathway mediated by the βγ subunits released from the $G_i$ heterotrimer. $G_i$-mediated *ras* and c-Raf kinase activation have been described (31). Our observations suggest that a $G_i$-activated c-Raf-independent pathway to MAP kinase also exists and is consistent with previous findings (17).

Taken together, the observations presented above suggest that the cloned thrombin receptor naturally expressed by mouse lung fibroblasts utilizes two distinct pathways to activate MAP kinase: one via a $G_q$-like G protein, protein kinase C, and c-Raf kinase; and a second via a $G_i$-like G protein and an unknown MEK kinase (Fig. 6). The use of several pathways by a single receptor cannot represent promiscuity due to receptor overexpression or to expression of the cloned thrombin receptor in an inappropriate cellular context in these untransfected fibroblasts. The biological importance of coupling to multiple pathways for modulation of the MAP kinase response pathway remains to be determined.

In summary, both thrombin and thrombin receptor agonist peptide stimulated DNA synthesis and activated an important mitogenic signaling pathway, the MAP kinase cascade, in fibroblasts derived from wild-type mouse lung. These responses were selectively lost in fibroblasts derived from $tr^{-/-}$ mouse lung. The cloned thrombin receptor is therefore both necessary and sufficient for thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. These cells provided an opportunity to define the pathways that the single cloned receptor utilizes to activate the MAP kinase cascade. Our findings indicate that two pathways, one dependent on protein kinase C and c-Raf and a second one dependent on a $G_i$-like G protein and c-Raf-independent, both participate in MAP kinase activation by the cloned thrombin receptor (Fig. 6).

The data presented above do not imply that the cloned thrombin receptor is the sole mediator of thrombin responses in all cell types. To the contrary, the $tr^{-/-}$ mouse unequivocally demonstrates that mouse platelets utilize a distinct thrombin

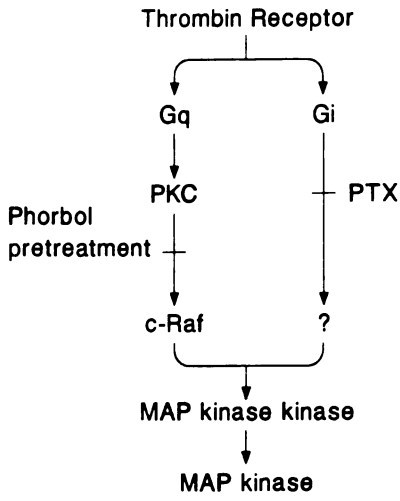


FIG. 6. **Schematic representation of thrombin receptor signaling to the MAP kinase cascade.** Our data suggest that the cloned thrombin receptor accounts for thrombin stimulation of MAP kinase in mouse lung fibroblasts. Even when naturally expressed by these cells, the single receptor appears to utilize two distinct pathways to activate MAP kinase. The cloned thrombin receptor can couple to $G_q$- and $G_i$-like G proteins. *Left side pathway*, in mouse lung fibroblasts, thrombin-induced phosphoinositide hydrolysis and c-Raf activation are pertussis-insensitive and presumably mediated by a $G_q$-like G protein. Protein kinase C (*PKC*) is activated by phosphoinositide hydrolysis. Elimination of phorbol-sensitive protein kinase C abolished c-Raf kinase activation but caused only partial inhibition of MAP kinase activation. This result implies the existence of a c-Raf-independent pathway in these cells. *Right side pathway*, inactivation of $G_i$-like proteins by pertussis toxin (*PTX*) treatment had no effect on c-Raf activation but caused partial inhibition of MAP kinase activation by thrombin. This second pathway presumably utilizes a MEK kinase distinct from c-Raf.

receptor and that distinct thrombin receptors mediate thrombin responses in different tissues (10). The observation that the cloned thrombin receptor is necessary for thrombin-induced fibroblast mitogenesis suggests that the thrombin receptor knockout mice are a valuable tool for investigating the role of thrombin in wound repair and other proliferative responses to vascular injury *in vivo*, especially since platelet activation and hemostasis are unperturbed in these mice.

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# Chapter 5

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# Protease-activated receptor 3 is a second thrombin receptor in humans

**Hiroaki Ishihara*, Andrew J. Connolly*†, Dewan Zeng*, Mark L. Kahn*, Yao Wu Zheng*, Courtney Timmons*, Tracy Tram* & Shaun R. Coughlin*‡**

* *Cardiovascular Research Institute and Daiichi Research Center, Departments of † Pathology and ‡ Medicine, University of California, San Francisco, HSW-831, 505 Parnassus Avenue, San Francisco, California 94143-0130, USA*

**Thrombin is a coagulation protease that activates platelets, leukocytes, endothelial and mesenchymal cells at sites of vascular injury, acting partly through an unusual proteolytically activated G-protein-coupled receptor[1–3]. Knockout of the gene encoding this receptor provided definitive evidence for a second thrombin receptor in mouse platelets and for tissue-specific roles for different thrombin receptors[4]. We now report the cloning and characterization of a new human thrombin receptor, designated protease-activated receptor 3 (PAR3). PAR3 can mediate thrombin-triggered phosphoinositide hydrolysis and is expressed in a variety of tissues, including human bone marrow and mouse megakaryocytes, making it a candidate for the sought-after second platelet thrombin receptor. PAR3 provides a new tool for understanding thrombin signalling and a possible target for therapeutics designed selectively to block thrombotic, inflammatory and proliferative responses to thrombin.**

A polymerase chain reaction (PCR)-based strategy yielded a new human complementary DNA encoding a putative G-protein-coupled receptor with 27% amino-acid sequence similarity to the cloned human thrombin receptor[3] (henceforth called PAR1) and 28% similarity to PAR2 (Fig. 1a). PAR2, a possible trypsin receptor, is the only other known member of the protease-activated receptor family[5]. The amino-terminal exodomain of the new receptor, designated PAR3, contained a possible thrombin cleavage site at residues K38/T39, followed by a sequence strikingly identical to a thrombin-binding sequence in the leech anticoagulant hirudin[6] (Fig. 1b). Analogous sequences in PAR1 mediate recognition and efficient cleavage by thrombin[7–10] (Fig. 1b); this cleavage unmasks a new amino terminus which serves as a tethered peptide ligand, binding intramolecularly to the body of PAR1 to effect transmembrane signalling[3,11,12]. These observations indicated that PAR3 was a new thrombin receptor that should be activated by cleavage of the K38/T39 peptide bond.

We confirmed that PAR3 was a thrombin substrate in a reaction in which 20 nM thrombin cleaved 80% of PAR3 expressed on the surface of Cos 7 cells within 5 min, which is comparable to PAR1 cleavage (Fig. 2). PAR3 cleavage was prevented by substitution of proline for threonine at position 39 (Fig. 2), the P1′ residue in the putative K38/T39 cleavage site (Fig. 1). To confirm the location of the thrombin cleavage site on PAR3, the amino-terminal exodomain of PAR3 (residues 21–94) was expressed in *Escherichia coli* as a soluble polypeptide and cleaved in solution. Even when incubated with 50 nM thrombin for 1 hour at 37 °C, only two cleavage products were detected on SDS–PAGE. Their size was consistent with cleavage at the K38/T39 peptide bond, and amino-terminal sequencing revealed only the original amino terminus and a single new amino terminus with the sequence TFRGA (Fig. 1). Thus thrombin selectively cleaves the peptide bond between K38 and T39 in PAR3's amino-terminal exodomain.

