Functional Interactions Between the Thrombin Receptor and the T-Cell Antigen Receptor in Human T-Cell Lines

By David E. Joyce, Yan Chen, Rochelle A. Erger, Gary A. Koretzky, and Steven R. Lentz

The proteolitically activated thrombin receptor (TR) is expressed by T lymphocytes, which suggests that thrombin may modulate T-cell activation at sites of hemostatic stress. We examined the relationship between TR function and T-cell activation in the Jurkat human T-cell line and in T-cell lines with defined defects in T-cell antigen receptor (TCR) function. Stimulation with thrombin or the synthetic TR peptide SFLLRN produced intracellular Ca^{2+} transients in Jurkat cells. As the concentration of TR agonist was increased, peak Ca^{2+} mobilization increased, but influx of extracellular Ca^{2+} decreased. TR signaling was enhanced in a TCR-negative Jurkat line and in T-cell lines deficient in the tyrosine kinase ick or the tyrosine phosphatase CD45, both of which are essential for normal TCR function. TCR cross-linking with anti-CD3 IgM desensitized TR signaling in Jurkat cells, but not in CD45-deficient cells. A proteinase-activated receptor (PAR-2)-specific agonist peptide, SLIGKV, produced small Ca^{2+} transients in both MEG-01 human megakaryocytic cells and Jurkat cells, but was less potent than the TR-specific agonist TFRIFD in both cell types. Like TR signaling, PAR-2 signaling was enhanced in TCR-negative or ick-deficient Jurkat clones. These findings provide evidence for functional cross-talk between proteolytically activated receptors and the TCR.

MATERIALS AND METHODS

Materials. Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). The human TR peptide SFLLRN was purchased from Bachem California (Torrence, CA). The Xenopus TR peptide TFRIFD were synthesized by Macromolecular Resources (Pittsburgh, PA). The anti-human TR monoclonal antibody (MoAb) ATAP2 was provided by Dr Lawrence Brass (University of Pennsylvania, Philadelphia, PA). The anti-CD3 MoAb 235 were provided by Dr Shu Man Fu (University of Virginia, Charlottesville, VA), and Dr Brian Link (University of Iowa, Iowa City, IA), respectively. Control MoAb MOPC195 and fluorescein isothiocyanate (FITC)-conjugated goat-antimouse IgG (γ specific) were purchased from Cappel Laboratories (Malvern, PA).

Cell lines. The human megakaryocytic cell line MEG-01 was provided by Dr Philip Majerus (Washington University, St Louis, MO). The human promyelocytic cell line HL60 was obtained from

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Dr Timothy Ley (Washington University). The human T-cell leukemia line Jurkat (clone E6-1); Jurkat variants J.RT-T3.5, J.CaM1/rep3, and J.CaM1/lck; and two subclones (HPB.45.1 and HPB.45.0) of the immature (CD4$^-$/CD8$^-$) human thymocytic cell line HPB.ALL were obtained from Dr Arthur Weiss (University of California, San Francisco, CA). J.RT-T3.5 is a radiation-induced Jurkat mutant that is surface TCR-negative.36 J.CaM1/rep3 and J.CaM1/lck are derived from J.CaM1,37 an ethyl methanesulfonate-induced Jurkat mutant that lacks lck expression, by transfection with empty vector or lck cDNA, respectively.38 J45.01 is a radiation-induced Jurkat mutant that is surface CD45 deficient.39 J45/CH11 is a clone of J45.01 transfected with an expression vector encoding a chimeric HLA-A2/CD45 protein containing the CD45 cytoplasmic domain.40 HPB.45.1 is a subclone of HBP.ALL that expresses normal levels of CD45. HPB.45.0 is a subclone of HPB.ALL that is CD45 deficient.41 All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.0 mmol/L glutamine.

Flow cytometry. Cells were incubated with the indicated MoAbs followed by an FITC-conjugated goat-antimouse IgG secondary antibody and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as described previously.42 Viable cells were gated by forward and side scatter and by propidium iodide staining.

