Proteinase-Activated Receptor-2: Key Role of Amino-Terminal Dipeptide Residues of the Tethered Ligand for Receptor Activation

Bahjat Al-Ani, Kristina K. Hansen, and Morley D. Hollenberg

ABSTRACT

Tryptic cleavage of proteinase-activated receptor-2 (PAR2) causes the unmasking of a tethered receptor-activating sequence, S37LIGRLDTP. . . . We sought to determine, in the amino-terminal sequence of the PAR2 tethered ligand, the key amino acid residues that are responsible for receptor activation. Using site-directed mutagenesis, nine PAR2 mutants with alanine substitutions in the first six amino acids of the tethered ligand, S37LIGRL42, . . . , were prepared: PAR2S37A, PAR2L38A, PAR2I39A, PAR2G40A, PAR2R41A, PAR2A37–38, PAR2A39–42, PAR2A37–39, and PAR2A37–42, along with the reverse-sequence construct, PAR2L37S38. These mutants, together with wild-type PAR2 (PAR2wt), were expressed in Kirsten virus-transformed rat kidney cells and were then assessed for receptor-mediated calcium signaling upon activation by trypsin and by receptor-activating peptides like SLIGRL-NH2. In addition, the release of the N-terminal receptor sequence that is cleaved from PAR2 by trypsin activation was monitored in the above cell lines using a site-targeted anti-receptor antibody. All PAR2 constructs were activated by SL-NH2, and all mutated tethered ligand sequences were unmasked by trypsin. However, differential activation of the receptor by trypsin in these mutants was observed: PAR2 mutants PAR2A37–38 and PAR2L37S38, in which the first two amino-terminal tethered ligand residues (S37L38) are either changed to alanines or reversed, yielded little or no response to trypsin, nor did PAR2A37–42. However, trypsin activated all other constructs. We conclude that the amino-terminal tethered ligand dipeptide sequence S37L38 plays a major role in the activation of PAR2.

Activation of rat proteinase-activated receptor-2 (PAR2) by trypsin, like PAR1 activation by thrombin (Vu et al., 1991), involves the proteolytic unmasking of an amino-terminal receptor sequence (S37LIGRLDTP. . . ) that acts as a receptor-activating tethered ligand (Vu et al., 1991; Nystedt et al., 1994). As with PAR1, PAR2 can be activated by short peptides (e.g., S1LIGRL-NH2) based on the tethered ligand sequence. These receptor-activating peptides (PAR2APs) can mimic the activation of PAR2 by trypsin in tissues and PAR2-expressing cells (Nystedt et al., 1994; Al-Ani et al., 1995; Böh m et al., 1996; Hollenberg et al., 1997; Dé ry et al., 1998; Hollenberg and Compton, 2002). Although the structure-activity relationships (SARs) for PAR2 activation by synthetic peptides based on the S1LIGRL-NH2 motif have been studied in some depth (Hollenberg et al., 1996, 1997; Kawabata et al., 1999; Maryanoff et al., 2001), there has yet to be a systematic SAR study to determine the key residues in the PAR2 revealed tethered ligand sequence that cause receptor activation. For the peptides, leucine at position 2 is essential for receptor activation, and the isoleucine at position 3 and the arginine at position 5 both contribute to peptide potency. Importantly, simply reversing the first two amino acids (L1SIGRL6-NH2) leads to a complete loss of activity, as does acylation of the amino terminus (N-acetyl-S1LIGRL-NH2). In contrast, the relative importance of these same residues in the proteolytically revealed tethered ligand has not yet been established. Furthermore, data obtained by us for PAR2 (Al-Ani et al., 1999a, 2002b) and by others for PAR1 (Blackhart et al., 2000) suggest that the soluble peptide agonists and the correspond-
ing tethered ligand sequences seem to interact differently with the body of the receptor. Based on the apparent discrepancies between our previous SAR work with the synthetic PAR2APs and our preliminary findings with the receptor mutants with changes both in extracellular loop-2 and in the tethered ligand of PAR2 (Al-Ani et al., 2002b), we decided to explore further the SAR profile for the tethered ligand sequence itself.