Does PAR3 cleavage by thrombin trigger signalling? Cos 7 cells transfected with PAR3 cDNA gave robust thrombin-stimulated phosphoinositide hydrolysis (Fig. 3a). Co-transfection with $\alpha_{16}$, a G-protein α-subunit expressed in haematopoietic cell lines[13], caused a 50–100% increase in the maximal PAR3-mediated response to thrombin in these cells (Fig. 3a). The half-maximal effective concentration ($EC_{50}$) for thrombin signalling by PAR3 in this system was ~0.2 nM, about twice that obtained with PAR1 and well within physiological thrombin concentrations (Fig. 3b). Mutation of the K38/T39 site to prevent receptor cleavage (Fig. 2) ablated PAR3 signalling (Fig. 3a), and thrombin rendered proteolytically inactive by the inhibitor PPACK[14] caused no signalling in PAR3-transfected cells at concentrations as high as 1 μM (not shown). Thus PAR3 mediates thrombin signalling and PAR3's K38/T39 peptide bond must be cleaved for activation of the receptor.

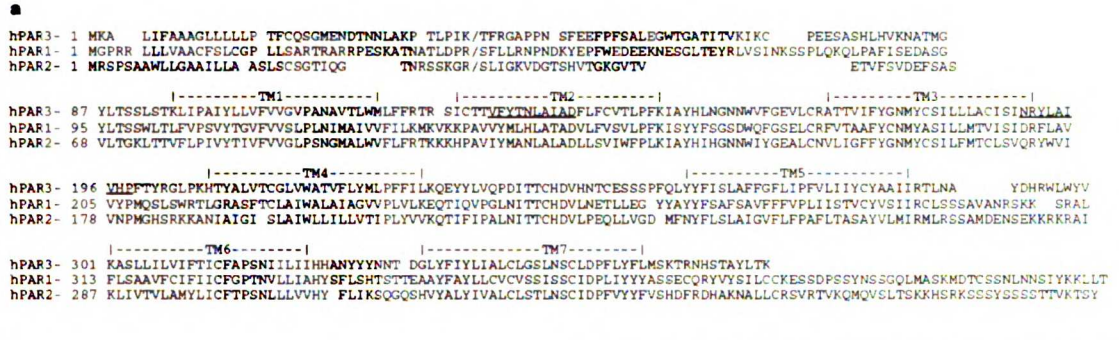
Several observations suggest that PAR3, like PAR1, uses thrombin's fibrinogen-binding exosite for receptor recognition[7–10]. γ-thrombin, which is defective in its fibrinogen-binding exosite[15], was 100 times less potent than α-thrombin (Fig. 3b). Similarly, incubation of α-thrombin with the fibrinogen-binding exosite blocker hirugen[16] right-shifted the dose–response curve by a factor of 100 (data not shown). Alanine substitutions at F48 and E49 in PAR3's hirudin-like sequence, residues predicted to dock with thrombin's fibrinogen-binding exosite by analogy with PAR1 (refs 7, 9) and hirudin[6] (Fig. 1), attenuated thrombin cleavage (Fig. 2) and right-shifted PAR3's concentration response to thrombin tenfold (data not shown).

How specific is PAR3 for thrombin compared with other proteases? We compared protease-triggered mobilization of radioactive calcium ($^{45}Ca$), which reflects phosphoinositide hydrolysis in

*Xenopus* oocytes expressing PAR3 or PAR1 (ref. 7). Thrombin caused strong responses in PAR3-expressing oocytes, whereas other arginine/lysine-specific serine proteases (factor Xa, trypsin, factor VIIa, tissue plasminogen activator or plasmin) had little or no activity (Fig. 3c). Chymotrypsin, elastase and cathepsin G were also inactive. Oocytes expressing PAR1 responded even more strongly to thrombin and were activated to some extent by preparations of factor Xa, plasmin or trypsin. Thus PAR3 is at least as specific for thrombin as the bona fide thrombin receptor PAR1.

Synthetic peptides that mimic the tethered ligands unmasked by thrombin cleavage of PAR1 or trypsin cleavage of PAR2 activate those receptors independently of receptor cleavage[3,5]. Surprisingly, peptides mimicking the putative tethered ligand of PAR3 (TFRGAP and TFRGAPPNS) or the PAR1- and PAR2-activating peptides SFLLRN and SLIGRL, showed little or no activity at PAR3 at concentrations as high as 100 μM (data not shown). PAR3's putative tethered ligand domain is required for signalling in that substitution of alanine for Phe 40 in PAR3 (Fig. 1b) yielded a receptor that failed to signal upon thrombin cleavage (Figs 2, 3). F40 in PAR3 is analogous to the critical F43 in PAR1's tethered ligand[17] (Fig. 1b). Cleavage of the K38/T39 peptide bond in PAR3 may trigger its activation by the same tethered-ligand mechanism[3,11] that is used by PAR1 and 2; failure of synthetic tethered ligand to activate PAR3 may be explained by a different avidity or structural context. We cannot yet exclude alternative mechanisms of activation: for example, PAR3 cleavage may release a constraint that maintains the receptor in an 'off' state.

Northern blot analysis revealed two PAR3 messenger RNA species migrating at 3.4 kilobases (kb) and 1.8 kb in human and at 2.8 kb and 1.8 kb in mouse (Fig. 4a). Hybridization with probes for coding as opposed to 3′ untranslated regions of mouse PAR3 cDNA and sequencing of 3′ rapid amplification of cDNA ends (RACE) products showed that the smaller mouse mRNA resulted from use of an alternative polyadenylation signal (ATTAAA) 340 nucleotides 3′ of the stop codon. The human cDNA sequence also contained a potential alternative polyadenylation signal (ACTAAA) 260 nucleotides 3′ of the stop codon. In humans, PAR3 mRNAs are expressed in bone marrow and a variety of other tissues (Fig. 4a), although it is not known which cell types are responsible for this expression. In mouse, PAR3 mRNA was detected in spleen, with a less intense signal in lung and brain (Fig. 4b). *In situ* hybridization revealed that PAR3 expression was high in megakaryocytes in mouse spleen and bone marrow (Fig. 4c, and data not shown) but was low in other mouse tissues under our exposure conditions (Fig. 4c).



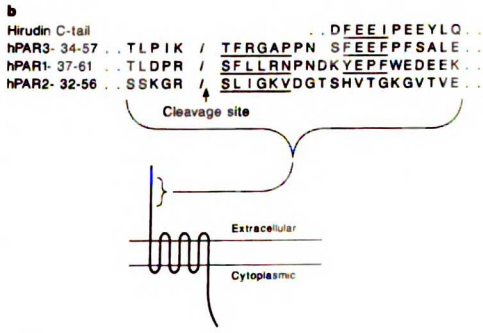




**Figure 1** Amino-acid sequence of human PAR3. **a**, Alignment with PAR1 and PAR2. Predicted transmembrane (TM) domains are overlined. Sequences corresponding to the degenerate PCR primers are underlined. **b**, Features of PAR amino-terminal exodomains. Cleavage sites are indicated; tethered ligand domains of PAR1 and PAR2 and putative tethered ligand domain of PAR3 are underlined. Also underlined is PAR3's hirudin-like domain (FEEIP). Note the similarity to the FEEIP and YEPFW sequences in hirudin and PAR1 that bind to thrombin's fibrinogen-binding exosite[1, 10].

