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

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ABSTRACT

Tryptic cleavage of proteinase-activated receptor-2 (PAR_2) causes the unmasking of a tethered receptor-activating sequence, S^{37}LIGRLDTP. . . . We sought to determine, in the amino-terminal sequence of the PAR_2 tethered ligand, the key amino acid residues that are responsible for receptor activation. Using site-directed mutagenesis, nine PAR_2 mutants with alanine substitutions in the first six amino acids of the tethered ligand, S^{37}LIGRL^{42}. . . , were prepared: $PAR_2S^{37}A$, $PAR_2L^{38}A$, $PAR_2I^{39}A$, $PAR_2G^{40}A$, $PAR_2R^{41}A$, $PAR_2A^{37–38}$, $PAR_2A^{39–42}$, $PAR_2A^{37,39–42}$, and $PAR_2A^{37–42}$, along with the reverse-sequence construct, $PAR_2L^{37}S^{38}$. These mutants, together with wild-type PAR_2 (PAR_2wt), 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-NH_2. In addition, the release of the N-terminal receptor sequence that is cleaved from PAR_2 by trypsin activation was monitored in the above cell lines using a site-targeted anti-receptor antibody. All PAR_2 constructs were activated by SL-NH_2, and all mutated tethered ligand sequences were unmasked by trypsin. However, differential activation of the receptor by trypsin in these mutants was observed: PAR_2 mutants $PAR_2A^{37–38}$ and $PAR_2L^{37}S^{38}$, in which the first two amino-terminal tethered ligand residues ($S^{37}L^{38}$) are either changed to alanines or reversed, yielded little or no response to trypsin, nor did $PAR_2A^{37,39–42}$. However, trypsin activated all other constructs. We conclude that the amino-terminal tethered ligand dipeptide sequence $S^{37}L^{38}$ plays a major role in the activation of PAR_2.

Activation of rat proteinase-activated receptor-2 (PAR_2) by trypsin, like PAR_1 activation by thrombin (Vu et al., 1991), involves the proteolytic unmasking of an amino-terminal receptor sequence (S^{37}LIGRLDTP. . .) that acts as a receptor-activating tethered ligand (Vu et al., 1991; Nystedt et al., 1994). As with PAR_1, PAR_2 can be activated by short peptides (e.g., S_1LIGRL$_6$-NH_2) based on the tethered ligand sequence. These receptor-activating peptides (PAR_2APs) can mimic the activation of PAR_2 by trypsin in tissues and PAR_2-expressing cells (Nystedt et al., 1994; Al-Ani et al., 1995; Böhm et al., 1996; Hollenberg et al., 1997; Déry et al., 1998; Hollenberg and Compton, 2002). Although the structure-activity relationships (SARs) for PAR_2 activation by synthetic peptides based on the S_1LIGRL$_6$-NH_2 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 PAR_2 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 (L_1SIGRL$_6$-NH_2) leads to a complete loss of activity, as does acylation of the amino terminus (*N*-acyl-S_1LIGRL$_6$-NH_2). 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 PAR_2 (Al-Ani et al., 1999a, 2002b) and by others for PAR_1 (Blackhart et al., 2000) suggest that the soluble peptide agonists and the correspond-

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ABBREVIATIONS: PAR, proteinase-activated receptor; PAR-APs, proteinase-activated receptor–activating peptides; SAR, structure-activity relationship; KNRK, Kirsten virus-transformed rat kidney cells; PAR_2wt, wild-type rat proteinase-activated receptor 2; SLAW-A, SLAWLLG-GPNSKGR; FACS, fluorescence-activated cell sorting; A23187, calcimycin.

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 PAR_2APs and our preliminary findings with the receptor mutants with changes both in extracellular loop-2 and in the tethered ligand of PAR_2 (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 ($S^{37}L^{38}$) would be crucial for receptor activation and that 2) Ile^{39} and Arg^{41} 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 $PAR_2L^{37}S^{38}$, having a reversal of the first two tethered ligand residues ($S^{37}L^{38} \rightarrow L^{37}S^{38}$). 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 PAR_2AP S_1LIGRL_6-NH_2. In addition, the calcium signaling assay was used to monitor, in wild-type PAR_2 (PAR_2wt) and in the mutant receptors, the activity of synthetic peptides having the same sequences as those of the mutated tethered-ligand sequences (e.g., A_1LIGRL_6-NH_2, corresponding to $A^{37}LIGRL^{42}$. . .).