We hypothesized that 1) the amino-terminal amino acids (S37L38) would be crucial for receptor activation and that 2) Ile39 and Arg41 would also contribute to receptor activation by the tethered ligand. To test these hypotheses, all of the first six amino acids at the amino terminus of the tethered ligand were replaced with alanines (Table 1). The wild-type receptor and the alanine-replacement receptor mutants were expressed in Kirsten-virus-transformed rat kidney (KNRK) cells (Al-Ani et al., 1999a), along with PAR2L37S38, having a reversal of the first two tethered ligand residues (S37L38 → L37S38). A calcium signaling assay (Al-Ani et al., 1999a; Kawabata et al., 1999) was used to assess activation of the wild-type and mutated receptors both by trypsin and by the PAR2AP S1LIGRL6-NH2. In addition, the calcium signaling assay was used to monitor, in wild-type PAR2 (PAR2wt) and in the mutant receptors, the activity of synthetic peptides having the same sequences as those of the mutated tethered-ligand sequences (e.g., A37LIGRL42...).

Added to the functional evaluation of the expressed receptor mutants, the efficiency of receptor cleavage by trypsin to unmask the tethered ligand was monitored for all PAR2 variants using an antibody (SLAW-A) that recognizes the sequence released at the R36/S37 cleavage/activation site. A maximum specific activity of 20,000 units/mg was used to calculate the approximate molar concentration of trypsin in the incubation medium (1 unit/ml = 2 nM).

**Preparation of PAR2 Constructs and their Expression in KNRK cells.** As previously documented (Saifeddine et al., 1996; Al-Ani et al., 1999a,b), rat PAR2 was cloned from kidney cDNA using the primer pairs: forward primer (containing a HindIII site and Kozak sequence shown in bold), 5'-TCAAGCTTCCACCATGCGAGTCTCACGGC-3'; reverse primer (containing Smal site shown in bold), 5'-CCGGGCTCACGAGGTATTAAC-3'. Then, the rat PAR2 cDNA, for which sequence verification was done (Sanger et al., 1977; DNA services facility at the University of Calgary) was subcloned further into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA), which was used to prepare all 10 receptor mutants shown in Table 1. The receptor mutants described in Table 1 were prepared using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. In PAR2A37, PAR2L37S38, PAR2G40A, PAR2I39A and PAR2R42A, residues S37, I39, G40, and R42 were changed to A, respectively; in PAR2A37S38, residues A37, S38, G40A, R42, and PAR2G40A, residues S37, I39, G40A, R42, and A37S38 were reversed to become I39S38. The wild-type and PAR2 mutants in pcDNA3 were then transfected into KNRK cells (American Type Culture Collection, Manassas, VA), as described previously (Al-Ani et al., 1999,a,b) to yield permanent cell lines for further study. Transfected cells (either vector alone or PAR2-containing vectors) were subcloned in 418–containing medium (0.6 mg/ml), and PAR2-expressing cells were isolated by fluorescence-activated cell sorting (FACS) with the use of the anti-receptor SLAW-A antibody as described elsewhere for a B5 anti-PAR2 antibody used by us previously (Kong et al., 1997; Al-Ani et al., 1999a,b). The SLAW-A antiserum recognizes the PAR2 receptor sequence that is released upon proteolytic activation of the receptor (SLAWLIG14G8PSNKR28GGYGC) (receptor antigenic sequences represented in bold; GGYGC added for radiolabeling and cysteine-coupling). In the cell lines so isolated, >80% of the population (flow cytometry) were found to exhibit reactivity with the SLAW-A antibody with an equivalent fluorescence intensity on a per-cell basis, in keeping with our previous work (Al-Ani et al., 1999a, 2002b). Cells were routinely propagated as described previously (Al-Ani et al., 1999a) in G-418 (0.6 mg/ml)-containing growth medium and were subcultured by re-suspension in calcium-free isotonic saline/EDTA solution, without the use of trypsin.

**Evaluating the Cleavage of PAR2 Variants by Trypsin.** PAR2 variant cell lines were grown to about 85% confluence. These clones possess an N-terminal sequence that is proximal to the receptor’s cleavage/activation sequence and therefore potentially released from the cell upon cleavage of PAR2 by trypsin at site Arg42. The rabbit solutions, prepared in 25 mM HEPES buffer, pH 7.4, were standardized by quantitative amino acid analysis to verify peptide concentration. Porcine trypsin (14,900 units/mg) was obtained from Sigma (St. Louis, MO). A maximum specific activity of 20,000 units/mg was used to calculate the approximate molar concentration of trypsin in the incubation medium (1 unit/ml = 2 nM).