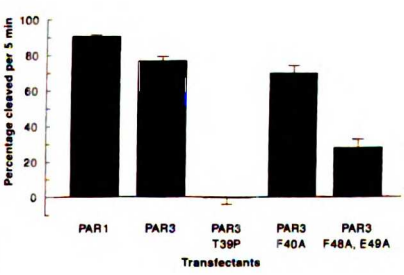



**Figure 2** Thrombin cleaves PAR3. Wild-type or mutant human PAR1 or PAR3 cDNAs encoding receptors displaying a Flag epitope (see Methods) amino to their thrombin cleavage sites were transiently expressed in Cos7 cells. The T39P mutation renders the predicted thrombin-cleavage site uncleavable, F40A hobbles PAR3's putative tethered ligand domain, and F48A, E49A mutates two residues predicted to contribute to thrombin binding (Fig. 1b). Surface expression levels, assessed as transfection-dependent binding of M1 anti-Flag antibody to the cell surface, were similar for all constructs examined. Cells were incubated for 5 min at 37 °C in the presence or absence of 20 nM thrombin. Receptor cleavage was detected as loss of the Flag epitope from the cell surface (expressed as a percentage of M1-antibody-specific binding lost upon thrombin treatment). Data shown are mean ± s.e.m. ($n = 3$). This experiment was replicated three times.

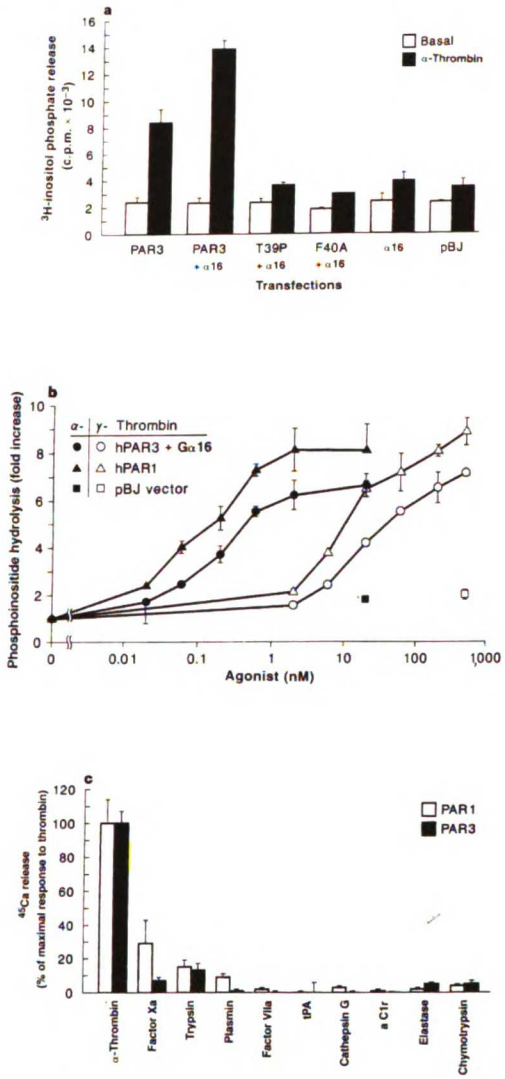



**Figure 3** PAR3 signalling. **a**, Wild-type and mutant PAR3 signalling in Cos 7 cells. Epitope-tagged human PAR1, PAR3, or the indicated mutants were transiently expressed in Cos 7 cells with or without $G\alpha_{16}$. Expression was similar for wild-type and mutant receptors with or without $G\alpha_{16}$ coexpression. Data shown are mean ± s.e.m. ($n$ = 3) $^3$H-inositol phosphates released per 120 min in the presence or absence of 20 nM α-thrombin[20]. Similar results were obtained when the incubation time was 15 min. **b**, Concentration responses. Phosphoinositide hydrolysis in response to the indicated concentration of α- or γ-thrombin was measured in Cos 7 cells transfected with PAR1 or PAR3 plus $G\alpha_{16}$, as in **a**. Data are mean ± s.e.m. ($n$ = 3). **c**, Specificity of PAR3 and PAR1. Protease-triggered $^{45}$Ca release per 10 min was measured in *Xenopus* oocytes expressing human PAR1 or PAR3. Proteases were at 20 nM except trypsin (0.5 nM). Data are mean ± s.e.m. ($n$ = 2) and are expressed as a percentage of the maximum response to thrombin. In the experiment shown, 20 nM thrombin caused 24- and eightfold increases in $^{45}$Ca release in PAR1- and PAR3-expressing oocytes, respectively, and surface expression of PAR1 was twice that of PAR3. No responses were seen in uninjected oocytes. Experiments in **a**, **b** and **c** were replicated three times.

Mouse platelets use a thrombin receptor that is distinct from PAR1 (ref. 4). The PAR3 expression found in mouse megakaryocytes indicates that PAR3 may mediate thrombin responses in mouse platelets. Determination of the signalling properties of mouse PAR3 and knockout of the PAR3 gene will test this idea. Unlike mouse platelets, human platelets use PAR1 (refs 3, 17–19) but thrombin's greater efficiency compared with PAR1-activating peptide at triggering some responses in human platelets suggests that they may use PAR1 and a second thrombin receptor[20–23].

Defining the signalling pathways and relative roles for PAR1 and PAR3 in human platelets, endothelium, leukocytes, mesenchymal and other cells will be important. The availability of different thrombin receptors with tissue-specific roles may allow more effective or selective disruption of thrombin signalling and help in development of pharmaceuticals aimed at interrupting thrombin's thrombotic, inflammatory and proliferative actions.

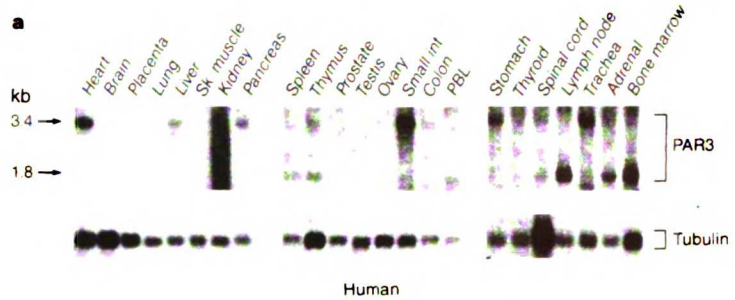
*Note added in proof*: A partial sequence of mouse PAR3 was recently deposited in the WashU-HHMI EST Project database. Accession no. mt07f02.r1. □