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 PAR_2 variants using an antibody (SLAW-A) that recognizes the sequence released at the R^{36}/S^{37} cleavage/activation site. A loss of reactivity with SLAW-A (immunocytochemistry) confirms the release of the N-terminal receptor sequence that masks the tethered ligand (Compton et al., 2001; Al-Ani et al., 2002a). Our data point to the key role played by the first two amino acids of the tethered ligand ($S^{37}L^{38}$), in contrast with other residues within the first six amino acids that play lesser roles.

Materials and Methods

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 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).

Preparation of PAR_2 Constructs and their Expression in KNRK cells. As previously documented (Saifeddine et al., 1996; Al-Ani et al., 1999a,b), rat PAR_2 was cloned from kidney cDNA using the primer pairs: forward primer (containing a HindIII site and Kozak sequence shown in bold), 5′-TC**AAGCTTCCACC**ATGCGAAGTCTCAGCCTGGC-3′; reverse primer (containing SmaI site shown in bold), 5′-**CCCGGG**CTCAGTAGGAGGTTTTAACAC-3′. Then, the rat PAR_2 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 QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. In $PAR_2S^{37}A$, $PAR_2L^{38}A$, $PAR_2I^{39}A$, $PAR_2G^{40}A$, and $PAR_2R^{41}A$, residues S^{37}, L^{38}, I^{39}, G^{40}, and R^{41} were changed to A, respectively; in $PAR_2A^{37–38}$, $PAR_2A^{39–42}$, $PAR_2A^{37,\ 39–42}$, and $PAR_2A^{37–42}$, residues $S^{37}L^{38}$, $I^{39}G^{40}R^{41}L^{42}$, $S^{37}I^{39}G^{40}R^{41}L^{42}$, and $S^{37}L^{38}I^{39}G^{40}R^{41}L^{42}$ were changed to A, respectively. In $PAR_2L^{37}S^{38}$, residues $S^{37}L^{38}$ were reversed to become $L^{37}S^{38}$. The wild-type and PAR_2 mutants in pcDNA3 were then transfected into KNRK cells (American Type Culture Collection, Manassas, VA), as described previously (Al-Ani et al., 1999a,b) to yield permanent cell lines for further study. Transfected cells (either vector alone or PAR_2-containing vectors) were subcloned in G-418–containing medium (0.6 mg/ml), and PAR_2-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-PAR_2 antibody used by us previously (Kong et al., 1997; Al-Ani et al., 1999a,b). The SLAW-A antiserum recognizes the PAR_2 receptor sequence that is released upon proteolytic activation of the receptor (^{5}**SLAWLLG**^{11}-**G**^{30}**PNSKGR**^{36}-GGYGGC) (receptor antigenic sequences represented in bold; GGYGGC added for radiolabeling and cysteine-coupling). In the cell lines so isolated, >80% of the populations (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 PAR_2 Variants by Trypsin. PAR_2 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 PAR_2 by trypsin at site Arg^{36}. The rabbit

TABLE 1

Wild-type and mutated PAR_2 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 PAR_2 variants are also shown.

