Structure-Activity Study of LVV-Hemorphin-7: Angiotensin AT₄ Receptor Ligand and Inhibitor of Insulin-Regulated Aminopeptidase

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ABSTRACT

The decapeptide LVV-hemorphin-7 binds with high affinity to the angiotensin IV (Ang IV) receptor (AT₄ receptor), eliciting a number of physiological effects, including cellular proliferation and memory enhancement. We have recently shown that the AT₄ receptor is identical to insulin-regulated aminopeptidase (IRAP) and that both LVV-hemorphin-7 and Ang IV inhibit the catalytic activity of IRAP. In the current study, a series of alanine-substituted and N- or C-terminally modified analogs of LVV-hemorphin-7 were evaluated for their abilities to compete with 125I-Ang IV binding in sheep adrenal and cerebellar membranes. Selected analogs were also analyzed for binding to recombinant human IRAP and inhibition of IRAP aminopeptidase activity. C-Terminal deletions of LVV-hemorphin-7 resulted in modest changes in affinity for IRAP, whereas deletion of the first three N-terminal residues abolished binding. Mono-substitutions of Tyr⁴ and Trp⁶ with alanine resulted in a 10-fold reduction in affinity. Competition binding studies using recombinant human IRAP demonstrated the same rank order of affinity as obtained for the ovine tissues. All LVV-hemorphin-7 analogs tested, except for Leu-Val-Val-Tyr, inhibit the cleavage of the synthetic substrate, leucine β-naphthylamide, by IRAP, with Kᵢ values between 56 and 620 nM. We find that the Val⁸ residue is crucial for LVV-hemorphin-7 binding to IRAP, whereas the C-terminal domain seems to play a minor role. The current study highlights the minimal residues necessary for binding and inhibition of IRAP and provides a basis to design peptidomimetic analogs for experimental and potentially clinical use.

A range of physiological functions are associated with Ang IV, including the facilitation of memory (Braszko et al., 1988; Wright et al., 1993, 1999), modulation of sodium uptake in the kidney (Hamilton et al., 2001), and vasodilatory effects (Haberl et al., 1991; Kramar et al., 1997, 1998). These actions are mediated by a specific binding site that has been termed the AT₄ receptor. We previously isolated an alternative AT₄ ligand, LVV-hemorphin-7 (LVVYPWTQRF), from the sheep cerebral cortex using a multistep procedure of reverse-phase and ion-exchange chromatography based on its ability to compete with 125I-Ang IV for the AT₄ receptor (Moeller et al., 1997). LVV-hemorphin-7 shares identical sequence to residues 30 to 39 of sheep β-globin and residues 32 to 41 of the β-, δ-, γ-, and ε-human globin. Various studies have demonstrated that LVV-hemorphin-7 mimics many biological actions of Ang IV. At the cellular level, LVV-hemorphin-7 stimulates DNA synthesis in SK-N-MC cells (Mustafa et al., 2001), whereas in hippocampal slices, the decapeptide enhances the potassium-evoked release of acetylcholine (Lee et al., 2001). We have recently demonstrated that central administration of LVV-hemorphin-7 enhances spatial learning (J. Lee, A. L. Albiston, A. M. Allen, F. A. Mendelsohn, S. E. Ping, G. L. Barrett, M. Murphy, M. J. Morris, S. G. McDowall, and S. Y. Chai, manuscript submitted for publication). We have identified the AT₄ receptor as the transmembrane enzyme insulin-regulated aminopeptidase (IRAP) via mass spectral analysis of tryptic peptides generated from AT₄ receptor purified from bovine adrenal membranes (Albiston et al., 2001). Analysis of the biochemical and pharmacological properties of IRAP confirm that it is the AT₄ receptor. We have also demonstrated that both Ang IV and LVV-hemorphin-7 inhibit the catalytic activity of IRAP, suggesting enzyme inhibition as one mechanism by which AT₄ ligands exert their effects (Albiston et al., 2001). For coherence, we describe the previously

ABBREVIATIONS: Ang IV, angiotensin IV; AT₄, angiotensin IV receptor; IRAP, insulin-regulated aminopeptidase; Leu-β-Na, Leu-β-naphthylamide; HEK, human embryonic kidney.
named AT$_4$ receptor agonists such as Ang IV and LVV-hemorphin-7 as AT$_4$ ligands and the AT$_4$ receptor as IRAP.