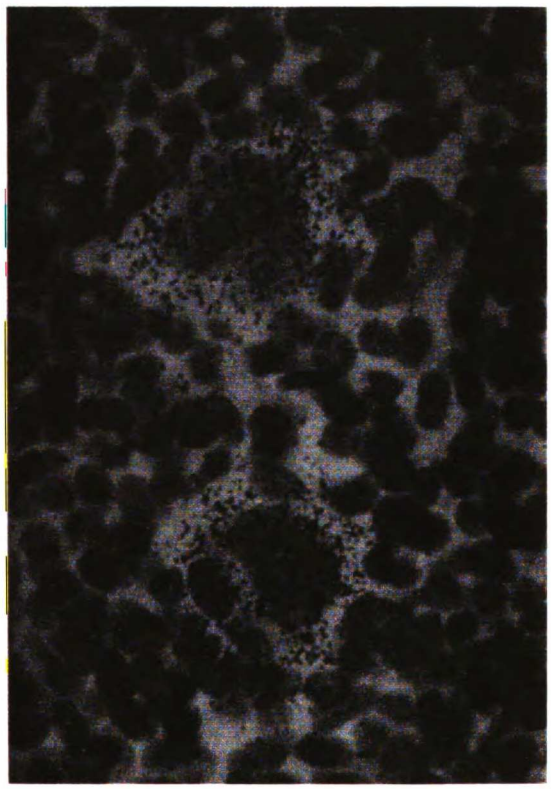
## Methods

**Cloning of PAR3.** Rat platelets were included among many RNA sources tested because they are a more abundant source of RNA than mouse platelets and, like mouse platelets, they do not respond to PAR1-agonist peptides (refs 4, 24, 25, and data not shown). Total RNA was prepared from rat platelets using Trizol reagent (Gibco BRL). cDNA was prepared using random hexamer primers and Superscript reverse transcriptase (Gibco BRL), then amplified using degenerate PCR primers. 11 'forward' and 13 'reverse' degenerate primers corresponding to a variety of sites relatively conserved among PAR1, PAR2, peptide, and glycoprotein hormone GPCRs[26] were tested in different pairwise combinations. Amplification of rat platelet cDNA with the primers 5′-GTI TAC ATG CTI (A/C)AC (C/T)TI GCI (A/C/G/T)TI GC(A/C/G/T) GA-3′ and 5′-GGA TAI ACI ACI GCI A(A/G/T)(A/G) (A/T)AI C(G/T)(A/C/G/T) TC-3′ yielded PAR3. Primers were used at 5 μM in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, with 50 U ml$^{-1}$ *Taq* polymerase. Temperature cycles were: 94 °C for 4 min; 30 cycles of 94 °C for 45 s, 39 °C for 60 s, 72 °C for 90 s; then 72 °C for 7 min. PCR products were subcloned using a TA cloning kit (Invitrogen). Clones with inserts of ~200 bp were grouped by restriction analysis and analysed by nucleic acid sequencing. One sequence predicted a novel G-protein-coupled receptor highly related to PAR1 and PAR2. This rat sequence was used to obtain mouse and human cDNA clones by a combination of standard PCR and hybridization techniques. The human PAR3 cDNA used for these studies was from a λgt10 small intestine cDNA library (Clonetech). The mouse cDNA used for *in situ* hybridization and northern blot analysis was from a spleen library in Lambda ZAP (Stratagene).

**PAR1 and PAR3 functional studies.** cDNA for epitope-tagged PAR3 analogous to Flag-epitope-tagged PAR1[27] was constructed to encode an N terminus with the sequence MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS/DYKDDDDVE-TF representing the prolactin signal peptide, the putative signal peptidase site (/), the Flag epitope DYKDDDD and junction VE fused to amino acid 17 in PAR3. Receptor cDNAs were subcloned into the mammalian expression vector pBJ1 or pFROG[3]. For receptor cleavage, Cos 7 cells were transfected using DEAE–dextran and thrombin-dependent loss of M1 antibody (Kodak) binding to the cell-surface Flag epitope was followed[10,27]. Over 95% of M1 antibody binding was transfection-dependent in this system. For identification of the cleavage site, PAR3 N-terminal exodomain residues 21–94 sandwiched between a translational start and hexahistidine tag (that is, MG, PAR3 21–94, VEHHHHHH) were expressed as a soluble polypeptide in *E. coli*, purified, and analysed before and after thrombin cleavage, as described for the analogous region in PAR (ref. 10). For signalling studies[3,28,29], *Xenopus* oocytes were microinjected with 12.5–25 ng receptor cRNA/oocyte and Cos 7 cells were transfected with 5 μg receptor cDNA in pBJ1 and/or 5 μg $G\alpha_{16}$ cDNA in pCDNA1 (InVitrogen) per 10-cm plate.

**PAR3 expression in mouse and human tissues.** Northern blots with ~2 μg mRNA loaded per lane (Clontech) were hybridized at high stringency with $^{32}$P-labelled probes for human or mouse PAR3. Probes were generated by random priming (Prime-It II kit; Stratagene) of PCR-amplified DNA fragments



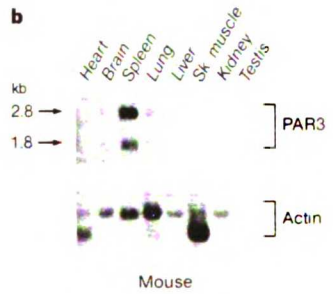




**Figure 4** Expression of PAR3 mRNA. Northern analysis of **a**, human, and **b**, mouse tissues. The smear in the human kidney lane is an artefact; little hybridization to kidney mRNA was seen in other northern blots. Arrows indicate two species of human or mouse PAR3 mRNA which probably result from use of alternative polyadenylation signals. **c**, *In situ* hybridization reveals PAR3 mRNA in mouse splenic megakaryocytes. Bright-field photomicrograph shows silver grains overlying large cells with multilobulated nuclei. Multiple tissues (spleen, brain, eye, thymus, heart, lung, liver spleen, pancreas, stomach, small intestine, colon, kidneys, bladder, uterus, ovary, testis, skeletal muscle, peripheral nerve and skin) were examined on the same slide. Only megakaryocytes in the spleen displayed clear-cut hybridization over background. Sense probe controls were negative for all cells.

corresponding to nucleotides 459–860 of human and 414–564 of mouse PAR3 cDNAs (A of start ATG, represents the first nucleotide). Hybridization for human tubulin or mouse β-actin controlled for lane loading. For *in situ* hybridization, anaesthetized adult C57BL/6 mice were perfusion-fixed with 4% paraformaldehyde. Organs were dissected free, immersion-fixed for 4 h in 4% paraformaldehyde, then embedded in paraffin. 5-μm sections were hybridized with a sense or antisense $^{35}$S-riboprobe transcribed *in vitro* from mouse PAR3 cDNA subcloned into the *Eco*RI site of pBluescript II SK$^-$. Hybridization, washing and development conditions were as described for mouse PAR1 (ref. 30). A 4-week exposure result is shown.

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Correspondence and requests for materials should be addressed to S.R.C. (e-mail: shaun_coughlin.src@quickmail.ucsf.edu). Sequences of mouse and human cDNAs for PAR3 have been deposited in GenBank, accession numbers U92971 and U92972.

# Chapter 6

{To be submitted as a Short Communication for the American Journal of Pathology}

## Mice lacking the thrombin receptor, PAR1, have normal skin wound healing.

Andrew J. Connolly[1], David Y. Suh[2], Thomas K. Hunt[2], and Shaun R. Coughlin[3]

Departments of Pathology[1], Surgery[2], and Medicine[3]
University of California, San Francisco.

### Abstract

In addition to its roles in coagulation, thrombin is a potent activator of numerous cell types involved in wound healing, including platelets, macrophages, fibroblasts, and endothelial cells (1-3). Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor that mediates many of the cellular activities of thrombin (4, 5). To test the role of this receptor *in vivo*, we had created mice with null mutations of the *par*1 gene (6). In this study, there was no difference found between wild-type and null mice in skin wound healing assays including closure of open wounds, tensile strength of healed incisions, wound histology, and collagen/DNA content of wound sponge implants. We conclude that PAR1 is not necessary for skin wound

healing in mice, possibly due to redundant signaling mechanisms for some thrombin-induced cellular responses.