PAR_2 Variant	Symbol	Trypsin Cleavage/Activation Site	PAR_2-Derived Peptides
PAR_2wt	●	…KGR^{36}/SLIGR^{41}LDTP…	SLIGRL-NH_2
$PAR_2S^{37}A$	■	…KGR/**A**LIGRLDTP…	ALIGRL-NH_2
$PAR_2L^{38}A$	▲	…KGR/S**A**IGRLDTP…	SAIGRL-NH_2
$PAR_2I^{39}A$	▼	…KGR/SL**A**GRLDTP…	SLAGRL-NH_2
$PAR_2G^{40}A$	×	…KGR/SLI**A**RLDTP…	SLIARL-NH_2
$PAR_2R^{41}A$	✚	…KGR/SLIG**A**LDTP…	SLIGAL-NH_2
$PAR_2A^{37–38}$	▶	…KGR/**AA**IGRLDTP…	AAIGRL-NH_2
$PAR_2A^{39–42}$	◀	…KGR/SL**AAAA**DTP…	SLAAAA-NH_2
$PAR_2A^{37,\ 39–42}$	⊗	…KGR/**A**L**AAAA**DTP…	ALAAAA-NH_2
$PAR_2A^{37–42}$	⊙	…KGR/**AAAAAA**DTP…	AAAAAA-NH_2
$PAR_2L^{37}S^{38}$	◆	…KGR/**LS**IGRLDTP…	LSIGRL-NH_2

polyclonal antiserum (SLAW-A) mentioned above was employed to monitor the disappearance of the signal (generated by the receptor sequence up to and including residue Arg^{36}) 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 cytospin 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 PAR_2-expressing KNRK cells. To quantify the disappearance of SLAW-A–reactive epitope by trypsin treatment, as monitored by immunocytochemistry, a morphometric analysis was used as described previously (Compton et al., 2001; Al-Ani and Hollenberg, 2003). In brief, several microscopic fields, comprising 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 PAR_2KNRK 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. PAR_2-transfected KNRK cells were loaded with the intracellular calcium indicator Fluo-3 (Molecular Probes Inc., Eugene, OR) at a final concentration of 22 μM (25 μg/ml) of fluo-3 acetoxymethyl ester (Kao et al., 1989; 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 luminescence spectrometer (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 3×10^5 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 PAR_2 tethered ligand mutants for trypsin activation, relative to the sensitivity of wild-type PAR_2, a ratio was calculated ($R_{EC,T}$) based on the concentration of trypsin required to cause a given calcium signal in the wild-type receptor (EC_{WT}) relative to the concentration of trypsin required to cause the *equivalent* calcium signal in the mutant PAR_2 receptor with an altered tethered ligand sequence (EC_{MUTANT}). Thus, for trypsin activation, $R_{EC,T} = EC_{WT}/EC_{MUTANT}$. Values of this ratio <1 denote a receptor that requires a higher concentration of trypsin to cause the same cellular response as for PAR_2wt 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 ($R_{EC,P}$) of the concentration of the wild-type peptide, SLIGRL-NH_2, required for a given calcium signal ($EC_{SLIGRL\text{-}NH2}$), relative to the concentration of test peptide ($EC_{PEPTIDE}$) 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 $R_{EC,T}$ and $R_{EC,P}$ 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 PAR_2 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-PAR_2wt. Not only did all cell lines exhibit equivalent average cell surface fluorescence and immunoreactivity (FACS) but $\geq$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 PAR_2 variant cell lines to the PAR_2AP SLIGRL-NH_2 was equivalent, with comparable EC_{50} values (3 to 10 μM) and maximal calcium signals at 50 μM SLIGRL-NH_2 that were 80% or greater than the signal generated by PAR_2wt (Fig. 1 and data not shown for constructs designated by ✕ and ✚ in Table 1).

Sensitivity of the PAR_2 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-NH_2, the next step was to measure their sensitivity to trypsin, reflecting the