**Peptides and Other Reagents.** All peptides were synthesized as carboxamides by solid-phase methods at the peptide synthesis facility [Dr. Denis McMaster, University of Calgary, Faculty of Medicine (Calgary, AB, Canada)]. High-performance liquid chromatography analysis, mass spectral analysis, and quantitative amino acid analysis confirmed the composition and purity of all peptides. Stock TABLE 1 Wild-type and mutated PAR2 tethered ligand sequences and cognate synthetic peptides

The sequences beginning at the cleavage/activation sites show the alanine and other replacements (bold type) in the tethered ligands unmasked by trypsin. The corresponding synthetic peptides are shown on the right. The symbols used in the figures for the PAR2 variants are also shown.

<table>
<thead>
<tr>
<th>PAR2 Variant</th>
<th>Symbol</th>
<th>Trypsin Cleavage/Activation Site</th>
<th>PAR2-Derived Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR2wt</td>
<td>●</td>
<td>.KGR37/SLIGRL42LDTF.</td>
<td>SLIGRL-NH2</td>
</tr>
<tr>
<td>PAR2S37A</td>
<td>▲</td>
<td>.KGR/ALIGRLDTF.</td>
<td>ALIGRL-NH2</td>
</tr>
<tr>
<td>PAR2L37S38</td>
<td>▲</td>
<td>.KGR/SILIGRLDTF.</td>
<td>SAIGRL-NH2</td>
</tr>
<tr>
<td>PAR2I39A</td>
<td>▲</td>
<td>.KGR/SILIGRLDTF.</td>
<td>SLIGRL-NH2</td>
</tr>
<tr>
<td>PAR2G40A</td>
<td>▲</td>
<td>.KGR/SILIGRLDTF.</td>
<td>SLIGAL-NH2</td>
</tr>
<tr>
<td>PAR2R42A</td>
<td>▲</td>
<td>.KGR/SLAIGRLDTF.</td>
<td>SLAGRL-NH2</td>
</tr>
<tr>
<td>PAR2A37–38</td>
<td>▲</td>
<td>.KGR/SLAIGRLDTF.</td>
<td>SLAGAL-NH2</td>
</tr>
<tr>
<td>PAR2A39–42</td>
<td>▲</td>
<td>.KGR/SLAAAADTF.</td>
<td>SLAGAL-NH2</td>
</tr>
<tr>
<td>PAR2A37–39</td>
<td>▲</td>
<td>.KGR/SLAAAADTF.</td>
<td>SLAGAL-NH2</td>
</tr>
<tr>
<td>PAR2A37–42</td>
<td>▲</td>
<td>.KGR/SLAAAADTF.</td>
<td>SLAGAL-NH2</td>
</tr>
<tr>
<td>PAR2L37S35</td>
<td>●</td>
<td>.KGR/SLIGRLDTF.</td>
<td>LSIGRL-NH2</td>
</tr>
</tbody>
</table>

Downloaded from molpharm.aspetjournals.org on June 17, 2017
polyclonal antiserum (SLAW-A) mentioned above was employed to monitor the disappearance of the signal (generated by the receptor sequence and including residue Arg36) upon trypsin treatment of all expressed mutants, thereby confirming trypsin cleavage (Compton et al., 2001; Al-Ani et al., 2002a). In brief, KNRK cells expressing the receptor constructs were exposed to 20 units/ml trypsin (40 nM) for 5 min at room temperature, and proteolysis was terminated by the addition of 1 μg/ml soya trypsin inhibitor. Cells were then harvested by a cytopin procedure in preparation for immunocytochemical detection of receptor with SLAW-A, comparing the receptor staining observed in cells both before and after trypsin treatment with reference to the disappearance of the SLAW-A immunoreactivity observed in control wild-type PAR2-expressing cell suspensions treated with 2 μl of a cell suspension of 200 or more fixed stained cells, were surveyed at random, and cells were scored as either SLAW-positive or SLAW-negative. The ratio of positive to negative cells in the untreated or trypsin-treated cells was then calculated. Upon trypsin treatment, a loss of over 80% of SLAW-A reactivity was routinely observed in all previous control experiments with wild-type PAR2-KNRK cells. The values obtained using the immunocytochemical approach agreed with data obtained using FACS analysis to document the removal of the SLAW-A epitope by trypsin (Compton et al., 2001; Al-Ani and Hollenberg, 2003).