## Introduction

Thrombin is a multifunctional serine protease generated at sites of vascular injury, including skin wounds. Active thrombin is sequestered in the fibrin clot where it is protected from neutralization by plasma proteinase inhibitors and may be released later during wound healing (7-9). In addition to its well-described roles in coagulation, thrombin directly activates a number of cell types that play important roles in wound healing. Thrombin causes aggregation and secretion of platelets, leading to their release of potent inflammatory mediators such as PDGF and serotonin (10). It activates endothelial cells leading to increased vascular permeability (11), increased adhesiveness for platelets and leukocytes (12), and release of platelet-derived growth factor (13) and monocyte chemotactic protein-1 (14). It is chemotactic for neutrophils (15), and monocytes (16). It stimulates proliferation of fibroblasts (17). Its actions on these numerous cell types suggest that thrombin may participate not only in hemostasis, but also inflammatory and proliferative responses to injury. In support of this idea, application of exogenous thrombin accelerates healing of rat skin incisions, as assessed by wound tensile strength, type I collagen deposition, and growth of capillaries in wounds by 7days postsurgery (18).

Two G protein-coupled receptors that mediate many of the cellular responses to thrombin have been cloned (4, 19). They are both activated by thrombin by a novel proteolytic mechanism in which thrombin cleaves the extracellular

amino terminus unmasking a new amino terminus which acts as a tethered ligand for the receptor. The first thrombin receptor, protease-activated receptors 1 (PAR1), was isolated by expression cloning and has been much studied over the last 8 years. The second thrombin receptor, PAR3, was cloned recently by homology to PAR1. Another protease-activated receptor, PAR2, exists that is activated by trypsin but not thrombin. PAR1 is widely expressed in human and mouse tissues, including in human platelets (4), monocytes (14), fibroblasts (5, 20), and endothelial cells (21). In mice, PAR1 is expressed in leukocytes, endothelial cells, smooth muscle cells, mesencymal cells, and neurons (22). In contrast, PAR3 expression was seen only in megakaryocytes in a broad *in situ* hybridization survey of mouse tissues (19).

Many of thrombin's known cellular activities are mediated by PAR1. Peptides that mimic the new receptor amino terminus unmasked by proteolysis are full agonists for cloned PARs expressed in Xenopus oocytes (19) and can be used to test the sufficient roles of PAR1. The PAR1-activating peptide is similar to thrombin in its *in vitro* activities on human platelets (4), fibroblasts (5), and endothelial cells (21, 23), suggesting that PAR1 mediates some of these responses to thrombin. The inflammatory properties of thrombin are also recapitulated by PAR1-activating peptides in *in vivo* models of leukocyte rolling in rat mesenteric blood vessels (24) and acute inflammation of the rat paw (25). Studies inhibiting PAR1 function have demonstrated that PAR1 is necessary for many of the cellular activities of thrombin. Anti-PAR1 antibodies are able to inhibit thrombin resonses in human platelets (26). Mouse lung fibroblasts and emryonic fibroblasts lose their responsiveness to thrombin when null mutations of the *par*1 gene are introduced(6, 17) .

To define the *in vivo* roles of PAR1, mice were produced with disruption of the PAR1 gene (6). These null mice are grossly normal and have no spontaneous bleeding defects. To test whether PAR1 is necessary for skin wound healing, mice with null mutations of the *par*1 gene were examined in this study.

## Materials and Methods

### Mice

The mice used for these studies were all healthy adult male mice of a mixed strain, each 50% C57BL/6 + 50% 129sV. They were raised and studied in microisolator cages in a mouse facility free from common pathogens. The *par*1 +/+ and *par*1 -/- mice grew at comparable rates and were matched for age (8-12 wks) and weight (25-35 grams). Mice had been genotyped by Southern blot (6) at 4 weeks of age and were genotyped once again after being sacrificed. Before all wounding studies, mice were anesthetized by inhalation of methoxyfluorane and the wound sites were carefully shaved. All wounds are full-thickness extending through the epidermis and dermis to the *panniculus carnosus*. After wounding, mice were separated into individual cages containing sterilized pressed-cellulose bedding to limit disruption or soiling of wounds. The wounds and general health of the mice were monitored each day; no complications were encountered.

### Area of Contracting Open Wounds

Two full-thickness punch biopsies ( 0.5 cm diameter, 2.0 cm apart) were made overlying the scapulas using circular skin bioptomes. Five mice were used

for each genotype. Compression with clean gauze was held on wounds for two minutes to achieve hemostasis. Wounds were neither dressed nor sutured. Each day for 11 days, the lengths and widths of the epidermal defects were measured and the wound areas calculated assuming an oval geometry.

## Wound Tensile Strength

Two full-thickness paramedian wound incisions (1.5 cm long, 2.5 cm apart) were made on the lower back using a scalpel. The wounds were made on the same ten mice used in the punch biopsy study. Compression with clean gauze was held on wounds for two minutes to achieve hemostasis, and the wounds were closed with two interrupted 4-0 nylon sutures spaced each 0.5 cm. After 11 days, the mice were euthanized and a transverse, full-thickness, 1.0 cm wide strip was taken through the center of each wound and mounted on a Sandblom tensiometer to assay breaking strength (27). Transverse, full-thickness, 1 cm-wide strips of skin were taken in a similar fashion from skin adjacent to the wounds in three mice of each genotype to act as control skin.

## Wound Histology

Two full-thickness paramedian wound incisions (1.0 cm long, 2.0 cm apart) were made overlying the scapulas using scissors. The wounds were neither dressed nor sutured. Two mice of each genotype were euthanized at the designated times (days 1, 3, 5, 7, 10). The wounds and surrounding skin were excised, fixed in 10% neutral buffered formalin, and processed for paraffin embedding. Level sections were cut from the midtransverse plane of each wound and stained with hematoxylin/eosin or Masson trichrome.

## Wound Implant Biochemical Assays

Implants were used as previously described (28, 29). Expanded PTFE tubing with pore size of 90 -120 um, internal diameter 1.2 mm and wall thickness 0.6 mm (Imprar, International Polymer Engineering, Tempe Ariz.) was cut into 3.5 cm lengths and guided percutaneously by a cutting needle into the subcutaneous tissue of the left and right flanks of five mice of each genotype. The implants were left in place for 10 days, before the mice were euthanized and the implants were removed by exposing one end and pulling the implant out. A 1.0 cm lenth of each implant was assayed for DNA content by the method of Burton (30); the remainder was assayed for total protein content by a ninhydrin assay (31) and hydoxyproline content by the method of Grant (32). To demonstrate the sensitivity of these assays to inhibitors of wound healing, a positive control group was added that consists of *par*1 +/+ mice injected with methylprednisolone (25 mg / kg body weight) at the time of surgery.

## Results

### Physical Assessment of Wounds

Wound healing was normal in *par*1 -/- mice in comparison with *par*1 +/+ mice matched for sex, age, weight, and strain. The area of wounds was followed after punch excisional biopsy for 11 days (Figure 1a). The general trend for both +/+ and -/- mice was a linear reduction in wound area until complete closure at 10-11 days. Skin tensile strength was measured in wounds and nearby control skin 11 days after incisions were made and closed by suturing (Figure 1b). The tensile strength of -/- wounds was indistinguishable from that of +/+ wounds. Control skin from +/+ and -/- mice had indistinguishable tensile strength.