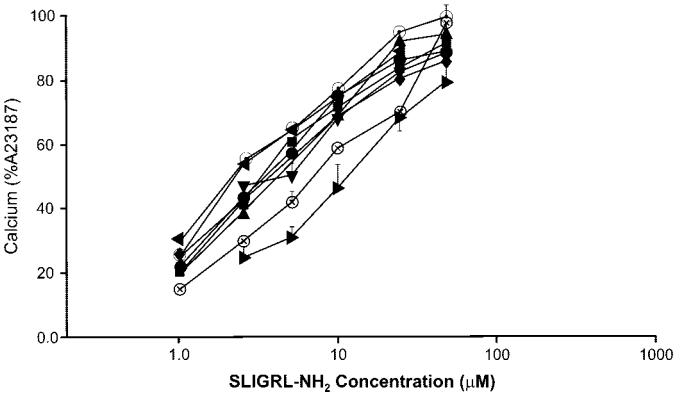

Fig. 1. Concentration-effect curves for SLIGRL-NH_2-stimulated calcium signaling in wild-type and mutated PAR_2-expressing KNRK cell lines. Permanent KNRK cell lines expressing the wild-type and mutated PAR_2 constructs with altered tethered ligand sequences (Table 1) were evaluated for their sensitivity to SLIGRL-NH_2 (calcium signaling) as outlined under *Materials and Methods*. The fluorescence emission (E_{530}) in response to increasing concentrations of SLIGRL-NH_2 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): ●, PAR_2wt; ■, $PAR_2S^{37}A$; ▲, $PAR_2L^{38}A$; ▼, $PAR_2I^{39}A$; ▶, PAR_2A^{37-38}; ◀, PAR_2A^{39-42}; ⊗, $PAR_2A^{37,39-42}$; ⊙, PAR_2A^{37-42}, and ◆, PAR_2 $L^{37}S^{38}$. 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 PAR_2wt (●). 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 $PAR_2L^{37}S^{38}$, which was otherwise fully responsive to SLIGRL-NH_2 (Fig. 2 and Table 2, ◆), and the essential lack of trypsin sensitivity of the construct with $A^{37}A^{38}$ substitutions ($PAR_2A^{37–38}$, ▶; Fig. 2). In contrast, changing only the first amino acid to alanine ($PAR_2S^{37}A$, ■; Fig. 2) resulted in a receptor with sensitivity toward trypsin that was the same as that of the wild-type receptor (PAR_2wt, ●; Fig. 2). In contrast, changing Leu^{38} to alanine at the second position of the tethered ligand ($PAR_2L^{38}A$: ▲, Fig. 2) caused a ~10-fold reduction in the sensitivity to trypsin but nonetheless resulted in a receptor that could still respond to trypsin with a maximal calcium response of ~75% of that observed for PAR_2wt. In addition to the crucial importance of the first two amino acids for tethered ligand activity, further alanine substitutions revealed the importance of residues Ile^{39} and Arg^{41} ($PAR_2I^{39}A$ and $PAR_2R^{41}A$, ▼ 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. . . ≈ SLAAAA. . . > 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 PAR_2wt ($R_{EC,T}$ values), are summarized in Table 2, expressed as a ratio ($R_{EC,T}$) of the concentrations of trypsin required to cause a calcium response in the wild-type receptor (EC_{WT}) relative to the concentration of trypsin required to generate the equivalent calcium signal in the receptor mutant (EC_{MUTANT}). Thus, $R_{EC,T} = EC_{WT}/EC_{MUTANT}$, where values <1.0 denote a receptor mutant with reduced sensitivity to trypsin.

Unmasking of Receptor Variants by Trypsin. Although all receptor variants expressed comparable amounts of functional cell surface receptors (responses to SLIGRL-NH_2 and FACS), it was essential for interpreting the trypsin sensitivity data (Fig. 2) to know that all variants were similarly cleaved by trypsin at Arg^{36} 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 PAR_2wt. 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 ($PAR_2A^{39–42}$) or no activity ($PAR_2L^{37}S^{38}$) upon trypsin activation. A similar removal of the epitope visualized by the SLAW-A antibody was also observed for all mutant PAR_2 cell lines (not shown). Morphometric analysis of the fixed stained cells revealed that, as for PAR_2wt, 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.