**Calcium Signaling Assay.** Measurements of trypsin and peptide-stimulated fluorescence emission (reflecting an increase in intracellular calcium from a baseline of about 30 nM to a peak of about 340 nM) were done with cells grown to about 85% confluence and disaggregated with calcium-free isotonic phosphate-buffered saline containing 0.2 mM EDTA. PAR2-transfected KNRK cells were loaded with the intracellular calcium indicator Fluor-3 (Molecular Probes Inc., Eugene, OR) at a final concentration of 22 μM (25 μM/ml) of fluo-3 acetoxyethyl ester (Kao et al., 1988; Minta et al., 1989), as described previously (Al-Ani et al., 1999a; Kawabata et al., 1999). Fluorescence measurements, reflecting elevations of intracellular calcium, were conducted at 24°C using an Aminco-Bowman series 2 luminance spectrophotometer (Spectronic Unicam, Rochester, NY), with an excitation wavelength of 480 nm and an emission recorded at 530 nm. The fluorescence signals caused by the addition of test agonists (trypsin or peptides, added to 2 ml of a cell suspension of about 104 cells/ml) were expressed as described previously (Al-Ani et al., 1999a; Kawabata et al., 1999; Compton et al., 2000), relative to the fluorescence peak height yielded by replicate cell suspensions treated with 2 μM concentrations of the ionophore A23187 (Sigma Chemical). Measurements were done using three or more replicate cell suspensions derived from two or more independently grown groups of cells. To express quantitatively the sensitivity of the PAR2 tethered ligand mutants for trypsin activation, relative to the sensitivity of wild-type PAR2, a ratio was calculated (RREC,T) based on the concentration of trypsin required to cause a given calcium signal in the wild-type receptor (ECWT) to relative to the concentration of trypsin required to cause the equivalent calcium signal in the mutant PAR2 receptor with an altered tethered ligand sequence (ECMUTANT). Thus, for trypsin activation, RREC = ECWT/ECMUTANT. Values of this ratio <1 denote a receptor that requires a higher concentration of trypsin to cause the same cellular response as for PAR2wt and is therefore less sensitive than the wild-type receptor. Similarly, as we have done previously (Hollenberg et al., 1997), we expressed the sensitivity of the receptors to the synthetic peptide analogs also as a ratio (RREC) of the concentration of the wild-type peptide, SLIGRL-NH2, required for a given calcium signal (ECWT/RECMUTANT), relative to the concentration of test peptide (ECPEPTIDE) required to generate the equivalent calcium response. Data for the wild-type receptor, denoted in the text by open symbols for peptide concentration-effect curves, are shown in Fig. 4; the peptide sensitivities of the mutant receptors are denoted by closed symbols in Fig. 4. The EC values were obtained along the linear portions of the concentration-response curves, like those shown in Figs. 2 and 4. Four to six points along the concentration-response curves were used to calculate the averages for the RREC and RREC values. Measurements done in this manner yielded average values, for which the standard error of the mean was less than 10% of the magnitude of the average.

**Results**

**Expression and Responsiveness of PAR2 Variants.** All receptor variants (Table 1) were expressed in KNRK cells as permanent cell lines, maintained in the presence of G-418. FACS analysis and immunocytochemical detection of the receptor using the SLAW-A antibody revealed that all mutant cell lines expressed a receptor density equivalent to that of the wild-type cell line, KNRK-PAR2wt. Not only did all cell lines exhibit equivalent average cell surface fluorescence and immunoreactivity (FACS) but ≥80% of all cells in each line were found to express the receptor by immunocytochemical morphometric analysis (data not shown). More importantly, the responsiveness (calcium signaling) of all PAR2 variant cell lines to the PAR2AP SLIGRL-NH2 was equivalent, with comparable EC50 values (3 to 10 μM) and maximal calcium signals at 50 μM SLIGRL-NH2 that were 80% or greater than the signal generated by PAR2wt (Fig. 1 and data not shown for constructs designated by ⚫ and ⬤ in Table 1).

**Sensitivity of the PAR2 Variants to Trypsin.** Given that the different cell lines expressed an equivalent cell surface abundance of functional receptor determined by FACS analysis and responsiveness to SLIGRL-NH2, the next step was to measure their sensitivity to trypsin, reflecting the