## Histologic Assessment of Wounds

The histology of healing wounds was similar for *par*1 -/- and +/+ mice at 1, 3, 5 and 7 days after wounds were made (Figure 2). In this model, 1 cm long incisions are made that gape 0.4 - 0.6 cm initially. The base of the wound is covered with blood clot and a fibrinopurulent exudate at 1 day. This is followed, in the sections at days 3 and 5, by macrophage infiltration and granulation tissue growth at the base and shoulders of the wound. The wound contracts and epidermis grows in from the edges, steadily displacing the eschar throughout days 1, 3, 5, and 7. The wounds had similar amounts of blood clot and neutrophil infiltration at 1 day, granulation tissue growth at 3 and 5 days, and epidermal growth throughout.

## Biochemical Assessment of Wounds

To assess the cellularity and collagen production of wounds in *par*1 -/- and +/+ mice, biochemical assays were performed on expanded PTFE implants that remained in the subcutaneous tissue of *par*1 +/+ and -/- mice for 10 days. The DNA content gives a measure of cellularity within the implant, the hydroxyproline content gives an estimate of collagen production, and total protein content assays amounts of both cellular and extracellular protein. No difference were seen between *par*1 +/+ and -/- mice in these assays. To demonstrate the sensitivity of these assays to inhibitors of wound healing, a positive control group was added that consists of *par*1 +/+ mice injected with methylprednisolone at the time of surgery. In this corticosteroid-treated group, reductions in DNA content and hydroxyproline content were more apparent than reductions in total protein, as has been noted before when this model has been used in corticosteroid treated rats (28).

## Discussion

Thrombin is generated in wounds and is a potent activator of cell types known to play key roles in wound healing. Many of the cellular responses to thrombin are mediated by PAR1, and we postulated that important aspects of wound healing could be mediated by thrombin stimulation of this receptor. In this study, wound healing was assessed physically, histologically, and biochemically, and no differences in healing were seen between *par*1 +/+ and -/- mice. This study definitively indicates that PAR1 is not necessary for skin wound healing in mice.

Subtle alterations could exist in wound healing of *par*1 -/- mice, however, the wound healing assays used in this study have proven to be sensitive to alterations in healing caused by various perturbations. There was demonstrated inhibition of wound healing in open wound closure and ePTFE implant models in ob/ob mice (29) and open wound closure and incision tensile strength models in db/db mice (33). In rats, wound healing as measured by these assays is altered by systemic administration of corticosteroids (28, 34), cyclosporine A (35), and anti-Thy1.2 (T cell) antibody (36).

We have not excluded the possibility that thrombin's cellular activities are important for wound healing, but are mediated by receptors other PAR1. Some thrombin-induced effects are not reproduced by PAR1-activating peptides in human endothelial cells (13, 37), hamster fibroblasts (38), and mouse platelets (39). However, comparisons between the agonist properties

of receptor-activating peptides and thrombin are tricky, given that peptides and thrombin activate the receptor by different mechanisms, differ in potency by at least four logs (6), and differ in pharmacokinetics *in vivo* and *in vitro* (40). The targeted disruption of the PAR1 gene in mice(6) demonstrated that at least one other protease-activated receptor existed in mice; this was subsequently cloned and named PAR3 (19). PAR3 is very restricted in its expression *in vivo*, as demonstrated by mouse multiple-tissue northern blots and by an *in situ* hybridization survey(19). The mRNA is expressed at measureable levels only in megakaryocytes and platelets of normal adult mice. It seems unlikely that PAR3 could play a significant role in wound healing given that mRNA for PAR3 was not found in healing incisions of mice by *in situ* hybridization (data not shown). However, it will be interesting to see if wound healing is normal in mice with null mutations of the *par*3 gene alone or in the *par*1 -/- background.

Some evidence exists that cells and wounds can also be stimulated by proteolytically-inactive preparations of thrombin that should not activate the cloned PARs. Upregulation of platelet-derived growth factor in human renal microvascular endothelium (41), human monocyte chemotaxis (16), and human neutrophil chemotaxis (15) can be stimulated by proteolytically-inactive thrombin. It is possible that inactive thrombin works *in vivo* by interacting with biologically important regulators of active thrombin, which could then act on PARs. This could be tested by studying whether inactive thrombin can accelerate wound healing in *par*1 -/-, *par*3 -/-, or prothrombin -/- mice.

The argument for a necessary role of thrombin in wound healing, other than in coagulation, is merely circumstantial. Selective reagents do not exist to define the relative importance of thrombin's roles in coagulation and cellular activation *in vivo* . Humans with complete deficiencies in prothrombin, i.e. aprothrombinemia, have not been identified (42). Prothrombin null mice, if viable, could provide a more direct test of the necessary roles of thrombin in wound healing. Skin wounds heal well in mice lacking fibrinogen , suggesting that the generation of fibrin is not necessary for skin wound healing. Comparisons of wounds in the prothromin -/- mice with the fibrinogen -/- mice could help to identify which cellular activation roles of thrombin are important in wound healing.