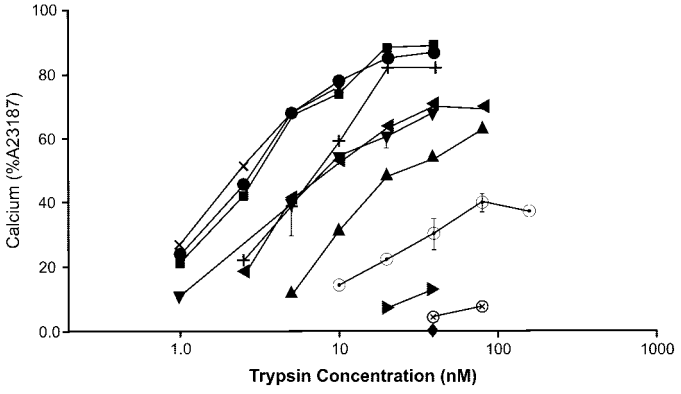

Fig. 2. Trypsin sensitivity of wild-type and mutated PAR_2-KNRK cell lines. Concentration-response measurements were done for trypsin-stimulated calcium signaling using the wild-type and mutated PAR_2-expressing KNRK cell lines with altered tethered ligand sequences (Table 1), as outlined in the legend of Fig. 1 for SLIRGL-NH2-stimulated calcium signaling. Cell lines are represented by the symbols (Table 1): ●, PAR_2wt; ■, $PAR_2S^{37}A$; ▲, $PAR_2L^{38}A$; ▼, $PAR_2I^{39}A$; ✕, $PAR_2G^{40}A$; ✚, $PAR_2R^{41}A$; ▶, $PAR_2A^{37–38}$; ◀, $PAR_2A^{39–42}$; ⊗, $PAR_2A^{37,39–42}$; ⊙, $PAR_2A^{37–42}$; and ◆, $PAR_2L^{37}S^{38}$. 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.

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 PAR_2-activating peptide sequence, S_1LIGRL_6-NH_2 (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 PAR_2wt 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-NH_2 (wild-type sequence), ALIGRL-NH_2, SAIGRL-NH_2, SLAGRL-NH_2, SLIARL-NH_2, SLIGAL-NH_2, AAIGRL-NH_2, SLAAAA-NH_2, and LSIGRL-NH_2 were tested for activity (calcium signal) in both PAR_2wt and in most of the receptor mutants having the cognate tethered ligand sequence. Because neither AAIGRL-NH_2 nor SLAAAA-NH_2 was found to be active in the calcium signaling assay (below), the peptides ALAAAA-NH_2 and AAAAAA-NH_2 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-NH_2 (▲, △), AAIGRL-NH_2 (▶, ▷), SLAAAA-NH_2 (◀, ◁), and LSIGRL-NH_2 (◆, ◇) were completely inactive both in PAR_2wt 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 EC_{50} 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-NH_2 ≈ SLIARL-NH_2 ≫ ALIGRL-NH_2 ≫ SLAGRL-NH_2 ≈ SLIGAL-NH_2, in contrast with the inactive peptides, SAIGRL-NH_2, AAIGRL-NH_2, SLAAAA-NH_2, and LSIGRL-NH_2. The relative potencies of these different sequences for activating PAR_2 in relation to the activity of SLIGRL-NH_2 were expressed quantitatively, as we have done previously (Hollenberg et al., 1997), as a ratio ($R_{EC,P}$) of the concentration

of SLIGRL-NH_2 required to cause a given calcium signal $EC_{SLIGRL\text{-}NH2}$, *relative* to the concentration of the peptide analog $EC_{PEPTIDE}$ required to generate the *equivalent* calcium response in either the wild-type receptor (PAR_2wt) or in the corresponding receptor mutant (i.e., $R_{EC,P} = EC_{SLIGRL\text{-}NH2} / EC_{PEPTIDE}$: Table 2).

Discussion

Our study is the first to examine systematically the structure-activity relationships for the activation of PAR_2 by its tethered ligand sequence. The main finding of our study was that the first two amino acids of the tethered ligand of rat PAR_2, 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 AAIGRL. . . , 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 PAR_2AP SLAAAA-NH_2 did not activate PAR_2wt.

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 PAR_2-activating peptide, SLIGRL-NH_2). Furthermore, the immunocytochemical analysis of trypsin-treated receptor-expressing cells revealed an equivalent removal of the sequence N-terminal to Arg^{36} that is visualized by SLAW-A. We had already identified the $R^{36}S^{37}$ site as the principle target for trypsin cleavage in the expressed receptor, resulting in the removal of the N-terminal epitope visualized by the SLAW-A antibody (Al-Ani and Hollenberg, 2003). Thus, both of the principal criteria required to interpret the trypsin sensitivity data were met, and the simple presence or absence of signaling generated by trypsin can be taken clearly to reflect the activity or lack thereof of a given tethered ligand sequence. That said, interpreting the *relative* activities of trypsin as reflecting the *relative* activities of the revealed tethered ligands in those receptor mutants that did yield calcium signals requires a further assumption that must be taken into account.

