Derivatized 2-Furoyl-LIGRLO-amide, a Versatile and Selective Probe for Proteinase-Activated Receptor 2: Binding and Visualization

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ABSTRACT

The proteinase-activated receptor-2 (PAR2)-activating peptide with an N-terminal furoyl group modification, 2-furoyl-LIGRLO-NH₂ (2fLI), was derivatized via its free ornithine amino group to yield [³H]propionyl-2fLI and Alexa Fluor 594-2fLI that were used as receptor probes for ligand binding assays and receptor visualization both for cultured cells in vitro and for colonic epithelial cells in vivo. The binding of the radiolabeled and fluorescent PAR2 probes was shown to be present in PAR2-transfected Kirsten normal rat kidney cells, but not in vector-alone-transfected cells, and was abolished by pretreatment of cells with saturating concentrations of receptor-selective PAR2 peptide agonists such as SLIGRL-NH₂ and the parent agonist 2fLI but not by reverse-sequence peptides such as 2-furoyl-OLRGIL-NH₂ that cannot activate PAR2. The relative orders of potencies for a series of PAR2 peptide agonists to compete for the binding of [³H]propionyl-2fLI (2fLI >> SLIGRL-NH₂ ≈ trans-cinnamoyl-LIGRLO-NH₂ > SLIGKV-NH₂ > SLIGKT-NH₂) mirrored qualitatively their relative potencies for PAR2-mediated calcium signaling in the same cells or for vasorelaxation in a rat aorta vascular assay. In the vascular assay, the potency of Alexa Fluor 594-2fLI was the same as 2fLI. We conclude that ornithine-derivatized 2fLI peptides are conveniently synthesized PAR2 probes that will be of value for future studies of receptor binding and visualization.

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ABBREVIATIONS: PAR, proteinase-activated receptor; Alexa Fluor 594, pyrano[3,4-g:5,6-g′]diquinolin-13-ium, 6-[2-carboxy-4(or 5)[[2,5-dioxo-1-pyrrolidinyl]oxycarbonyl] pheryl]-1,2,2,10,11-hexamethyl-4,8-bis(sulfomethyl)-succinimidyl ester; Alexa Fluor 594-2fLI, 2-furoyl-LIGRLO(N-Alexa Fluor 594)-O-NH₂; Alexa Fluor 594-2fOL, reverse-sequence receptor-inactive 2-furoyl-N-Alexa Fluor 594-OLRGIL-NH₂; 2fLI, 2-furoyl-LIGRLO-NH₂; 2fO or 2fOL, 2-furoyl-OLRGIL-NH₂; [³H]propionyl-2fLI, 2-furoyl-LIGRLO(N-[³H]propionyl)-O-NH₂; PAR-AP, PAR-activating peptide; KNRK, Kirsten normal rat kidney; IC₅₀, concentration of binding competitor for which specific ligand binding is inhibited by 50%; R̄IC₅₀, R̄IC₅₀CGD, and R̄IC₅₀CGD, relative IC₅₀s for radioligand binding, Alexa-594 2fLI binding, and vascular bioassay, respectively, normalized to the value for SLIGRL-NH₂, 1.0; HPLC, high-performance liquid chromatography; OCT, ornithine carbamyl transferase; HEK, human embryonic kidney; A23187, calcimycin; tc, trans-cinnamoyl.
tems (Vergnolle, 2004, 2005; Hansen et al., 2005, 2007; Cenac et al., 2007; Ramachandran and Hollenberg, 2008).

Despite the expanding interest in the pathophysiology of PARs, it has not been possible to assess its abundance and location in live cells because of the lack of reliable and conveniently synthesized radiolabeled and fluorescent receptor binding probes. In our previous work, we developed a [\(^3\)H]propionyl derivative of the receptor-selective PAR\(_2\)-activating peptide, trans-cinnamoyl (tc)-LIGRLO-NH\(_2\) (Al-Ani et al., 1999), that was adequate for a ligand binding assay but not suitable as a fluorescently labeled probe for receptor visualization because of its relatively low receptor affinity. Furthermore, our work revealed that, unlike SLIGRLO-NH\(_2\), the trans-cinnamoyl peptide was able to activate receptors in addition to PAR\(_2\) and was therefore not optimal as a receptor probe. Therefore, we developed and evaluated in depth the receptor selectivity of a higher potency PAR\(_2\) agonist, 2-furoyl-LIGRLO-NH\(_2\) (2fLI), that like the trans-cinnamoyl derivative was designed to have a C-terminal ornithine that can be readily derivatized via its side-chain amino group (McGuire et al., 2004). Comparable compounds lacking the C-terminal ornithine (2-furoyl-LIGKV-NH\(_2\) and 2-furoyl-LIGRLO-NH\(_2\)) were also studied by others (Ferrell et al., 2003; Kawabata et al., 2004). From previous work by us and by others evaluating the structure-activity relationships for PAR\(_1\) and PAR\(_2\)-activating peptides (Bernatowicz et al., 1996; Hollenberg et al., 1997; Maryanoff et al., 2001), we knew that only the first five amino acids of the PAR-activating peptides are critical for receptor activation and that C-terminal substitutions are readily introduced into peptides that retain full PAR\(_2\)-activating activity. Given that result, we hypothesized that the 2fLI peptide derivatized on the side chain amino group of its ornithine would be a good receptor probe for both ligand binding and receptor visualization studies. Therefore, we synthesized ornithine-substituted [\(^3\)H]propionyl-2fLI and Alexa Fluor 594-2fLI and tested their suitability as PAR\(_2\) probes. The equivalence of the biological activity of Alexa Fluor 594-2fLI with that of 2fLI was tested in a vascular endothelium-dependent PAR\(_2\)-activated relaxation bioassay. These derivatives were then used 1) for a cultured cell binding assay and 2) for visualizing PAR\(_2\) by fluorescence microscopy using cultured KNRK cells that were transfected or not with rat PAR\(_2\) (Al-Ani et al., 1999) and by fluorescence microscopy of fixed colon tissue that had been exposed in vivo to Alexa Fluor 594-2fLI.