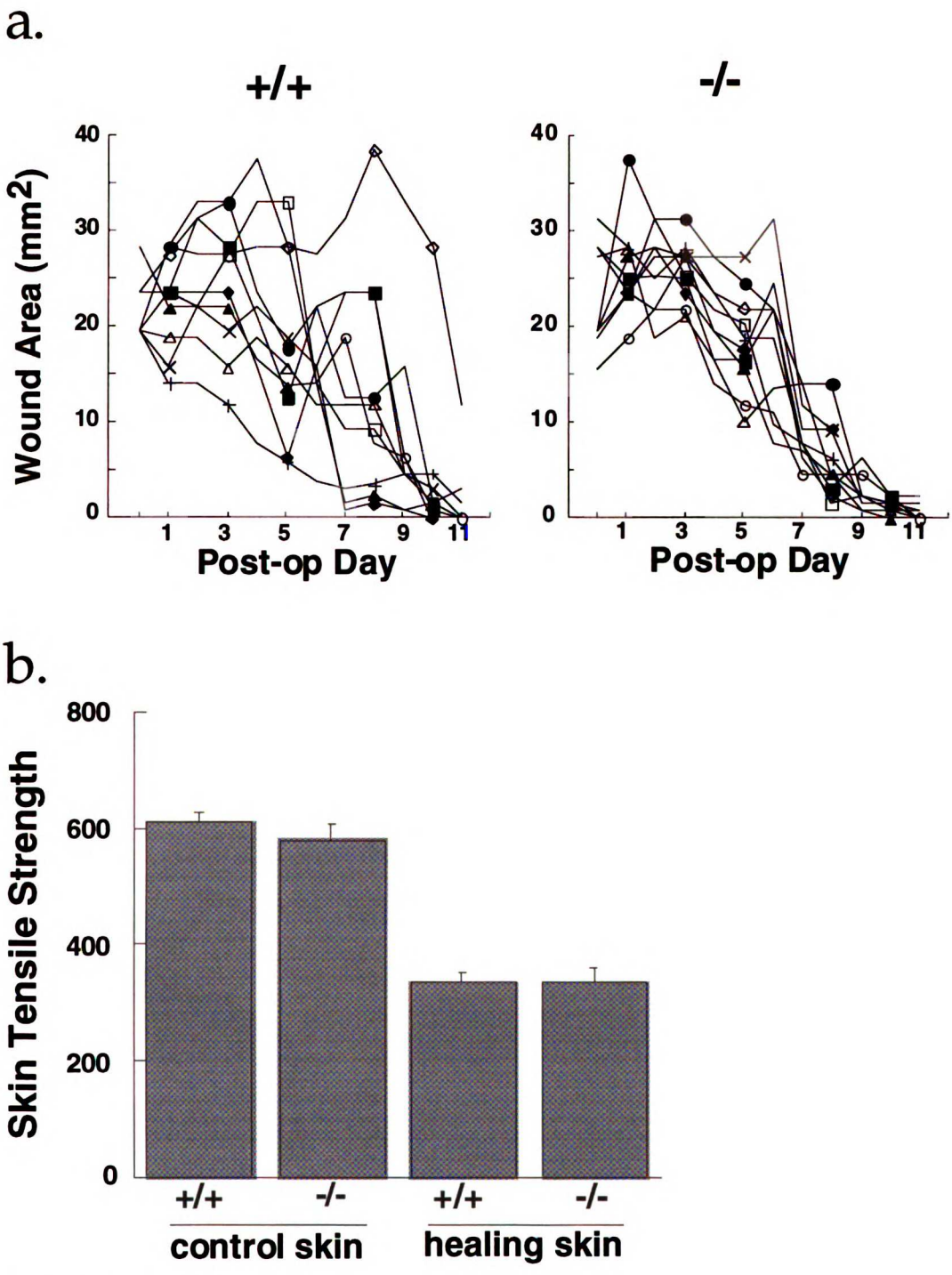


**Figure 1.** Analysis of wound closure and tensile strength in healing skin wounds in *par*1 +/+ and *par*1 -/- mice. **a:** Wound areas measured daily after excisional biopsy of back skin. **b:** Tensile strength of wound skin and nearby control skin in mice 11 days days after incisions with primary closure by suturing.

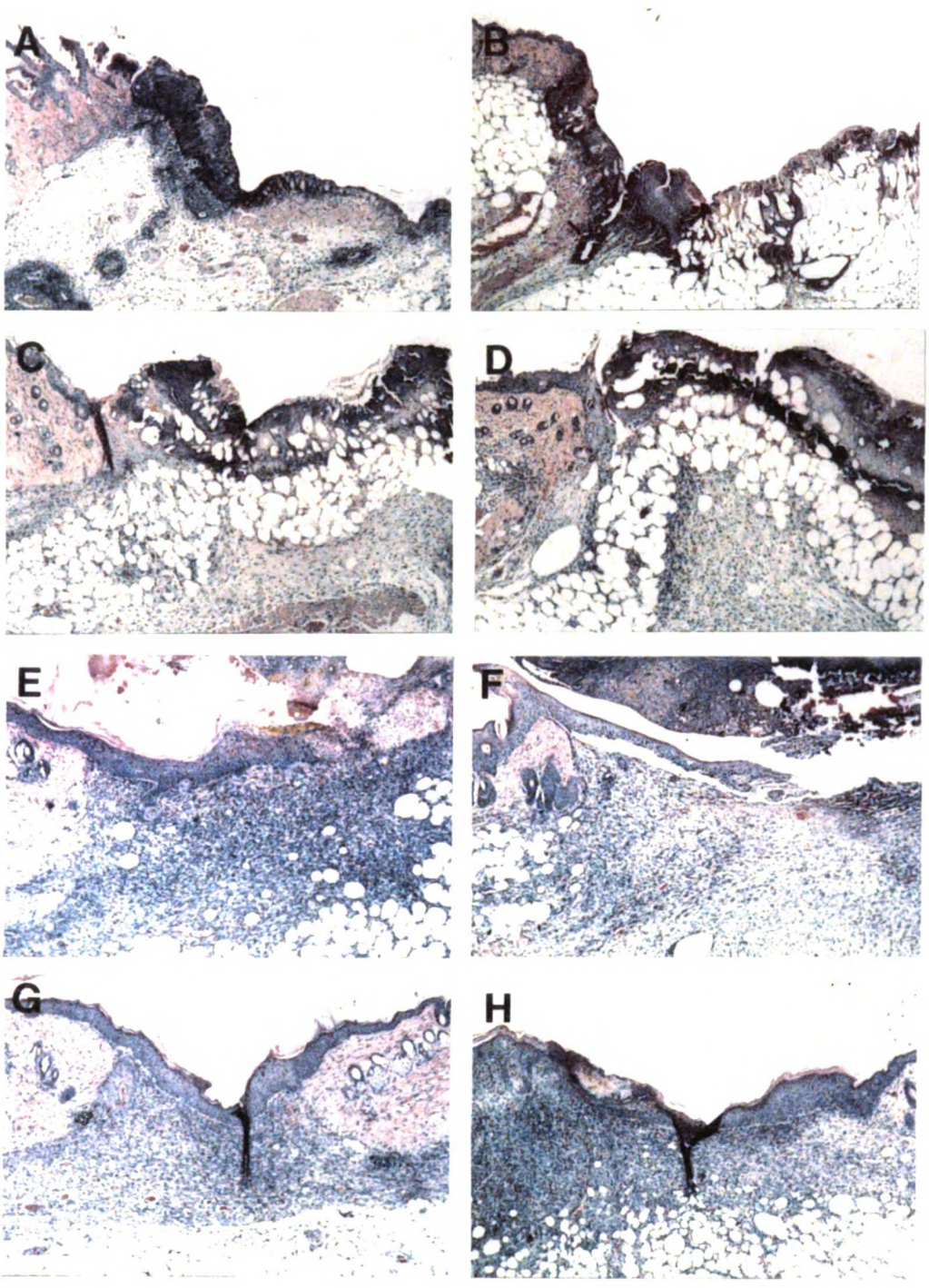


**Figure 2.** Histology of healing skin incisions in par1 +/+ and par1 -/- mice. Mid-transverse sections were taken from par1 +/+ (A,C,E,G) and par1 -/- (B,D,F,H) mice at 1 (A,B), 3 (C,D), 5 (E,F), and 7 (G,H) days after wounding. Four wounds from two mice were studied in each group at each time point with similar results. Tissue sections were stained with hematoxylin and eosin; each photographic field covers 1.7 x 1.2 mm of tissue section.

| Mice | DNA content | Protein content | HP content |
|---|---|---|---|
| *par*1 +/+ | 45.5 +/- 14.0 | 1190 +/- 129 | 21.0 +/- 5.2 |
| *par*1 -/- | 43.9 +/- 18.8 | 1090 +/- 238 | 21.1 +/- 6.0 |
| *par*1 +/+ with MePrednis. | 17.6 +/- 2.4 | 911 +/- 162 | 14.8 +/- 5.5 |

**Table 1.** Analysis of DNA content, total protein content, and hydroxyproline content in expanded PTFE implants 10 days after implantation in subcutaneous tissue of the flank. Results are expressed as ug/cm of ePTFE implant. To demonstrate sensitivity of these assays to inhibitors of wound healing, methylprednisolone was given to three *par*1 +/+ mice when the wound was made.