The assumption that must be made to use the $R_{EC,T}$ values to reflect the relative activities of the tethered ligand mutants is that the rate of trypsin cleavage must be the same for all receptor mutants and that this rate must not be limiting for generating a calcium signal. We were able with the immunocytochemical/morphometric approach to determine successfully that the extent of trypsin cleavage was the same for all mutants, within the time frame of signaling (5 min); unfortunately, however, we could not accurately measure the precise rate constants for cleavage for each construct because of the technical limitations of our measurements of SLAW-A epitope removal. That said, the finding of equivalent cleavage of all PAR_2 variants within 5 min strongly supports the assumption that the cleavage rates were comparable. Moreover, the clustering of the $R_{EC,T}$ values for 1) PAR_2wt, $PAR_2S^{37}A$, and $PAR_2G^{40}A$, and 2) $PAR_2I^{39}A$, $PAR_2R^{41}A$, and PAR_2A^{39-42} (Fig. 2 and Table 2) with mutations either at or downstream of the trypsin cleavage/activation site supports the hypothesis that the trypsin cleavage rate is not a factor in interpreting the data.

Given that the two key criteria for interpreting the relative trypsin sensitivities were essentially met, we used the $R_{EC,T}$ values (Table 2) to reflect the relative activities of the revealed tethered ligands. There were major discrepancies between the SARs for activation of the receptor by the tethered ligand sequences (Fig. 2 and Table 2) as opposed to the SARs for the soluble PAR-APs (Fig. 4 and Table 2). For instance, the sequences S^{37}LAAAA and S^{37}AIGRL. . . were quite active as tethered ligands (◀, ▲; Fig. 2), whereas the peptides S_1LAAAA_6-NH_2 and S_1AIGRL_6-NH_2 were devoid of activity (◀, ◁, ▲, △; Fig. 4). Furthermore, the tethered ligand sequence, A^{37}LIGRL. . . , was as active as the wild-type sequence, whereas the activity of the peptide, A_1LIGRL_6-NH_2, was considerably reduced (about 10-fold) compared with SLI-

TABLE 2

Relative activities of PAR_2 tethered ligand sequences and their cognate synthetic peptides

The relative activity values for activation by trypsin ($R_{EC,T}$) or for activation by the synthetic peptides ($R_{EC,P}$) as carboxamides corresponding to the cognate tethered ligands were calculated from the data shown in Figs. 2 and 4, according to the formulae: $R_{EC,T} = EC_{WT}/EC_{MUTANT}$ and $R_{EC,P} = EC_{SLIGRL\text{-}NH2}/EC_{PEPTIDE}$, as outlined under *Materials and Methods* (Hollenberg et al., 1997). Thus, the value for the wild-type sequence is unity, whereas values <1 denote a tethered ligand or peptide with an activity less than that of the wild-type sequence. Alanine and other substitutions in the tethered ligand sequence are shown in bold type. The symbols denote the sequences of the cognate PAR-APs used to activate either the wild-type (open symbols) or mutant receptor (closed symbols) as shown in Fig. 4. The closed symbols are also used for the trypsin activation data (Fig. 2) with the same tethered ligand sequences.