Materials and Methods

Peptides and Other Reagents

All peptides were synthesized as carboxy amides (>95% purity, assessed by HPLC and mass spectrometry) by Dr. Denis McMaster (Peptide Core Facility at University of Calgary, Calgary, AB, Canada). Unless indicated otherwise, all remaining chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Peptides were dissolved in buffer, pH 7.4, containing 25 mM HEPES. [\(^3\)H]Propionyloxyacetic acid was from Moravek Biochemicals (Brea, CA; 110 Ci/mmol); Alexa Fluor 594 was from Invitrogen (Carlsbad, CA); and dinonyl phthalate was from [\(^3\)H]Ligand Binding Assay

The binding of [\(^3\)H]propionyl-2fLI to PAR\(_2\)-expressing KNRK cells was measured by a Microfuge centrifugation method (Beckman Coulter, Fullerton, CA) that pellets the cell-bound ligand below an oil-water interface, as described previously (Cuatrecasas and Hollenberg, 1976; Hollenberg and Cuatrecasas, 1976). For routine binding assays, suspensions of KNRK cells (transfected or not with rat PAR\(_2\)) (approximately 10\(^7\) cells/ml in a total volume of 50 \(\mu\)l) were aliquoted into 1-ml polypropylene Titertube microtest tubes (8.8 \(\times\) 4.5 cm)
Monitoring Ligand Binding and Binding-Competition by Fluorescence Microscopy

Measurements with Cultured Cells. To visualize PAR2 by fluorescence microscopy in a cultured cell system, KNRK cells transfected or not with rat PAR2 were grown to approximately 60% confluence as monolayers on Multiwell glass bottom 35-mm diameter Petri dishes (P35G-0-14-C; MatTek Corporation). In keeping with the binding study, cells were first washed free of growth medium and incubated for 2 min on ice or at room temperature, with or without an excess of nonfluorescent binding probe (20–50 μM in a total volume of 2 ml), at which point Alexa Fluor 594-2fLI (50–500 nM) was then added. The binding reaction was allowed to proceed for 60 min either on ice or at room temperature, and unbound fluorescence was removed by a rapid wash (2–3 × 1 ml) followed by visualization of bound Alexa Fluor 594-2fLI.

Bound fluorescence was visualized with an Olympus IX70 inverted microscope with a 10× (tissue) or 20× (KNRK cells) objective coupled to a CCD camera (Qimaging, Surrey, BC, Canada) and Volocity image capture software (Improvision, Waltham, MA). Fluorescence was activated at a wavelength of 555 nm, with emission recorded at 617 nm. Fluorescence observed in the absence of unlabelled PAR2 competitor was compared with the fluorescence in the presence of competitor. Measurements were done with five or more independently grown cell monolayers. The amount of fluorescent probe bound was documented both by observation of individual cells and by morphometric analysis of the visualized cells. The relative fluorescence observed in the absence or presence of increasing amounts of unlabelled PAR2 peptide competitor was quantified by integrating the total fluorescence signal yield (NIH ImageJ software) from randomly selected microscopic fields (each approximately 100 × 100 μm area) containing up to a total of 100 or more cells. The binding-competition curves obtained from these fluorographic morphometric measurements were constructed by plotting the ratio of fluorescence observed in the presence of increasing amounts of nonfluorescent PAR2 peptide competitor relative to the signal observed in the absence of competitor.