Equilibrium binding. Direct equilibrium binding with $^{125}$I-labeled anti-TR MoAb ATAP2 was performed by a modification of methods described previously.31,43 ATAP2 IgG was radioiodinated in the University of Iowa Diabetes and Endocrinology Research Center using lactoperoxidase. Total binding was measured by incubating $10^6$ cells for 60 minutes at 4°C in RPMI 1640, 50 mmol/L HEPES, pH 7.3, and 1.0% bovine albumin containing 0 to 25 μg/mL $[{}^{125}I]ATAP2$, followed by sedimentation of cells through 40% sucrose to separate cell-bound from free IgG. Cell-associated $[{}^{125}I]^{-}$-
Table 1. Quantitation of Cell-Surface TR Expression

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>[125I]ATAP2 Bound (fmol/10^6 cells)</th>
<th>Surface TR (receptors/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>0 ± 43</td>
<td>0 ± 26,000</td>
</tr>
<tr>
<td>MEG-01</td>
<td>186 ± 21</td>
<td>112,000 ± 12,000</td>
</tr>
<tr>
<td>Jurkat</td>
<td>115 ± 38</td>
<td>69,000 ± 23,000</td>
</tr>
<tr>
<td>JRT-3.5</td>
<td>113 ± 24</td>
<td>68,000 ± 14,000</td>
</tr>
<tr>
<td>J.CaM1/rep3</td>
<td>171 ± 27</td>
<td>103,000 ± 16,000</td>
</tr>
<tr>
<td>J.CaM1/lck</td>
<td>136 ± 34</td>
<td>82,000 ± 20,000</td>
</tr>
<tr>
<td>J45.01</td>
<td>158 ± 29</td>
<td>95,000 ± 18,000</td>
</tr>
<tr>
<td>J45/CH11</td>
<td>148 ± 39</td>
<td>89,000 ± 23,000</td>
</tr>
</tbody>
</table>

Direct equilibrium binding of anti-TR MoAb ATAP2 was performed by incubating 10^6 cells with 125I-labeled ATAP2 for 60 minutes at 4°C. Values represent mean ± SE of three to five separate determinations.

ATAP2 was measured in a γ counter. Nonspecific binding was determined by adding a 100-fold excess of unlabeled ATAP2 IgG to the incubation mixture and also by performing binding studies with HL60 cells, which do not express TR. Specific binding of [125I]ATAP2 to MEG-01 cells was concentration-dependent and saturable, with a kd of 0.37 ± 0.23 µg/mL and R_max of 130,000 ± 30,000 binding sites per cell (mean ± SD) determined by Scatchard analysis. This number of thrombin receptors per cell is comparable to that reported for other hematopoietic cell lines. For quantitative comparison of TR surface expression on mutant T-cell lines, specific binding was measured with each cell line using a saturating concentration (1.5 µg/mL) of [125I]ATAP2.

Measurement of intracellular [Ca^2+]. Cells were loaded with the pentaacetoxymethyl ester of Indo-1 (Molecular Probes, Eugene, OR) as described, washed, and resuspended at 6 × 10^6 cells/mL in 25 mmol/L HEPES, pH 7.4, 1.0 mmol/L Na_2HPO_4, 125 mmol/L NaCl, 5.0 mmol/L KCl, 0.5 mmol/L MgCl_2, 1.0 mmol/L CaCl_2, 0.1% glucose, and 1.0 mg/mL bovine serum albumin. Continuous Indo-1 fluorescence was measured using a spectrofluorimeter (Spex Industries, Edison, NJ), with excitation at 334 nm and detection at 400 nm. The fluorimeter was calibrated for each determination by complete lysis with 0.1% Triton-X 100, followed by chelation of Ca^2+ with 16 mmol/L EGTA. Increases in intracellular [Ca^2+] were calculated using the formula [Ca^2+] = K_app(R-R_min)/(R_max-R), where K_app for Ca^2+ binding to Indo-1 = 0.25 µmol/L.

RESULTS

Cell-surface thrombin receptor expression. Cell-surface TR expression was detected by flow cytometry and quantitated by equilibrium binding using the MoAb ATAP2, which recognizes both cleaved and uncleaved forms of human TR. In agreement with previous studies, TR was not detected on the surface of the human promyelocytic cell line HL60, but strong TR surface expression was observed on the human megakaryocytic cell line MEG-01 (Fig 1 and Table 1). TR also was detected on the surface of Jurkat cells and on the Jurkat variants J.RT-T3.5, JCaM1/lck, JCaM1/rep3, J45/CH11, and J45.01. Jurkat cells expressed approximately 40% less surface TR than MEG-01 cells, and all of the Jurkat cell lines expressed similar levels of TR.