Receptor	Symbols	Sequence	Relative Activity	
			$R_{EC,T}$	$R_{EC,P}$
PAR_2wt	○	SLIGRL	1	1
$PAR_2S^{37}A$	■, □	**A**LIGRL	0.92	0.095
$PAR_2G^{40}A$	✕,⊠	SLI**A**RL	1.2	0.98
$PAR_2I^{39}A$	▽	SL**A**GRL	0.42	0.025
$PAR_2R^{41}A$	✚, ✙	SLIG**A**L	0.39	0.025
PAR_2A^{39-42}	◀,◁	SL**AAAA**	0.37	N.A.
$PAR_2L^{38}A$	▲, △	S**A**IGRL	0.14	N.A.
PAR_2A^{37-42}	⊙	**AAAAAA**	0.032	N.D.
PAR_2A^{37-38}	▶,▷	**AA**IGRL	0.019	N.A.
$PAR_2A^{37,\ 39-42}$	⊗	**A**L**AAAA**	N.A.	N.D.
$PAR_2L^{37}S^{38}$	◆, ◇	**LS**IGRL	N.A.	N.A.

N.A., not active; N.D., not done.

GRL-NH_2 (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 (Arg^{41}) 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 ($PAR_2L^{37}S^{38}$) or replacing the first two residues with alanine (PAR_2A^{37-38}), 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 Ser^{37} and Leu^{38} residues changed to alanines (PAR_2A^{39-42}), the revealed tethered ligand ($S^{37}LAAAA. . .$) was still able to cause a substantial activation of the receptor (Fig. 2, ◀). The interaction of the $S^{37}L^{38}$ 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-