Visualizing PAR2 in Colonic Tissues in Vivo. Although the vascular assays were done with rat-derived tissues to match data obtained with the rat PAR2-expressing KNRK cells, a murine in vivo model, rather than the rat, was employed to minimize the amount of reagents required for the experiments. Male C57Bl6 mice (6–8 weeks) were purchased from Charles River Laboratories (Montreal, QC, Canada). All procedures were approved by Institutional Animal
Care Committee (University of Calgary). The Alexa Fluor 594-2fLI probe (10 μg in 0.1 ml of 10% v/v ethanol/10% v/v Tween 80 in 0.9% NaCl) and its reverse peptide sequence fluorescent analog were administered intracolonically through a catheter inserted at a distance of approximately 4 to 5 cm from the anus to three groups of C57Bl/6 male mice, as described previously (Nguyen et al., 2003). The receptor probe was in solution either with or without a 10-fold excess (100 μg) of nonlabeled 2-FLI. One hour after the intracolonic administration of the fluorescent receptor probe or its reverse-sequence fluorescent control peptide, the animals were lightly anesthetized with sodium pentobarbital (approximately 100 mg/kg i.p.) and then immediately perfused via the heart with 5 ml of saline, followed by 5 ml of 10% formalin for fixation of tissues in vivo. Upon fixation by cardiac perfusion, animals were sacrificed, and colonic tissue at the site of peptide administration was harvested (within 5 min) and fixed further overnight in 10% formalin. Fixed tissues were prepared for microscopic analysis by directly embedding in paraffin or were placed in 20% v/v sucrose for another 24 h at 4°C before embedding in ornithine carbamyl transferase compound (OCT; Miles, Elkhart, IN). OCT-embedded tissues were then cryosectioned at 10 μm, washed in isonic phosphate-buffered saline and mounted in Prolong mounting medium (Invitrogen) for microscopic analysis. Parafin-embedded sections were sectioned at 5 μm, deparaffinized by two 5-min washes in xylene, and were mounted directly with Cytoseal xylene based mounting media (Richard-Allan Scientific, Kalamazoo, MI) to observe the fluorescence signal or were stained with hematoxylin and eosin as per standard protocols on a Fisher Histometric Slide Stainer (Thermo Fisher Scientific, Waltham, MA) before mounting with Cytoseal. CY-3 fluorescence in the sections was monitored as outlined above with an excitation wavelength of 555 nm and an emission wavelength recorded at 617 nm. For morphometric analysis of the fluorescence present at specific tissue locations (e.g., over the luminal epithelial layer), the fluorescence yield (arbitrary units) was measured over equal tissue areas, and the integrated values were compared. The background fluorescence yield for the biologically inactive Alexa Fluor 594-2fLI was considered as “nonspecific” labeling. The value for the Alexa Fluor 594-2fLI fluorescence was subtracted from the fluorescence observed for 594-2fLI (in the absence or presence of competing nonfluorescent 2fLI) to calculate the “specific” receptor-related fluorescence.

**Rat Aorta Relaxation Assay**

The endothelium-dependent rat aorta relaxation assay that reflects the activation of endothelial PAR2 was done using tissue derived from male Sprague-Dawley albino rats (200–250 g) essentially as described previously (Al-Ani et al., 1995; Hollenberg et al., 1997; McGuire et al., 2004). In brief, segments of aorta rings were isolated from animals that had been killed by decapitation and were mounted in an organ bath (4-ml plastic cuvette). Tissues were continuously maintained in a standard physiological solution buffer containing 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH2PO4, 1.2 mM MgCl2, 11 mM d-glucose, 25 mM NaHCO3, and 2.5 mM CaCl2 that was bubbled with a 95%/5% O2/CO2 gas mixture to maintain the buffer at pH 7.4. Maintained in a standard physiological salt solution buffer containing an organ bath (4-ml plastic cuvette). Tissues were continuously maintained in a standard physiological salt solution buffer containing 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH2PO4, 1.2 mM MgCl2, 11 mM d-glucose, 25 mM NaHCO3, and 2.5 mM CaCl2 that was bubbled with a 95%/5% O2/CO2 gas mixture to maintain the buffer at pH 7.4. Rings of rat aorta were suspended vertically by two metal hooks; the lower hook was connected to an immovable support in 4-ml plastic cuvettes containing the standard gassed physiological solution. Ten rings were measured using Statham or Grass force transducers. Tissues were contracted by exposure to phenylephrine (1 μM), and the relaxant response to PAR2-activating peptides was measured as a percentage (%Ach) of the relaxation caused in the same tissue by 10 μM acetylcholine.

**Calcium Signaling Measurements**

The calcium signaling assay that monitors PAR2 activation in PAR2-transfected KNRK cells was done essentially as described previously for cultured HEK and PAR-expressing KNRK cells. (Al-Ani et al., 1999; Kawabata et al., 1999; Compton et al., 2000). Cells harvested in an isotonic EDTA-containing dissociation medium without the use of trypsin were incubated in a solution of serum-free Dulbecco’s modified minimal essential medium that contained 0.25 mM sulfinpyrazone and 22 μM Fluo-3 acetoxyethyl ester (Invitrogen) for 25 min at room temperature. Cells were then resuspended in a buffered solution, pH 7.4, that contained the 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl2, 10 mM glucose, 20 mM HEPES, and 0.25 mM sulfinpyrazone. Light emission at 530 nm using a 480-nm excitation wavelength was monitored using an Amino Bowman Series 2 Luminescence spectrometer (Thermo Spectronic, Madison, WI). Cell suspensions (2 ml) in 4-ml cuvettes were mixed continuously with a magnetic stirrer and maintained at 25°C. The response that resulted from the addition of a test agonist was standardized relative to the peak fluorescence elicited by the addition of calcium ionophore (2 μM A23187).