Thrombin receptor signal transduction. TR signal transduction was measured in Jurkat and MEG-01 cells using the calcium sensitive fluor Indo-1. In Jurkat cells, the addition of thrombin or the human TR peptide SFLLRN produced dose-dependent, transient increases in intracellular [Ca^2+], with higher doses of thrombin or SFLLRN producing responses of higher peak amplitude and shorter duration (Fig 2). Chelation of extracellular Ca^2+ with EGTA did not affect the peak amplitude of the Ca^2+ transient, but shortened its duration in Jurkat cells stimulated with 5 nmol/L thrombin or 40 µmol/L SFLLRN (Fig 2A and C). Chelation of extracellular Ca^2+ had little effect on the duration or amplitude of the calcium transient in Jurkat cells stimulated with 50 nmol/L thrombin or 40 µmol/L SFLLRN (Fig 2B and D). TR agonists also stimulated Ca^2+ transients in MEG-01 cells (Fig 3). Compared with Jurkat cells, MEG-01 cells were...
more sensitive to low doses of thrombin and SFLLRN, and the Ca\(^{2+}\) transients were more sustained. Moreover, chelation of extracellular Ca\(^{2+}\) dramatically shortened the duration the Ca\(^{2+}\) transient in MEG-01 cells stimulated with either low or high concentrations of thrombin or SFLLRN. These findings indicate that Jurkat cells express functional TR and suggest that TR coupling to calcium influx differs between T cells and megakaryocytic cells.

**Thrombin receptor signaling in Jurkat mutants.** To determine whether signal transduction through TR is altered in T cells lacking a functional TCR signaling pathway, we examined Ca\(^{2+}\) mobilization in response to TR agonists in the Jurkat variant J.RT-T3.5, which does not express TR, and in two Jurkat variants with defined defects in protein tyrosine phosphorylation, JCaM1/rep3 and J45.01. J.CaM1/rep3 is deficient in the *src* family protein tyrosine kinase *lck*, and J45.01 is deficient in the protein tyrosine phosphatase CD45. Both *lck* and CD45 are essential for normal signal transduction through TCR. Compared with Jurkat, JRT3.5 exhibited greater increases in peak intracellular [Ca\(^{2+}\)] in response to increasing concentrations of thrombin or SFLLRN (Fig 4A). Enhanced sensitivity to thrombin or SFLLRN also was observed in the *lck*-deficient cell line JCaM1/rep3 (Fig 4B) and the CD45-deficient cell line J45.01 (Fig 4C). Enhanced sensitivity to TR agonists was lost in cell lines JCaM1/lck and J45/CH11, in which TCR function had been reconstituted by stable transfection with *lck* or the phosphatase domain of CD45, respectively (Fig 4B and C). Thus, Jurkat TR signal transduction was sensitized in the absence of TCR or in the presence of a dysfunctional TCR signaling pathway.

To determine whether the correlation between TCR dysfunction and TR sensitization was unique to the Jurkat cell line, we compared responses to TR agonists in CD45\(^{+}\) and CD45\(^{-}\) subclones of the human immature thymocytic cell line HPB.ALL. HPB.45.1 is a CD45\(^{+}\) subclone with a normal TCR signaling pathway, and HPB.45.0 is a CD45-deficient subclone with defective TCR signaling function. Like CD45-deficient Jurkat cells, CD45-deficient HPB.45.0 cells were hyperresponsive to thrombin (Fig 5). HPB.45.0 cells also showed enhanced responses to the TR agonist peptides (Fig 5). These findings suggest that TCR-mediated signal transduction, rather than merely TCR internalization, is necessary for desensitization of TR.

**PAR-2 signaling in MEG-01 and Jurkat cells.** In most of the Jurkat cell lines, SFLLRN consistently produced larger peak calcium transients than thrombin. This suggested that Jurkat cells may contain a second SFLLRN-responsive receptor such as PAR-2. To distinguish between responses mediated by TR and those mediated by PAR-2, Ca\(^{2+}\) mobilization assays were performed with the *Xenopus* TR peptide TFRIFD, which activates human TR but not PAR-2, or the
To directly test the possibility that the discrepancy between maximal calcium responses to SFLLRN and thrombin was caused by stimulation of PAR-2 by SFLLRN, Jurkat cells were stimulated simultaneously with 50 nmol/L thrombin and 120 μmol/L SLIGKV (Fig 8). There was no significant difference in the amplitude of the peak Ca\(^{2+}\) transient between cells stimulated with thrombin alone and those stimulated with both thrombin and SLIGKV. In each case, the peak response was markedly less than that seen after stimulation with 40 μmol/L SFLLRN. This raises the possibility that Jurkat cells may contain additional SFLLRN-responsive receptors other than TR and PAR-2.48