IRAP belongs to the M1 family of zinc metallopeptidases that is characterized by the zinc binding motif HEXXH(X)$_{18}$E and the exopeptidase motif GXMEN. IRAP is a type II membrane-spanning protein such that when at the plasma membrane the catalytic site is extracellular (Keller et al., 1995). The enzyme was initially defined as specifically cleaving the N-terminal amino acid CysXaa-, in which the half-cystine residue is involved in a disulfide loop, notably in oxytocin or vasopressin, but in vitro has also been demonstrated to cleave a range of peptides not containing disulfide loops (Matsumoto and Mori, 1998; Matsumoto et al., 2000). Our preliminary studies indicate that AT$_4$ ligands are not cleaved by IRAP (R. A. Low, T. Mustafa, S. Ye, S. G. McDowall, S. Y. Chai, and A. L. Albiston, manuscript submitted for publication).

Considering the wide-ranging effects mediated by AT$_4$ ligands, an understanding of the structural requirements for the ligand-enzyme interaction will be beneficial for the design of metabolically stable inhibitors of IRAP. The critical amino acids required for Ang IV binding to IRAP have been identified (Sardinia et al., 1993, 1994; Krishnan et al., 1999). The presence of an amino-terminal valine, and more precisely, a primary $\alpha$-amine in the $\lambda$-amino acid conformation in position 1, seems to be important in the binding process. Glycine substitutions at positions 1, 2, or 3 of Ang IV greatly reduce affinity for IRAP, whereas substitutions at positions 4, 5, or 6 of Ang IV have little effect (Sardinia et al., 1993). Moreover, N-terminal elongation of Ang IV results in a marked reduction in affinity, whereas C-terminally extended peptides bind to the receptor with an affinity similar to that of the native ligand (Sardinia et al., 1993). Thus, the N-terminal residues of the Ang IV peptide are critical for receptor binding, whereas the C-terminal portion plays a less critical role.

Interestingly, despite a similar binding affinity for IRAP, LVV-hemorphin-7 (LVVYPWTQRF) shares little sequence homology to Ang IV (VYIHPF). Considering that this biologically active peptide is more stable than Ang IV (Moeller et al., 1999), LVV-hemorphin-7 may be a useful template for the design of peptidomimetics targeting the IRAP protein. In the current study, we set out to determine the structural requirements of LVV-hemorphin-7 binding to IRAP. To achieve this aim, a series of N- and C-terminally modified and alanine-substituted analogs of LVV-hemorphin-7 were screened for their abilities to compete for $^{125}\text{I}$-Ang IV binding in sheep adrenal and cerebellar membranes. Moreover, selected truncated LVV-hemorphin-7 analogs were also analyzed for their ability to bind to and inhibit the recombinant form of human IRAP.

Materials and Methods

Synthesis and Preparation of Peptides

Truncated analogs of LVV-hemorphin-7 and Val-Tyr-Pro-motif extended peptides were synthesized by Mimotopes (Clayton, Victoria, Australia). The N-terminally extended analogs and Ala-substituted analogs of LVV-hemorphin-7 were synthesized in the peptide laboratory at the Howard Florey Institute (University of Melbourne, Parkville, Victoria, Australia), using the continuous flow Fmoc methodology (Wade et al., 2001). Peptides were dissolved in 0.05 M acetic acid and stored as 1 mM stock solutions at $-20^\circ\text{C}$. The fluorescent substrate Leu-$\beta$-naphthylamide (Leu-$\beta$-NA), its cleavage product $\beta$-naphthylamine, and other reagents were purchased from Sigma Chemical (Castle Hill, NSW, Australia).

Tissue Samples

Sheep adrenal glands and cerebellum obtained from the abattoir were frozen in isopentane on dry ice at $-40^\circ\text{C}$ and stored at $-80^\circ\text{C}$.

Expression of Human IRAP in Human Embryonic Kidney (HEK) 293T Cells

HEK293T cells were transiently transfected with either pcI-IRAP (a gift from M. Tsuchimoto, Department of Obstetrics and Gynaecology, Nagoya University School of Medicine, Japan) or empty vector using LipofectAMINE transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Membrane Preparation.