**Results**

**Optimization and Validation of the Binding Assay.** Preliminary work (not shown) established that, at room temperature, binding equilibrium was reached at approximately 1 h and that equilibration took approximately 2 h at 4°C. Binding was linear with respect to cell number in the range of 105 to 108 cells/ml. Thus, the routine binding-competition experiment used approximately 107 cells/ml with an equilibration time of 1 h and that equilibration took approximately 2 h at 4°C. Binding was linear with respect to cell number in the range of 105 to 108 cells/ml. Thus, the routine binding-competition experiment used approximately 107 cells/ml with an equilibration time of 1 h and that equilibration took approximately 2 h at 4°C. Furthermore, the binding of radiolabeled [3H]propionyl-2fLI was done at 4°C, with an equilibration time of 2 h. Table 1 shows, for a representative experiment, the binding of radiolabeled [3H]propionyl-2fLI in the absence and presence of an excess (100–200 μM) of either unlabeled SLIGRL-NH2 or unlabeled 2-furoyl-LIGRLO-NH2. Although these two PAR2-selective agonists were able to compete for the binding of radiolabeled probe, the reverse-sequence-peptide that could not activate PAR2 (2-furoyl-OLIGIL-NH2) (McGuire et al., 2004) did not compete for binding. Furthermore, the binding of radioligand by cells that had been transfected with vector alone (KNRK-pcDNA3, 1770 ± 80 CPM) (Table 1, fourth line, experiment 1) was comparable to and even lower than the nonspecific binding level detected in the rat PAR2-expressing KNRK cells incubated with radiolabeled ligand probe in the presence of an excess of unlabeled PAR2-activating peptide (KNRK-PAR2 + SLIGRL-NH2, 2760 ± 90 cpm) (Table 1, second line, experiment 1). Thus, the binding proved to be

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**TABLE 1**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Peptide Added (100 μM)</th>
<th>Counts per Min Bound (Mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNRK-PAR2</td>
<td>None</td>
<td>4360 ± 200</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>SLIGRL-NH2</td>
<td>2760 ± 90</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>2f-LIGRLO-NH2</td>
<td>2100 ± 100</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>2f-OLIGIL-NH2</td>
<td>4400 ± 240</td>
</tr>
<tr>
<td>KNRK-pcDNA3</td>
<td>None</td>
<td>1770 ± 80</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>None</td>
<td>500 ± 16</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>2f-LIGRLO-NH2</td>
<td>230 ± 19</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>Alexa Fluor 594–2fLI</td>
<td>220 ± 13</td>
</tr>
<tr>
<td>KNRK-PAR2</td>
<td>Reverse Alexa Fluor 594–2fLI</td>
<td>520 ± 70</td>
</tr>
</tbody>
</table>

Binding of [3H]2fLI in the absence and presence of excess unlabeled peptide (240,000 cpm per sample; 300,000 cpm added; incubation for 1 h at room temperature).
both peptide- and receptor-specific, and the degree of non-specific (presumably nonreceptor) binding was of a sufficiently low magnitude to enable further studies. Binding competition studies done at either room temperature or at 4°C yielded comparable results; therefore, routine binding competition experiments were conducted at room temperature to optimize the convenience of the assay.

**Binding-Competition Curves for Unlabeled Ligands and Binding Curve for Radiolabeled Binding Probe.** The ability of several PAR2-activating peptides to compete for the binding of radiolabeled 2fLI is illustrated in Fig. 1, in which the relative IC$_{50}$s for binding competition (Table 2) revealed a relative affinity order of 2-furoyl-LIGRLO-NH$_2$ > SLIGRL-NH$_2$ = trans-cinnamoyl-LIGRLO-NH$_2$ > SLIGKV-NH$_2$ > SLIGKT-NH$_2$ > TFLLR-NH$_2$. LSIGRL-NH$_2$ and 2-furoyl-OLRGIL-NH$_2$ were not active. Quantitatively, the relative affinities of these peptides (relative IC$_{50}$s for radioligand binding: R$_{IC50}$, normalized to the binding of the most widely used PAR$_2$-activating peptide, SLIGRL-NH$_2$, set equal to 1.0) were 0.03:1.0:0.5:7.8:6.6 (Table 2). A value for the R$_{IC50}$ greater than 1.0 denotes a peptide with an affinity lower than that of SLIGRL-NH$_2$. Qualitatively, this order of binding affinities and the lack of binding competition by the two scrambled peptide analogs are exactly in keeping with the relative potencies of the PAR$_2$-APs and the lack of activity of the control peptides in the PAR$_2$ calcium signaling assays (Al-Ani et al., 1999; Kawabata et al., 1999) and vascular assays (see below and McGuire et al., 2004) that we had previously used to evaluate the same peptides.

The binding isotherm for increasing concentrations of radiolabeled ligand done at 4°C was constructed by a curve-fitting procedure, in keeping with very early work describing the binding of radiolabeled atropine to the muscarinic receptor in guinea pig intestinal smooth muscle (Paton and Rang, 1965). The data revealed a biphasic curve (Fig. 2) with a high-affinity site clearly evident as a plateau of binding in the range of concentrations from approximately 50 to 120 nM. The curve-fitting analysis showed that this site, with an apparent affinity (half-maximal binding) of approximately 50 nM, was present in an abundance of approximately 60,000 sites per cell. The lower affinity binding in the region up to the micromolar range of radiolabeled ligand probe was also ligand-specific (i.e., competed for by unlabeled ligand). This lower affinity binding displayed an abundance of between 450 to 500 × 10$^3$ sites per cell. Scatchard analysis of the data were in general accord with the information that can be obtained directly from the binding curve (Fig. 2), indicating a complex two-site binding process with a submicromolar high-affinity site of low abundance and a micromolar affinity site with a 10-fold higher abundance. However, because the Scatchard plot analysis (not shown) was not linear at either high or low concentrations of radioligand, a detailed analysis was considered inappropriate. The interpretation of the nonlinear Scatchard plots was therefore left for a separate study.