**DISCUSSION**

In addition to its role as a central enzyme in blood coagulation, thrombin functions as a potent agonist for a variety of target cells. Current models of thrombin function propose that thrombin generated in response to vascular injury or increased vascular permeability mediates the coordinated activation of hemostatic and proliferative processes during tissue repair. Recent demonstrations that T lymphocytes express TR15-19 suggest that thrombin may modulate T-cell activation at sites of hemostatic stress. In agreement with these earlier studies, we detected functional TR on the surface of the Jurkat human T-cell leukemia line. Compared with MEG-01 human megakaryocytic cells, Jurkat cells expressed approximately 40% less surface TR and were less sensitive to stimulation with thrombin. Moreover, we observed different patterns of TR coupling to Ca\(^{2+}\)
Fig 6. Effect of TCR stimulation on TR Ca\(^{2+}\) responses and cell-surface expression. Jurkat cells (A) or J45.01 cells (B) were incubated for 16 hours with either control medium (●) or the IgM anti-CD3 MoAb 235 (□), and Ca\(^{2+}\) responses were measured in response to 50 nmol/L thrombin or 40 μmol/L SFLLRN. Values represent the mean ± SD of triplicate determinations. To measure effects of TCR stimulation on cell-surface expression, flow cytometry was performed using the negative control MoAb (dotted lines), the antihuman TR MoAb ATAP2 (solid lines), or the IgG anti-CD3 MoAb OKT3 (dashed lines). (C) Unstimulated Jurkat cells; (D) unstimulated J45.01 cells; (E) Jurkat cells stimulated with anti-CD3; and (F) J45.01 cells stimulated with anti-CD3.

Fig 7. Ca\(^{2+}\) responses to specific PAR-2 and TR agonists. Indo-1-loaded cells were stimulated with either the PAR-2–specific agonist SLIGKV (120 μmol/L; solid line) or the TR-specific agonist TFRIFD (120 μmol/L; dotted line), and intracellular [Ca\(^{2+}\)]\(\_i\) was measured using a spectrofluorimeter. (A) MEG-01; (B) Jurkat; (C) J.RT-T3.5 (TCR-negative); and (D) J.CaM1/rep3 (lck-deficient).
influx in Jurkat and MEG-01 cells. Stimulation of Jurkat cells with low concentrations of TR agonists (5 nmol/L thrombin or 4 μmol/L SFLLRN) produced measurable Ca\(^{2+}\) influx from the extracellular medium as well as Ca\(^{2+}\) mobilization from intracellular stores. Stimulation with 10-fold higher concentrations of thrombin or SFLLRN increased peak Ca\(^{2+}\) mobilization, but decreased Ca\(^{2+}\) influx. In comparison, MEG-01 cells exhibited sustained Ca\(^{2+}\) influx in response to stimulation with either low or high concentrations of TR agonists. Unlike previous studies in osteoblasts,\(^{14}\) we did not observe agonist-dependent differences in TR-stimulated Ca\(^{2+}\) influx in Jurkat cells, and Ca\(^{2+}\) influx was not inhibited by pretreatment with the serine/threonine phosphatase inhibitor calyculin A (not shown). This suggests that TR coupling to Ca\(^{2+}\) influx in Jurkat cells may be regulated by mechanisms other than receptor phosphorylation.

Our results with mutant T-cell lines provide evidence for functional cross-talk between the TR and TCR signaling pathways. TR Ca\(^{2+}\) signaling was enhanced in a TCR-negative cell line and in T-cell lines deficient in the protein tyrosine kinase lck or the protein tyrosine phosphatase CD45. Thus, unlike signal transduction through TCR, signal transduction through TR does not require lck or CD45. Conversely, TR signal transduction appears to be sensitized in cells lacking an intact TCR signaling pathway. The basal activation state of the TCR signaling pathway is thought to represent a dynamic equilibrium between positive and negative regulatory signals.\(^{47}\) Our observation that TR responsiveness is enhanced in cell lines lacking an intact TCR pathway implies that TR signaling is tonically inhibited by basal activation through the TCR pathway. This suggests that desensitized (tolerized) T cells may be more responsive to thrombin than resting T cells.