---

**Fig. 1.** Concentration-effect curves for SLIGRL-NH2-stimulated calcium signaling in wild-type and mutant PAR2-expressing KNRK cell lines. Permanent KNRK cell lines expressing the wild-type and mutated PAR2 constructs with altered tethered ligand sequences (Table 1) were evaluated for their sensitivity to SLIGRL-NH2 calcium signaling as outlined under Materials and Methods. The fluorescence emission (Emax) in response to increasing concentrations of SLIGRL-NH2 was expressed as a percentage (% A23187) of the signal generated with 2 μM of the ionophore, A23187. Cell lines are represented by these symbols (Table 1): ⚫, PAR2wt; ⬤, PAR2S37A; ⬤, PAR2L38A; ⬤, PAR2A37; ⬤, PAR2A39. Values ± S.E.M. (bars) represent the averages of measurements made with three or more separately grown cell samples. For symbols without error bars, the magnitude of the bars fell within the size of the symbol. Values for those receptor mutants not shown for purposes of clarity (⚫, ⬤) all fell within the region of the response curve shown for PAR2wt (⚫). Because of overlapping values, the symbols shown for some constructs (⚫, ❄, ❄, ❄) are all present throughout the cluster of curves shown, but are clearly visible in only portions of the graph.
activity of the revealed tethered ligand (Fig. 2). Most striking was the complete lack of activity of trypsin in PAR₂L³⁷S⁵⁸, which was otherwise fully responsive to SLIGRL-NH₂ (Fig. 2 and Table 2, ■), and the essential lack of trypsin sensitivity of the construct with A³³A³⁸ substitutions (PAR₂A³⁷–³⁸; ▲; Fig. 2). In contrast, changing only the first amino acid to alanine (PAR₂S³⁷A, ▼; Fig. 2) resulted in a receptor with sensitivity toward trypsin that was the same as that of the wild-type receptor (PAR₂wt, ◆; Fig. 2). In contrast, changing Leu⁴⁰ to alanine at the second position of the tethered ligand (PAR₂L³⁸A; ▲, Fig. 2) caused a ~10-fold reduction in the relative activity of the construct with ~75% of that observed for PAR₂wt. In addition to the crucial importance of the first two amino acids for tethered ligand activity, further alanine substitutions revealed the importance of residues Ile³⁹ and Arg⁴¹ (PAR₂A³⁹A and PAR₂R³⁴A, ▼ and ◆; Fig. 2).

Overall, the relative potencies of trypsin, reflecting the relative activities of the sequences as tethered ligands unmasked by proteolysis, were (Fig. 2 and Table 2): SLIGRL . . . ≈ ALIGRL . . . ≈ SLIARL . . . > SLAGRL . . . > SLIGAL . . . > SLIAAA . . . > SAIGRL . . . > AAAAAA . . .; all of the AAIGRL . . ., ALAAAA . . ., and LSIGRL . . . tethered ligand sequences exhibited little or no activity (Fig. 2). Quantitatively, the sensitivities of all receptor variants toward trypsin, relative to PAR₂wt (Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑἐlectron瀛 ordinance of measure -

Unmasking of Receptor Variants by Trypsin. Although all receptor variants expressed comparable amounts of functional cell surface receptors (responses to SLIGRL-NH₂ and FACS), it was essential for interpreting the trypsin sensitivity data (Fig. 2) to know that all variants were similarly cleaved by trypsin at Arg⁴⁶ to reveal the tethered ligand sequence. To assess the unmasking of the tethered ligand sequence in all variants, cells were first exposed to trypsin for 5 min at a trypsin concentration (40 nM; 20 units/ml) sufficient to expose the tethered ligand and generate a maximum calcium signal in PAR₂wt. Cleavage was monitored as outlined under Materials and Methods, with the SLAW-A antibody that detects only the receptor sequence removed by trypsin. As shown in Fig. 3, trypsin was able efficiently to remove the epitope detected by SLAW-A from the wild-type receptor that is fully activated by trypsin, as well as from receptor variants that showed either reduced sensitivity (PAR₂A³⁹–⁴³) or no activity (PAR₂L³⁷S⁵⁸) upon trypsin activation. A similar removal of the epitope visualized by the SLAW-A antibody was also observed for all mutant PAR₂ cell lines (not shown). Morphometric analysis of the fixed stained cells revealed that, as for PAR₂wt, brief trypsin treatment eliminated SLAW-A reactivity from 80% or more of all of the mutant receptor-bearing cells. Comparable results were obtained using FACS analysis of the trypsin-treated cells (not shown). Thus, trypsin treatment caused an equivalent cleavage and exposure of the tethered ligand in all receptor mutants.