**Lack of Effect of 2-Furoyl-OLRGIL-NH$_2$ in a PAR$_2$ Calcium Signaling Assay.** Although the reverse-sequence peptide, 2-furoyl-OLRGIL-NH$_2$, did not compete for the binding of radiolabeled ligand at concentrations up to approximately 200 μM, a small degree of binding competition was observed at concentrations of the unlabeled peptide in the submillimolar range (not shown), suggestive of potential binding at a site distinct from the receptor's tethered ligand binding site. This situation could reflect observations with PAR$_1$, wherein a PAR-activating peptide binding site distinct from the tethered ligand binding-activation site has been identified (Blackhart et al., 2000). The possibility was therefore considered that the reverse sequence 2fOL peptide, which did not compete for radioligand binding at the receptor activation site (i.e., the site occupied by the tethered ligand), might potentially bind to a nonactivation site in order to be a receptor antagonist in cells via an allosteric receptor mechanism (i.e., acting via a site distinct from the main binding site targeted by our assay). This working hypothesis was examined by assessing the ability of the reverse 2fLI peptide either to cause a calcium signal on its own at very high concentrations (200–400 μM) in a KNRK-PAR$_2$ calcium signaling assay or to block the ability of a PAR$_2$ peptide agonist to generate a calcium signal. As illustrated in Fig. 3, the reverse sequence peptide, 2fO, neither caused a calcium signal on its own nor blocked calcium signaling triggered by 2fLI. Comparable results were obtained either with human PAR$_2$-expressing HEK cells or with rat PAR$_2$-expressing KNRK cells (not shown). This result validated further the use of the reverse 2fO peptide to serve as a “control” peptide for the vascular bioassay and for the ligand binding and fluorescence visualization studies.

**Activity of Alexa Fluor 594-2fLI and Other PAR$_2$-Activating Peptides in the Aorta Relaxation Assay.** To validate the Alexa Fluor 594-2fLI for use in the receptor visualization studies, we first wished to measure its biologi-
LIGRLO-NH₂ either after (left-hand trace) or before (right-hand trace) 
pressing KNRK cells was monitored in response to 10⁻¹⁰ M 
NH₂ 
binding, and the relative EC₅₀s for vascular relaxation (RrIC₅₀, RaIC₅₀, and RbEC₅₀, respectively) were calculated using the value for SLIGRL-NH₂ as the reference.

### TABLE 2

Relative IC₅₀s for binding and relative EC₅₀s for relaxation

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Symbol</th>
<th>IC₅₀, Radioligand Binding</th>
<th>IC₅₀, Alexa Fluor 594–2fLI Binding</th>
<th>EC₅₀, Vascular Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLIGRL-NH₂</td>
<td>○</td>
<td>7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>tc-LIGRLO-NH₂</td>
<td>●</td>
<td>7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2-Furoyl-LIGRLO-NH₂</td>
<td>△</td>
<td>0.2 (0.03)</td>
<td>0.2 (0.02)</td>
<td>0.34 (0.2)</td>
</tr>
<tr>
<td>SLIGKV-NH₂</td>
<td>◻</td>
<td>40</td>
<td>5.7</td>
<td>3.5</td>
</tr>
<tr>
<td>SLIGKT-NH₂</td>
<td>□</td>
<td>60</td>
<td>8.6</td>
<td>15 (6.7)</td>
</tr>
<tr>
<td>Alexa Fluor 594–2fLI</td>
<td>○</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>LRGILS-NH₂</td>
<td>▽</td>
<td>Not active</td>
<td>Not active (not active)</td>
<td>Not active</td>
</tr>
<tr>
<td>2-furoyl-OLRGIL-NH₂</td>
<td>△</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not done.