Maximal calcium mobilization in response to TR agonist peptides was somewhat lower in the lck-deficient cell line (JCaM1/rep3) than in TCR-deficient or CD45-deficient cell lines (Fig 4). Unlike the TCR- and CD45-deficient cell lines, the JCaM1 mutant retains a small amount of residual responsiveness to TCR stimulation.\(^{37}\) Therefore, the slightly lower peak [Ca\(^{2+}\)]\(_i\), response to TR agonists in this cell line may reflect a higher basal flux through the TCR signaling pathway. An alternative explanation for differential TR-mediated calcium responses in CD45-deficient and lck-deficient cell lines is that TR signaling may be regulated by src family kinases other than lck, which in turn may be subject to regulation by the CD45 phosphatase.

TR agonists have been reported to potentiate distal T-cell activation responses induced by TCR ligation,\(^{15,19}\) which suggests that effects of thrombin on T-cell activation may vary depending on the sequence in which T cells encounter TR and TCR agonists. Stimulation with thrombin before antigen presentation may enhance T-cell proliferation and interleukin-2 production, whereas stimulation with antigen before thrombin exposure may desensitize to TR stimulation, contributing to downregulation of the proliferative response.

TCR-generated signals may modulate signal transduction through TR by multiple mechanisms. In agreement with previous studies,\(^{19}\) we observed diminished TR signal transduction and decreased TR surface expression in Jurkat cells that had been activated by TCR cross-linking. This observation raised the possibility that enhanced sensitivity to TR agonists in cells with a defective TCR pathway may result from increased surface TR expression. However, we detected no significant differences in TR surface expression in cell lines with intact (Jurkat, JCaM1/lck, and J45/CH11) or dysfunctional (JRT3.5, JCaM1, and J45.01) TCR signaling pathways (Fig 1 and Table 1). This implies that the TCR pathway desensitizes TR signal transduction through mechanisms distinct from modulating TR surface expression. We also observed that TR signaling remained intact in CD45-deficient Jurkat cells after internalization of TCR with a cross-linking anti-CD3 MoAb. This observation provides evidence that signaling through TCR, rather than TCR internalization, is required for desensitization of TR.

The recent discovery that SFLLRN activates both TR and PAR-2\(^{26,27}\) raised the possibility that differential responsiveness to thrombin and SFLLRN in Jurkat cells could be caused by stimulation of PAR-2 by SFLLRN. This possibility was tested using the PAR-2-specific agonist peptide SLIGKV.\(^{29}\) We found that SLIGKV produced small Ca\(^{2+}\) transients in MEG-01 cells, and also in several Jurkat clones, suggesting that these cells express both PAR-2 and TR. Responses to SLIGKV were likely mediated by PAR-2, rather than by cross-reactivity with TR, for the following reasons. (1) Using a Xenopus oocyte system, Blackhart et al\(^{27}\) found that SLIGKV, even at millimolar concentrations, did not activate human TR. (2) Compared with Ca\(^{2+}\) transients produced by TFRIFD, which develop immediately upon stimulation, Ca\(^{2+}\) transients produced by SLIGKV are delayed in onset and more gradual in slope (Fig 8). (3) Human PAR-2
mRNA has been detected by Northern hybridization in spleen and blood leukocytes and in Jurkat cells. Like TR, PAR-2 appears to be modulated by the TCR pathway, because responses to SLIGKV were enhanced in both TCR-negative and lck-deficient Jurkat clones.

The low levels of functional PAR-2 relative to TR in Jurkat and MEG-01 cells contrast with those reported in human keratinocytes, which respond better to PAR-2-specific agonists than to TR-specific agonists. These findings argue that PAR-2 activation is an unlikely explanation for the greater sensitivity of Jurkat cells to SFLLRN compared with thrombin. Enhanced responsiveness to SFLLRN in Jurkat cells also is unlikely to be caused by expression of PAR-3, a recently reported third member of the protease-activated receptor family, since PAR-3 does not respond to SFLLRN. However, we cannot exclude the possibility that Jurkat cells express other SFLLRN-sensitive receptors in addition to TR and PAR-2.

In summary, this study shows that signal transduction through proteolytically activated receptors is influenced by the activation state of T cells. These observations support the model that thrombin provides a functional link between hemostasis and immune activation, two highly regulated processes that have coevolved in response to environmental stress.

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