Membranes were prepared as described previously (Mustafa et al., 2001). In brief, tissues and transfected cells were suspended in 50 mM Tris- HCl pH 7.4, homogenized for 10 s, and centrifuged at 600 g for 5 min at 4°C to remove cellular debris. The supernatant was incubated for 20 min at 65°C followed by centrifugation at 50,000 g for 20 min at 20°C. Membranes were resuspended in 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride buffer containing 100 $\mu$M phenylmethylsulfonyl fluoride, 20 $\mu$M bestatin, 100 $\mu$M phenanthroline, and 0.1% bovine serum albumin.

Western Blot Analysis of IRAP in Ovine Adrenal and Cerebellum Membranes

Ovine adrenal and cerebellum crude membranes (200 $\mu$g of total protein) were run on SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a Protran BA nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and immunodetected using an in-house rabbit anti-IRAP polyclonal antibody (raised against amino acids 25–47 of human IRAP). The primary antibody was detected using horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Chemicon International, Temecula, CA); enhanced chemiluminescence was used to detect conjugated horseradish peroxidase activity and was captured using a luminescent image analyzer LAS-1000 plus (FujiFilm, Kanagawa, Japan).

Binding Assays

Competition. Crude membranes (20 $\mu$g for transfected cells and 65 $\mu$g for ovine tissues) of protein were incubated with 0.5 $\mu$Ci/ml of $^{125}$I-Ang IV and increasing concentrations ($10^{-12}$–$10^{-5}$ M) of unlabeled peptide, for 2 h at 37°C. Bound and free radioligand was separated using the standard filtration method as described previously (Moeller et al., 1997). The radioligand binding data were analyzed using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA) to determine the $IC_{50}$ value for each analog. LVV-hemorphin-7 or Ang IV was included for each set of experiments serving as controls.

Saturation. Binding studies were carried out by incubating transfected cell membranes (2 $\mu$g) in the presence of increasing concentrations (1–12,000 pM) of $^{125}$I-Ang IV, and nonspecific binding was determined in the presence of 10 $\mu$M of unlabeled Ang IV; $K_s$ and $B_{max}$ values obtained by Scatchard analysis. $K_s$ values were obtained using the equation $IC_{50} = K_s(1 + [S]/K_s)$.

Enzyme Inhibition Assay

For enzyme activity assays, cell membranes were prepared as described above, omitting EDTA in the harvesting buffer. The membrane pellet was resuspended in 20 mM HEPES, 255 mM sucrose, 100 mM NaCl, pH 7.4, with protease inhibitors (10 $\mu$g/ml aprotinin, 10 $\mu$M leupeptin, 1 $\mu$M pepstatin, and 1 $\mu$M phenylmethylsulfonyl fluoride), snap frozen on dry ice, and stored at $-70^\circ\text{C}$ for up to 3 months.

Aliquots of crude membranes were thawed, centrifuged at 9000g
in a tabletop Microfuge at 4°C for 15 min, and the supernatant discarded. Membranes were resuspended in Tris-buffered saline (25 mM Tris-HCl, 125 mM NaCl, pH 7.4) containing 1% Triton X-100 at a protein concentration of 1 mg/ml, and rotated gently for at least 5 h at 4°C to solubilize membrane proteins. After solubilization, the membranes were pelleted by centrifugation as described above, the supernatant stored at 4°C, and used in assays within 24 h.

IRAP activity was monitored by the increase in fluorescence after cleavage of Leu-β-NA. Assays were performed in black 96-well microtiter plates: each well contained 2 μg of human IRAP-HEK293T-solubilized membrane protein, 25 μM Leu-β-NA, and the peptide of interest in a final volume of 200 μl of Tris-buffered saline. Reactions proceeded at 37°C for 30 min in a thermostated /Max fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA), before reading the fluorescence (λexcitation = 320 nm, λemission = 420 nm). The ability of each peptide to inhibit IRAP was determined over a range of peptide concentrations (0.01–10 μM), with each concentration being assayed in triplicate in two separate experiments.