### Visualizing PAR₂ in Receptor-Transfected Cells: Receptor Specificity

As shown in Fig. 5, A and C, incubation activity relative to the parent peptide, 2fLI. Because its fluorescence interferes with measurements using Fluor-3, the Alexa Fluor 594-2fLI could not be assessed for its biological activity in the calcium signaling assay. Therefore, the biological activity of the Alexa Fluor 594-2fLI peptide was evaluated in the aorta relaxation assay, along with the activities of the other PAR₂-APs that were studied concurrently in the binding assay. In the vascular assay, in keeping with previous data (McGuire et al., 2004), the relative relaxant potencies of the peptides were 2-furoyl-LIGRLO-NH₂ ≫ SLIGRL-NH₂ ≫ trans-cinnamoyl-LIGRLO-NH₂ > SLIGKV-NH₂ > SLIGKT-NH₂ (Fig. 4). As for the calcium signaling assay (Fig. 3), the reverse-sequence peptide, 2-furoyl-OLRGIL-NH₂, was neither an agonist nor an antagonist in the vascular assay (not shown). The relaxant activity of Alexa Fluor 594-2fLI was indistinguishable from that of 2fLI itself (Fig. 4, first curve on the left, open diamonds). This order of peptide potencies was the same as what we had observed previously for the calcium signaling responses in PAR₂-expressing KNRRK cells (Al-Ani et al., 1999) and, qualitatively, also reflected the relative IC₅₀s of the peptides in the [³H]2fLI binding assay (Fig. 1; Table 2). The relative biological potencies of the different peptides, normalized to that of SLIGRL-NH₂ (EC₅₀ peptide/EC₅₀ SLIGRL-NH₂: RbEC₅₀), were 1.0:1.0:1.0:1.0:1.0 (Table 2). A value for the RbEC₅₀ greater than 1.0 denotes a peptide with a potency lower than that of SLIGRL-NH₂. These relative biological potencies differed quantitatively from the relative IC₅₀s measured in the radioligand binding assay (RbEC₅₀: 0.03:1.0:1.0:5.7:8.6; Table 2). In keeping with the potent biological activity of the Alexa Fluor 594-2fLI, we also observed that the fluorophore-substituted peptide competed as efficiently for the binding of [³H]propionyl-2fLI as did the PAR₂-activating peptide, 2f-LIGRLO-NH₂, in the ligand binding assay (Table 1, experiment 2). The reverse-sequence Alexa Fluor 594 derivative (Alexa Fluor 594-2fOL) failed to compete for the binding of the radioligand probe (Table 1, last line).
tion of cell monolayers with Alexa Fluor 594-2fLI (50–500 nM) on ice permitted a visualization of the receptor with the same membrane-localized distribution (Fig. 5A, white arrowheads) that we have seen previously in fixed cells by an immunohistochemical approach using our B5 anti-PAR2 antiserum (Al-Ani et al., 1999). It is interesting that when the same experiment was done at room temperature, the fluorescently labeled peptide was observed to internalize into an apparently lysosomal and perinuclear location (Fig. 5B, white arrowheads; data not shown). These results indicated that receptor internalization could be visualized with the Alexa Fluor 594-2fLI probe. However, given that the primary aim of our study was to validate the fluorescent PAR-activating peptide as a ligand probe for PAR2 and not to evaluate receptor dynamics, the precise location of the internalized receptor was not studied in detail. The receptor was visualized only in PAR2-expressing cells and was not labeled either in cells that had been transfected with vector alone (Fig. 5D) or in monolayers that had been pretreated with an excess concentration (20 μM) of the PAR2-selective peptide agonist SLIGRL-NH2 (Fig. 5F) or 2fLI (data not shown). It is significant that the partial reverse sequence PAR2 peptide, LSI-GRL-NH2 (20 μM), that cannot activate PAR2 failed to reduce the binding of Alexa Fluor 594-2fLI (Fig. 5E). Likewise, the reverse sequence PAR2-inactive peptide, 2-furoyl-OL-RGIL-NH2, did not compete for the binding of the fluorescently labeled probe (Fig. 6).

**Binding-Competition Curves for Unlabeled Ligands Measured Using Alexa Fluor 594-2fLI Fluorescence.** To establish further the receptor selectivity of the Alexa Fluor 594-2fLI, we evaluated the relative potencies of the parent peptide, 2fLI and SLIGRL-NH2, for competing with the binding of the Alexa Fluor 594 derivative using a fluorescence readout. Binding competition was quantified by measuring the reduction in fluorescence caused by increasing concentrations of nonfluorescent peptides, as outlined under Materials and Methods. As shown by the binding-competition curves in Fig. 6 and the summarized data in Table 2, the relative IC50s for the ability of the unlabeled peptides to reduce the fluorescence signal qualitatively reflected accurately 1) their relative ability to compete (or not) for the binding of [3H]2fLI and 2) their relative biological activities in the vascular relaxation assay (Fig. 4). In KNRK cells that were transfected with empty vector, the very low fluorescence signal that could be observed was not competed for by the nonfluorescent peptide (Fig. 6, open circles at top) and was thus considered to represent a low level of nonspecific binding. Thus, the receptor specificity of binding of Alexa Fluor 594-2fLI was established unequivocally with the use of the PAR2-nonexpressing cells and by the binding-competition data (competition with 2fLI and SLIGRL-NH2 but not by LSIGRL-NH2 or 2-furoyl-OLRGIL-NH2).

**Visualizing PAR2 in Vivo.** Having validated the Alexa Fluor 594-2fLI receptor probe in the cultured KNRK cells expressing PAR2, we next turned to its use to visualize the receptor in vivo. One hour after its intracolonic administration (a time in keeping with the beginning of the acute inflammatory response of the colon to a PAR2-activating peptide) (Cenac et al., 2002), the fluorescence signal yielded by the colonic administration of Alexa Fluor 594-2fLI was found primarily in epithelial cells at the villus tips (Fig. 7, top right panel, Alexa-2fLI, white arrowheads). The fluorescent derivative of the reverse peptide sequence (Alexa Fluor 594-2fOL) in general yielded only a minimal signal (Fig. 7, right middle panel, Alexa-2fOL). However, in the paraffin-embedded specimen, a fluorescence signal was observed for the

![Fig. 5. Visualizing PAR2 in KNRK cells with Alexa Fluor 594-2fLI.](image)