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# Chapter 7

## Summary

In this thesis, a molecular genetic analysis of thrombin receptor function in mice was undertaken to clarify the role of PAR1, *in vitro* and *in vivo*. We initially postulated that most, if not all, responses to thrombin were mediated by PAR1 and that this receptor mediates many important responses to injury *in vivo*.

These studies have demonstrated that PAR1 is necessary and sufficient for *in vitro* responses to thrombin in some cells, such as fibroblasts. To our surprise, mouse platelets use a second receptor to respond to thrombin. The second thrombin receptor, PAR3, was cloned by degenerate PCR and it was shown to be activated by thrombin by a mechanism similar to that of PAR1.

The *in vivo* studies revealed that hemostasis is normal in *par*1 -/- mice, presumably due to the presence of PAR3 in mouse platelets. Recently, Dr. Ishihara used anti-PAR3 antibodies to demonstrate that PAR3 is indeed necessary for thrombin responses in mouse platelets (manuscript in preparation). Skin wound healing is normal in *par*1 -/- mice. This is less likely to be explained by redundancy provided by PAR3. PAR3 expression was seen by *in situ* hybridization only in megakaryocytes in adult mouse tissues, including the skin wounds themselves. However, low level expression of PAR3 cannot be excluded. Examination of *par*3 -/- mice and double *par*1 -/- : *par*3 -/- could address the relative roles of PAR3 and PAR1 directly.

Examination of other animal models is pending. The *par*1 -/- mice have been crossed into an apoE -/- background and a complete analysis of the role of PAR1 in atherosclerosis awaits sufficient numbers of mice. In addition,

half of the mice die at embryonic day E9-10.5 for reasons that are unclear. The lethality occurs at a stage when development of the circulatory system is critical. It is provocative that the phenotype of the *par*1 -/- mice resembles the phenotype of mice with null mutations of tissue factor (66) or coagulation factor V (67), all with partial embryonic lethality during E9-10.5. This raises the possibility that thrombin generation and stimulation of PAR1 is necessary for appropriate development of the circulatory system. A carefully controlled comparison of the different mutant mice could help to clarify this issue.

One goal in this molecular genetic analysis was to reveal *in vivo* functions of PAR1 that could be extrapolated to human biology. The human PAR3 transcript was also cloned and its expression pattern *in vivo* was studied by multiple tissue northern blot in Chapter 5. The pattern of expression of human PAR3 is much broader than that of mouse PAR3. In situ hybridization or immunohistochemistry of human tissues will be necessary to clarify which cell type is expressing PAR3. By Dr. Ishihara's antibody blocking studies, it appears that thrombin responses are mediated almost entirely by PAR1 in human platelets, while they are mediated almost entirely by PAR3 in mouse platelets. The mouse embryo findings also may not apply to human embryonic development, given that human embryos do not require the rapid development of a vitelline circulation at stages analogous to mouse E9-10.5. However, the embryonic role may suggest roles in the adult such as vascular remodeling or angiogenesis. One clear extrapolation from mouse to human is that more than one thrombin receptor exists and that the two cloned receptors have distinct patterns of expression.

Further study of the *par*1 -/- mice (and *par*3 -/- mice) in various animal models is warranted. Beyond their basic scientific value, it is our hope that *in vivo* studies may reveal that thrombin receptors are desirable therapeutic

targets. We are hopeful that avid thrombin receptor antagonists with specificity for either PAR1 or PAR3 could be developed if sufficient effort were devoted. In general, excellent specific antagonists can be developed for G protein-coupled receptors. Thus, it will be important to define the relative importance of the two thrombin receptors in thrombotic, inflammatory and proliferative responses *in vivo*.

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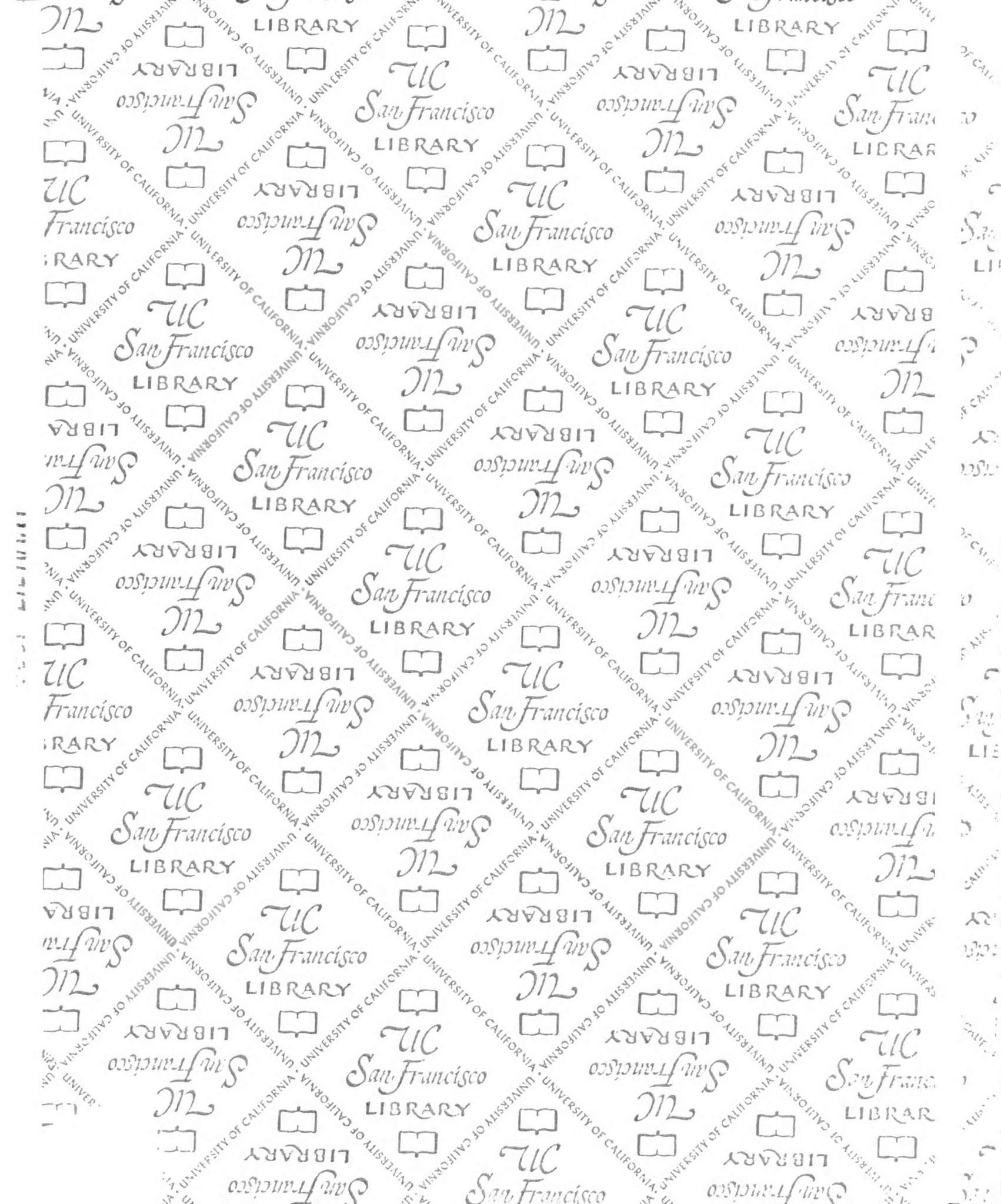
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