Activity of Tethered Ligand Sequences as Soluble Peptides. Although in previous work, we and others had obtained structure-activity data for alanine substitutions in the receptor-selective PAR₂ activating peptide sequence, S₅₆LIGRL₄₋₉-NH₂ (Hollenberg et al., 1996, 1997; Maryanoff et al., 2001), it was necessary in the present study to evaluate again the activity of the synthetic peptides corresponding to the mutated tethered ligand sequences not only in PAR₂wt but also in the receptor mutants possessing the corresponding tethered ligand sequence with the ‘alanine walk’ mutations. Thus, as outlined in Table 1 and Fig. 4, nine synthetic peptides, SLIGRL-NH₂ (wild-type sequence), ALIGRL-NH₂, SAIGRL-NH₂, SLAGRL-NH₂, SLIARL-NH₂, SLIGAL-NH₂, AAIGRL-NH₂, SLAAAA-NH₂ and LSIGRL-NH₂ were tested for activity (calcium signal) in both PAR₂wt and in most of the receptor mutants having the cognate tethered ligand sequence. Because neither AAIGRL-NH₂ nor SLAAAA-NH₂ was found to be active in the calcium signaling assay (below), the peptides ALAAAA-NH₂ and AAAAANH₂ were presumed to be inactive and were not tested in the interests of economy. As shown in Fig. 4, at concentrations in the range of 200 to 400 μM, the peptides SAIGRL-NH₂ (▲, △), AAIGRL-NH₂ (◆, ◆), SLAAAA-NH₂ (▲, ▼), and LSIGRL-NH₂ (◆, ◆) were completely inactive both in PAR₂wt and in the receptor mutants possessing the same sequence as the mutated tethered ligand. In contrast, the other peptide analogs displayed relative potencies that clustered in three groups, with EC₅₀ values in the ranges of 3, 35, and 120 μM, as summarized in the next paragraph.

The relative order of potencies of the peptides for activating the receptor (Fig. 4 and Table 2) was: SLIGRL-NH₂ > SLIARL-NH₂ > ALIGRL-NH₂ > SLAGRL-NH₂ > SLIGAL-NH₂; in contrast with the inactive peptides, SAIGRL-NH₂, AAIGRL-NH₂, SLAAAA-NH₂, and LSIGRL-NH₂. The relative potencies of these different sequences for activating PAR₂ in relation to the activity of SLIGRL-NH₂ were expressed quantitatively, as we have done previously (Hollenberg et al., 1997), as a ratio (Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑἐlectron瀛 ordinance of measure -

![Fig. 2. Trypsin sensitivity of wild-type and mutated PAR₂KNRK cell lines.](image-url)
of SLIGRL-NH₂ required to cause a given calcium signal 
EC₅₀ SLIGRL-NH₂, relative to the concentration of the peptide 
alog EC₅₀ PEPTIDE required to generate the equivalent cal-
cium response in either the wild-type receptor (PAR₂wt) or 
in the corresponding receptor mutant (i.e., Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler} of Table 2).

**Discussion**

Our study is the first to examine systematically the structure-activity relationships for the activation of PAR₂ by its tethered ligand sequence. The main finding of our study was that the first two amino acids of the tethered ligand of rat PAR₂, in tandem, play a key role in receptor activation. This conclusion was supported by the data indicating that the revealed tethered ligand SLAAAA . . . was able to generate a substantial calcium signal in response to trypsin, whereas the receptor mutants with revealed tethered ligands AAIIGRL . . ., ALAAAA . . ., and LSIGRL . . . generated little or no calcium signal in response to trypsin. Thus, the SL . . motif on its own as a tethered ligand was sufficient to generate a substantial receptor signal. This activity for the tethered ligand would not have been predicted because the soluble PAR₂AP SLAAAA-NH₂ did not activate PAR₂wt.

To interpret the sensitivity to trypsin (calcium signal) as reflecting the activity of the tethered ligand, it was important to establish two key criteria: 1) that the receptor mutants were expressing an equivalent abundance of functional cell surface receptor and 2) that the tethered ligand in all of the receptor variants was unmasked by trypsin with comparable efficiency. Our data indicate that equivalent densities of functional receptor were indeed expressed in all cell lines (FACS analysis and comparable sensitivities to the PAR₂-functional receptor were indeed expressed in all cell lines). Furthermore, the immunocytochemical analysis of trypsin-treated receptor-expressing nocytochemical analysis of trypsin-treated receptor-expressing (FACS analysis and comparable sensitivities to the PAR₂-functional receptor were indeed expressed in all cell lines).