![Fig. 6. Competition for Alexa Fluor 594-fLIGRLO-NH2 binding by PAR2-APs. The competition by increasing concentrations of nonfluorescent PAR-APs for the binding of fluorescently labeled Alexa Fluor 594-2-furoyl-LIGRLO-NH2 (Fluo-fLI) was measured as outlined under Materials and Methods. The binding was expressed as a percentage (%Max) of the maximal fluorescence pixels monitored, corrected for background fluorescence observed in the presence of an excess of nonfluorescent 2-furoyl-LIGRLO-NH2.](image)
reverse-sequence peptide (2fOL) in the muscular layer. This signal was not observed in the OCT-processed sections (data not shown) and was presumed to represent nonspecific uptake of the fluorescent probe. The fluorescence signal of Alexa Fluor 594-2fLI at the villus tips was clearly diminished when the fluorescent receptor probe was administered along with a 10-fold molar excess of nonfluorescent 2fLI (Fig. 7, bottom right panel, Competition). Morphometric analysis of the fluorescence signal in the area of the epithelial cells (as done for the Alexa Fluor 594-labeled KNRK cells) showed a reduction of approximately 60% in the signal when the 10-fold excess of unlabeled peptide was added along with the fluorescent probe (Fig. 7, histograms, bottom panel, Competition). Because of the marked inflammatory effects of 2fLI and its derivatives in the mouse colon at 1 h (data not shown), we were not able to use a larger excess (e.g., 100-fold) of the nonfluorescent 2fLI. This reduction in fluorescence in the presence of unlabeled 2fLI shown for a representative experiment in Fig. 7 (Fig. 7, histogram on right) ranged from approximately a 40 to 80% competition by the unlabeled peptide (data not shown). Only a very low fluorescence signal in the epithelial cell area was observed for the reverse-sequence receptor-inactive probe, Alexa Fluor 594-2fOL (Fig. 7, middle histogram, bottom panel). The tissues visualized by the hematoxylin-eosin staining procedure (Fig. 7, left panels) show the location of the fluorescence in relation to the intestinal lumen and in relation to the different intestinal cells.

**Discussion**

Receptor Selectivity and Utility of Derivatized 2-Furoyl-LIGRLO-NH₂ for Monitoring PAR₂. The goal of our study was to validate the derivatized PAR₂ ligand, 2-furoyl-LIGRLO-NH₂, as a receptor probe. The main finding of our study was that the N-acetylated, ornithine-substituted derivatives of this PAR₂ agonist are potent and selective ligand probes for monitoring the receptor by ligand binding and fluorescence microscopy approaches. Both as a ligand for binding studies and as a fluorescent receptor probe, the ornithine-substituted analogs of 2fLI displayed the ligand specificity expected for PAR₂: 1) in terms of the competition for binding of the ornithine-substituted derivatives by recognized PAR₂-activating peptides, with an order of affinities (IC₅₀(8)) of 2-furoyl-LIGRLO-NH₂ >> SLIGRL-NH₂ = trans-cinnamoyl-LIGRLO-NH₂ > SLIKGKV-NH₂ > SLIKGT-NH₂ >> TFLLR-NH₂ that was the same as that for the EC₅₀(8) for activation of PAR₂ in either a calcium signaling or aorta relaxation assay; 2) the lack of binding of the derivatized 2-fLI analogs to KNRK cells that do not express the receptor, in comparison with receptor-expressing cells; 3) the lack of binding competition by the PAR-inactive scrambled sequences, LSIGRL-NH₂ and 2-furoyl-OLRGIL-NH₂; 4) a lack of fluorescence labeling of PAR₂-expressing cells or tissues exposed to the reverse-sequence biologically inactive Alexa Fluor 594-2fOL; and 5) the reduced fluorescence signal from the cultured PAR₂-expressing KNRK cells or the in vivo colon tissues exposed to the active fluorescent receptor probe combined with an excess of nonfluorescent 2-fLI that would compete for receptor binding. It is noteworthy that the Alexa Fluor 594-2fLI derivative demonstrated a biological potency in the aorta relaxation assay that was the same as that of unsubstituted 2fLI and that the derivatized peptide was able to compete effectively for the binding of radiolabeled 2fLI to the PAR₂-expressing KNRK cells. These data demonstrated that the ornithine side chain can be substituted with a relatively large substituent without affecting the biological activity or receptor binding activity of the peptide sequence. Furthermore, qualitatively, the relative potencies (IC₅₀(8)) of 2fLI and SLIGRL-NH₂ for reducing the binding of the fluorescent receptor probe were exactly in agreement with their relative potencies in the vascular and [³H]2fLI binding assays. Thus, we anticipate that this fluorescent PAR₂ probe will prove to be of value for visualizing the receptor in intact tissues in a variety of settings and to follow the dynamics and locations of receptor internalization.

It can be pointed out that, quantitatively, the relative IC₅₀(8) of the peptide agonists in the radioligand binding assay, normalized to the IC₅₀(8) of SLIGRL-NH₂ = 1.0 (2-furoyl-LIGRLO-NH₂, SLIGRL-NH₂, trans-cinnamoyl-LIGRLO-NH₂, SLIKGKV-NH₂).
SLIGKT-NH₂:: 0.03:1.0:0.1:5.7:8.6) (Table 2, R_EC50 values) did not match exactly the relative EC₅₀ of the same peptides in the vascular bioassay (Table 2, R_EC50 values), normalized to the EC₅₀ of SLIGRL-NH₂ = 1.0 (0.2:1.0:1.0:1.9:6.7). This quantitative difference in the relative values (Table 2, R_EC50 versus R_EC50 values) between the binding IC₅₀ and bioassay EC₅₀ points to differences in the relative peptide intrinsic activities/efficacies, as defined by Stephenson (1956) and Ariens et al. (1957), in the bioassay. The distinct intrinsic activities/efficacies would not be reflected by the radioligand binding assay. These potential differences between the agonist peptides in terms of their bioassay efficacies merit study in the future.