Inhibitor constants (K_i) for competitive inhibitors were calculated from the relationship IC_{50} = K_i(1 + [S]/K_m), where K_m for Leu-β-NA was previously determined from kinetic experiments to be 32.3 μM (R. A. Lew, T. Mustafa, S. Ye, S. G. McDowall, S. Y. Chai, and A. L. Albiston, manuscript submitted for publication).

**Statistics**

The IC_{50} value for each peptide was determined and expressed as the mean ± S.E.M. (GraphPad Prism; GraphPad Software Inc.). Statistical differences between IC_{50} values for the various peptides were determined by one-way analysis of variance (GraphPad Prism).

### TABLE 1

**Binding affinities (IC_{50}) of truncated analogs of LVV-hemorphin-7 for IRAP**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sheep Adrenal IC_{50} (nM)</th>
<th>Sheep Cerebellum IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (LVV-hemorphin-7)</td>
<td>17.6 ± 6.2</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>C-Terminal deleted peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg</td>
<td>6.8 ± 1.0</td>
<td>10.7 ± 1.1</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln</td>
<td>34.0 ± 5.7</td>
<td>23.2 ± 2.1</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln</td>
<td>8.9 ± 2.3</td>
<td>3.9 ± 1.9</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr</td>
<td>13.3 ± 0.58</td>
<td>5.9 ± 1.9</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro</td>
<td>46.1 ± 13.8</td>
<td>46.0 ± 7.1***</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro</td>
<td>185.9 ± 82.3***</td>
<td>189.6 ± 60.4***</td>
</tr>
<tr>
<td>N-Terminal deleted peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>14.7 ± 3.2</td>
<td>35 ± 6.1</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>0.98 ± 0.3*</td>
<td>0.50 ± 0.1*</td>
</tr>
<tr>
<td>Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>N.D. (&gt;μM)</td>
<td>N.D. (&gt;μM)</td>
</tr>
</tbody>
</table>

N.D., not detectable.

*P < 0.05, **P < 0.01, ***P < 0.001: significantly different from LVV-hemorphin-7

### TABLE 2

**Binding affinities (IC_{50}) of Val-Tyr-Pro-extended analogs for IRAP**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sheep Adrenal IC_{50} (nM)</th>
<th>Sheep Cerebellum IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val-Tyr-Pro-extended peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-Tyr-Pro</td>
<td>N.D. (&gt;μM)</td>
<td>N.D. (&gt;μM)</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp</td>
<td>17.2 ± 4.5*</td>
<td>19.0 ± 3.6*</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr</td>
<td>1.9 ± 0.9</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln-Arg</td>
<td>4.1 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>0.98 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro</td>
<td>46.1 ± 13.8*</td>
<td>46.1 ± 7.1*</td>
</tr>
<tr>
<td>Val-Tyr-Ile-His-Pro-Phe (Ang IV)</td>
<td>2.9 ± 0.8</td>
<td>1.9 ± 0.6</td>
</tr>
</tbody>
</table>

N.D., not detectable.

* Significantly different from Ang IV (P < 0.05).
TABLE 3

Binding affinities (IC<sub>50</sub>) of alanine-substituted LVV-hemorphin-7 analogs for IRAP

Competition binding studies on <sup>125</sup>I-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of alanine-substituted analogs were performed as described under Materials and Methods (n = 3 for each peptide).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sheep Adrenal IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Sheep Cerebellum IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine-substituted peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>26 ± 7.2</td>
<td>18.6 ± 6.5</td>
</tr>
<tr>
<td>Leu-Val-Val-Ala-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>236.4 ± 22.4*</td>
<td>221.7 ± 61.5*</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Ala-Trp-Thr-Gln-Arg-Phe</td>
<td>55.7 ± 15.1</td>
<td>57 ± 21.3</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Ala-Gln-Arg-Phe</td>
<td>273.9 ± 61.1*</td>
<td>140 ± 12.3*</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Ala-Gln-Arg-Phe</td>
<td>19.9 ± 7.7</td>
<td>9.4 ± 3.2</td>
</tr>
</tbody>
</table>

* Significantly different from LVV-hemorphin-7 (P < 0.05).