The assumption that must be made to use the Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler} was considerably reduced (about 10-fold) compared with SLI-

**Table 2** Relative activities of PAR₂ tethered ligand sequences and their cognate synthetic peptides

The relative activity values for activation by trypsin (Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler) or for activation by the synthetic peptides (Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler) as carboxamides corresponding to the cognate tethered ligands were calculated from the data shown in Figs. 2 and 4, according to the formulae: Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler = ECₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler / ECₑₑₑₑₑₑₑₑₑₑₑₑ euler. Thus, the value for the wild-type receptor sequence, whereas the peptides S₁LAAAA-NH₂ and S₁AAIGRL-NH₂ were devoid of activity (Fig. 4). Furthermore, the tethered ligand sequence, A³⁷LIGRL . . ., was as active as the wild-type sequence, whereas the activity of the peptide, A₁LIGRL-NH₂, was considerably reduced (about 10-fold) compared with SLI-

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Symbols</th>
<th>Sequence</th>
<th>Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑ euler</th>
<th>Rₑₑₑₑ euler</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR₂wt</td>
<td>○</td>
<td>SLIGRL</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PAR₂S³⁷ A</td>
<td>●, □</td>
<td>AIGRL</td>
<td>0.92</td>
<td>0.095</td>
</tr>
<tr>
<td>PAR₂G³⁷ A</td>
<td>●, ●</td>
<td>SLARL</td>
<td>1.2</td>
<td>0.98</td>
</tr>
<tr>
<td>PAR₂F³⁹ A</td>
<td>▽</td>
<td>SLAAA</td>
<td>0.42</td>
<td>0.025</td>
</tr>
<tr>
<td>PAR₂R³⁳ A</td>
<td>●, △</td>
<td>SLIGRL</td>
<td>0.39</td>
<td>0.025</td>
</tr>
<tr>
<td>PAR₂L³⁸ A</td>
<td>△, ▽</td>
<td>S₁LAAAA</td>
<td>0.37</td>
<td>N.A.</td>
</tr>
<tr>
<td>PAR₂A³⁸ - ⁴²</td>
<td>△, ▽</td>
<td>S₁AAIGRL</td>
<td>0.14</td>
<td>N.A.</td>
</tr>
<tr>
<td>PAR₂A³⁷ - ⁴²</td>
<td>△, ▽</td>
<td>S₁LAAAA</td>
<td>0.032</td>
<td>N.D.</td>
</tr>
<tr>
<td>PAR₂A³⁶ - ⁴²</td>
<td>△, ▽</td>
<td>S₁AAIGRL</td>
<td>0.019</td>
<td>N.A.</td>
</tr>
<tr>
<td>PAR₂L³⁸ - ⁴²</td>
<td>△, ▽</td>
<td>S₁LAAAA</td>
<td>N.A.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PAR₂L³⁷ S³⁸</td>
<td>●, ▽</td>
<td>LSIGRL</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not active; N.D., not done.
GRL-NH₂ (Fig. 4 and Table 2). Thus, in keeping with previous results obtained by us and by others (Blackhart et al., 2000; Al-Ani et al., 2002b), our new data point out more emphatically that the SAR data obtained for activation of the proteinase-activated receptors only by the synthetic peptides cannot be used as a basis for understanding the tethered ligand mechanism.

In our previous study (Al-Ani et al., 2002b), we focused primarily on potential interactions between the fifth residue of the revealed tethered ligand (Arg⁴¹) and acidic residues in extracellular loop-2. Our data provided evidence against such an interaction but did not at all establish the key tethered ligand residues essential for receptor activation. Clearly, simply reversing the first two residues of the revealed tethered ligand (PAR₂L³⁷S³⁸) or replacing the first two residues with alanine (PAR₂A³⁷⁻³⁸), leaving the other tethered ligand residues unchanged, substantially reduced or completely abrogated the ability of the revealed tethered ligand to activate the receptor (Fig. 2, ◀ and ◁). However, with all other amino acids except the Ser⁷⁷ and Leu²⁸ residues changed to alanines (PAR₂A⁹⁹⁻⁴²), the revealed tethered ligand (S³⁷LAAAA... ) was still able to cause a substantial activation of the receptor (Fig. 2, ◁). The interaction of the S³⁷L²⁸ motif with the body of the receptor would therefore seem to be both sufficient and necessary to activate the receptor. Nonetheless, substituting alanine for serine at the N termi-