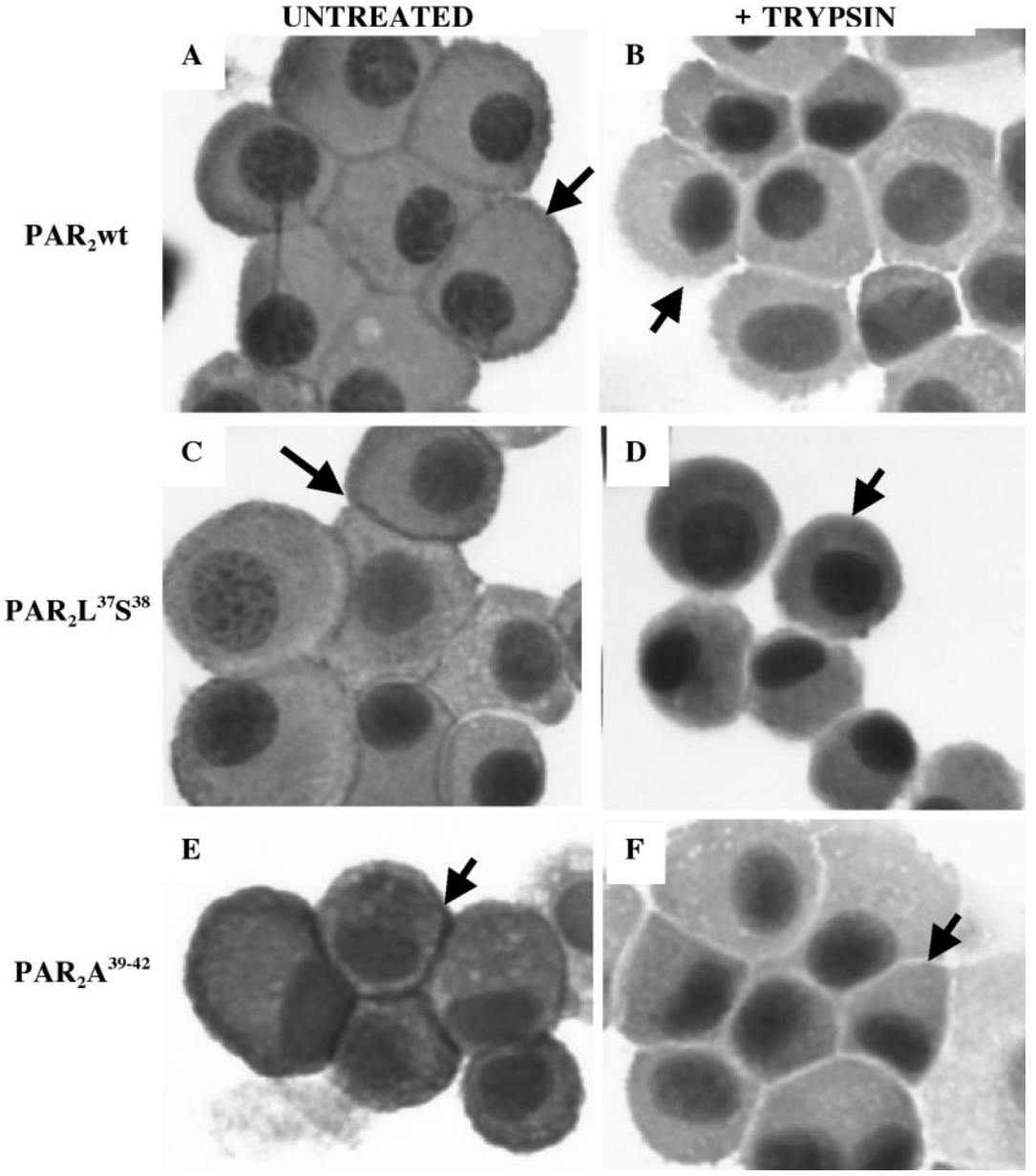

Fig. 3. Trypsin-catalyzed removal of the N-terminal PAR_2 sequence that masks the tethered ligand. KNRK cell lines expressing either wild-type PAR_2 (A and B) or receptors with mutated tethered ligand sequences (C–F) were either treated (B, D, F) or not (A, C, E) for 5 min at room temperature with 40 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, PAR_2wt; C and D, $PAR_2L^{37}S^{38}$; E and F, PAR_2A^{37-38}. 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 ($PAR_2S^{37}A$) led to a sequence with an activation profile equivalent to that of the wild-type tethered ligand (compare ● 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^{38} with alanine ($PAR_2L^{38}A$, ▲; 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_2APs 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}LAAAA. . . was essentially inactive, given that sequences S^{37}LAAAA. . . 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}LAAAA. . . (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_2Aps 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^{39} and Arg^{41} side chains. That said, the activity of PAR_2A^{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.

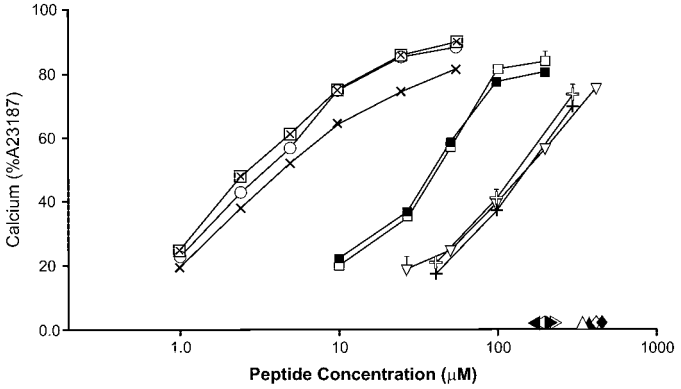

Fig. 4. Activation of calcium signaling in wild-type and mutated PAR_2 variants by synthetic peptides corresponding to the revealed tethered ligands. Both wild-type (open symbols) and mutated receptors (solid symbols) with the different tethered ligand sequences shown in Table 1 were exposed to increasing concentrations of the synthetic peptides (as carboxamides) representing either the wild-type tethered ligand sequence, SLIGRL-NH_2 (○) or the sequences of the corresponding mutated tethered ligands. Increases in intracellular calcium were monitored, as outlined in the legend to Fig. 1, as a percentage (%A23187) of the fluorescence emission (E_{530}) caused by 2 μM concentrations of the ionophore A23187. Peptides corresponding to the altered tethered ligand sequence were tested either in PAR_2wt (open symbols) or in the cognate receptor with the corresponding tethered ligand sequence (closed symbols). The synthetic peptides evaluated were: ○, SLIGRL-NH_2; ✕, ⊠, SLIARL-NH_2; ■, □, ALIGRL-NH_2; ✚, ✙, SLIGAL-NH_2; ▽, SLAGRL-NH_2; ▲, △, SAIGRL-NH_2; ▶, ▷, AAIGRL-NH_2; ◀, ◁, SLAAAA-NH_2; and ◆, ◇, LSIGRL-NH_2. Values represent the averages ± S.E.M. (bars) 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.

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