It is clear that the apical aspect of the intestinal colonic villus epithelial cells was labeled in vivo, as we have found previously using an anti-receptor antibody probe (Kong et al., 1997; Cenac et al., 2002; Nguyen et al., 2003). Future studies could investigate the detailed expression of PAR2 with this fluorescent probe at different time points after its intraluminal administration and correlate these observations with the appearance of pathophysiologic features, such as altered smooth muscle contractility, enteric nervous system activation, increased intestinal permeability, or generation of hyper-sensitivity (Cenac et al., 2007). Whereas the effects of administering PAR₂-activating peptides in vivo have been studied in a variety of different organs (including the intestine, the lungs, the heart, the liver, and the musculoskeletal system), one important question still remains unanswered in those in vivo models. Which cell types are the target for PAR₂ activation and the resulting pathophysiological consequences? The use of the probe we have developed in the present study should be a valuable tool to answer this question.

Although the Alexa Fluor 594-2fLI or [3H]2fLI probes might in principle bind to non-PAR₂ sites, the “net” receptor signal (either binding or fluorescence) calculated by subtracting from the signal detected in the absence of SLIGRL-NH₂ or 2-fLI, the signal observed in the presence of a large molar excess of a nonlabeled PAR₂ agonist, can be taken to reflect accurately the presence of PAR₂ in tissues or cells of interest (Fig. 7, histogram). Our new data complement and support a study that appeared upon completion of our work (Kanke et al., 2006), demonstrating the utility of a [3H]-labeled derivative of 2-furoyl-LIGRL-NH₂ to serve as a ligand binding reagent for PAR₂. Our data indicate that the affinity and selectivity of the ornithine-substituted derivatives (both trimethylated and labeled with Alexa Fluor 594) are essentially equivalent to that of [3H]-labeled 2-furoyl-LIGRL-NH₂. In that previous study, the radioligand was synthesized by the catalytic introduction of [H] into the leucine side chains of dehydro-Leu-2-furoyl-LIGRL-NH₂. Our data indicate that the introduction of a substituent on the side-chain amino group of the ornithine in 2-fLI does not alter the receptor selectivity of the ligand and yields a receptor probe equivalent to [3H]2-furoyl-LIGRL-NH₂ via a more versatile synthetic route that will be more accessible to most laboratories than the tritium gas-palladium catalysis procedure. Our curve-fitting analysis of the binding isotherm (Fig. 2) reveals a plateau in binding in the middle of the curve (between approximately 50 and 120 nM: Fig. 2), indicating a lower capacity binding site with a somewhat higher affinity (approximately 50 nM) than the one previously reported (120 nM) (Kanke et al., 2006). The higher affinity site we report here was not observed by Kanke et al., (2006). Our finding of curvilinearity of the Scatchard plot at the extremes of the concentration range that we used was not explored further. However, to us, the curvilinearity sounds a cautionary note in accepting the Scatchard plot analysis uncritically, without a more in-depth evaluation than what was warranted by our study and aimed mainly at validating the receptor probe. We chose to show the direct binding isotherm deconstructed by simple curve fitting as evidence for the two-site binding curve to emphasize its nature but not to reinterpret the data as possibly done using a detailed mathematical analysis. Because binding at both relatively low (i.e., below 100 nM) and high (i.e., in the micromolar range) concentrations of radioligand were competed for by the unlabeled ligand (i.e., ligand-specific), the data may well reflect the multiplicity states that a G-protein-coupled receptor can exhibit, depending on its effector interactions (Cuatrecasas and Hollenberg, 1976; DeLean et al., 1978). We suggest that other ornithine-derivatized analogs of 2fLI (e.g., biotinylated or affinity column-coupled) will display a receptor affinity and selectivity comparable to the two derivatives we describe here as “proof of principle.” Thus, labeling the peptide with an iodinated acylating reagent, according to the procedure described by Bolton and Hunter (1973) could in principle yield a PAR₂ probe suitable for examining the higher affinity binding site that we have observed in more depth. Furthermore, the ornithine-derivatized peptide could provide for the synthesis of useful receptor affinity columns. Given the binding of the PAR₂-activating peptides to receptor sites that are in some ways distinct from those that bind the tethered ligand (Al-ANI et al., 2004), we predict that the 2fLI probes we describe here will interact both with the nascent and proteolytically activated form of PAR₂. Our continuing work is directed to testing this hypothesis.

Conclusion. The ornithine-derivatized PAR₂-activating peptides that we describe provide a number of advantages over the ligand binding probe prepared by catalytic substitution of dehydro-leucine with tritium. The ornithine-substituted PAR₂ probes are not only more readily synthesized than those prepared by catalytic tritium substitution but our synthesis platform is quite versatile in providing for the preparation of a variety of different receptor probes, two of which we illustrate in this series of experiments. This principle can be used for the development for other receptor probes, for example, to study chemokines receptors like CXCR3 (Vergote et al., 2006). It is clear that the PAR₂-targeted reagents with ornithine substitutions yield ligands that are PAR₂-specific and of sufficiently high affinity to be used in ligand binding and receptor visualization studies. Our data show that the receptor binding of such ornithine-substituted ligands to PAR₂ can be assessed by the usual binding competition paradigms that have been generalized for measurements of receptor binding in other systems (Hollenberg and Cuatrecasas, 1976). We suggest that the reagents and synthesis platform we describe will be of value, not only for the design of PAR₂-targeted reagents but also for the synthesis of comparable receptor probes for PAR₁ and 4. It is our intent to use such reagents for the further study of the molecular pharmacology and physiology of PAR₂ in both cultured cell and intact tissue settings in vivo.
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