Fig. 3. Trypsin-catalyzed removal of the N-terminal PAR₂ sequence that masks the tethered ligand. KNRK cell lines expressing either wild-type PAR₂ (A and B) or receptors with mutated tethered ligand sequences (C–F) were either treated (B, D, F) or not (A, C, E) for 5 min at room temperature with 40 nM trypsin, followed by the addition of soya trypsin inhibitor to stop proteolysis. Cells were then harvested for immunocytochemical analysis using the SLAW-A antibody, as described under Materials and Methods. The arrows in A, C, and E show the ring of receptor staining visualized in untreated cells; the arrows in B, D, and F point to the disappearance of the N-terminal receptor epitope detected by the SLAW-A antibody upon exposure of the tethered ligand by trypsin cleavage. A and B, PAR₂wt; C and D, PAR₂L³⁷S³⁸; E and F, PAR₂A⁹⁹⁻⁴². Slides like the ones shown were used for morphometric analysis to quantify the exposure of the tethered ligand sequence by trypsin for all receptor mutants.
nus of the revealed tethered ligand (PAR$_2$S$^{37}$A) led to a sequence with an activation profile equivalent to that of the wild-type tethered ligand (compare $\bullet$ and ■ in Fig. 2). Other amino acids that can substitute for the S$^{37}$L$^{38}$ motif of the revealed tethered ligand to yield full receptor activation remain to be determined. In this regard, substitution of the Leu$^{37}$ with alanine (PAR$_2$L$^{37}$A, ▶; Fig. 2) led to a considerable loss of activity of the tethered ligand. Based on our data, one can suggest that hydrophobic residues at positions 2 and 3 of the tethered ligand may interact in a complementary pocket of the remainder of the receptor to trigger signaling.

In contrast with the discrepancies already mentioned, the SAR data for the tethered ligand sequences do parallel, to some degree, the SAR profile for the PAR$_2$-activating peptides (Hollenberg et al., 1996, 1997; Maryanoff et al., 2001). For instance, neither the peptide LSIGRL-NH$_2$ nor the tethered ligand sequence L$^{37}$SIGRL... was able to cause receptor activation. Furthermore, the previous SAR data for the synthetic peptides pointing to the importance of the isoleucine at the third position and the arginine at the fifth position (Hollenberg et al., 1996, 1997; Maryanoff et al., 2001), are mirrored by the reduced activity of S$^{37}$LAGRL... and S$^{37}$LIGAL... as tethered ligands. Where concordant, the SAR data for the tethered ligand mutants and the soluble PAR$_2$APs add support to our hypothesis that Ile$^{39}$ and Arg$^{41}$ play important roles in the tethered ligand activation process. This information bears directly on the future development of much needed PAR$_2$ antagonists.

It was unexpected that the tethered ligand sequence A$^{37}$AAAAA... was essentially inactive, given that sequences S$^{37}$AAAAA... and A$^{37}$LIGRL... both showed activity. Furthermore, it was surprising that the tethered sequence A$^{37}$AAAAA... was able to activate the receptor (Fig. 2, ◽), albeit with a substantially lower activity than that of the sequence, S$^{37}$AAAAA... (Fig. 2, ◦). It seems that although there are specific steric requirements for an efficient activation of the receptor (the S$^{37}$L$^{38}$ motif), there may also be ‘negative’ constraints built into the tethered ligand that can be removed by the homogeneous replacement of all six tethered ligand residues by alanine.

Taken together, our data highlight the primary importance of the first two tethered ligand amino acids, SL, as critical for receptor activation. This conclusion could not have been reached based on the SAR data obtained with the soluble PAR$_2$APs alone. Furthermore, our study indicates the contributions (albeit secondary) of the third and fifth (Ile$^{39}$ and Arg$^{41}$) residues for tethered ligand activity. Thus, for the design of potential PAR$_2$ antagonists, the pharmacophores of the SL motif would seem to be paramount, in concert with the Leu$^{37}$ and Arg$^{41}$ side chains. That said, the activity of PAR$^{37-42}$ with alanine replacements at all six tethered ligand residues should sound a cautionary note, suggesting that the proteolytic exposure of the tethered ligand may remove a prior structural constraint that enables the SL motif, to trigger signaling efficiently. It will be of considerable interest in future work to determine whether the first two amino acids of the PAR$_1$ tethered ligand are similarly critical for receptor activation.

Acknowledgments

We are grateful to Dr. Mahmoud Saiedfenne for his technical assistance with the conduct of some of the experiments described herein and to Laurie Robertson of our Faculty Flow Cytometry Core Facility for assistance with the FACS analyses.

References


**Address correspondence to:** Morley D. Hollenberg, Department of Pharmacology and Therapeutics, University of Calgary Faculty of Medicine, 3330 Hospital Dr. N.W., Calgary, AB Canada T2N 4N1. E-mail: mhollenb@ucalgary.ca