Fig. 2. Competition binding of <sup>125</sup>I-Ang IV binding to HEK293T cells transfected with pCI-IRAP. Crude membranes were prepared, as described under Materials and Methods, and inhibition of <sup>125</sup>I-Ang IV binding to IRAP by peptides LVV-H<sub>7</sub> (■), LVVYPWT (▲), VYPWTQRF (□), VYPWT (●), LVVY (▲), and VYP (●) was performed. Values are the mean ± S.E.M. of three experiments performed in duplicate. B/Bo × 100 = percentage of available binding sites occupied.

Results

Affinity for Central and Peripheral IRAP Site. Western blot analysis clearly demonstrates a size difference between the sheep cerebellum and adrenal gland IRAPs as has previously been described for both the rat and bovine central and peripheral tissues (Keller et al., 1995; Zhang et al., 1999). The molecular mass of ovine adrenal IRAP is 165 kDa and ovine cerebellum IRAP is 145 kDa (Fig. 1). However, there were no statistically significant differences in the IC<sub>50</sub> values obtained, between the cerebral membranes and adrenal IRAP, for any of the peptides tested (Tables 1-4).

C-Terminal Deletions of LVV-Hemorphin-7. Deletions of the C-terminal residues Phe<sup>10</sup>, Arg<sup>9</sup>, Gln<sup>8</sup>, and Thr<sup>7</sup> (Leu<sup>1</sup>-Val<sup>2</sup>-Val<sup>3</sup>-Tyr<sup>4</sup>-Pro<sup>5</sup>-Trp<sup>6</sup>) from the full-length LVV-hemorphin-7 peptide did not significantly affect their affinities for IRAP, except for a modest decrease in affinity with deletion of the Arg<sup>9</sup> residue in cerebellar membranes. Subsequent removal of the Trp<sup>6</sup> and Pro<sup>5</sup> (Leu<sup>1</sup>-Val<sup>2</sup>-Val<sup>3</sup>-Tyr<sup>4</sup>) resulted in rightward shifts in the competition binding curves for both adrenal and cerebellar IRAP (5-10-fold for Leu<sup>1</sup>-Val<sup>2</sup>-Val<sup>3</sup>-Tyr<sup>4</sup>-Pro<sup>5</sup> and 23-50-fold for Leu<sup>1</sup>-Val<sup>2</sup>-Val<sup>3</sup>-Tyr<sup>4</sup>-Pro<sup>5</sup>-Trp<sup>6</sup>) relative to LVV-hemorphin-7 (P < 0.01) (Table 1).

N-Terminal Deletions of LVV-Hemorphin-7. Deletion of the Leu<sup>1</sup> residue of LVV-hemorphin-7 (Val<sup>2</sup>-Val<sup>3</sup>-Tyr<sup>4</sup>-Pro<sup>5</sup>-Trp<sup>6</sup>-Thr<sup>7</sup>-Gln<sup>8</sup>-Arg<sup>9</sup>-Phe<sup>10</sup>) did not significantly affect its affinity for IRAP (Table 1). Serial deletion of the Val<sup>2</sup> residue (Val<sup>1</sup>-Tyr<sup>4</sup>-Pro<sup>5</sup>-Trp<sup>6</sup>-Thr<sup>7</sup>-Gln<sup>8</sup>-Arg<sup>9</sup>-Phe<sup>10</sup>) resulted in a 10-fold increase in the affinity for IRAP with respect to LVV-hemorphin-7 (P < 0.05). Subsequent removal of the Val<sup>3</sup> residue resulted in abolition of binding to IRAP (Table 1).

Val-Tyr-Pro-Motif Extended Peptides. Previous structure-activity studies with Ang IV revealed that the minimum requirement of binding to IRAP is the tripeptide VYI, which binds to IRAP with weak affinity (IC<sub>50</sub> = 0.48 μM) (Sardina et al., 1993). We therefore investigated the effect of sequential N-terminal or C-terminal extension of the tripeptide Val-Tyr-Pro, using amino acid sequences from LVV-hemorphin-7. In both the sheep adrenal and cerebellar membranes, Val-Tyr-Pro failed to compete for <sup>125</sup>I-Ang IV binding even at concentrations of >10 μM. The addition of Trp to the C terminus of the peptide (Val-Tyr-Pro-Trp) increased the affinity in both adrenal and cerebellar membranes (IC<sub>50</sub> = 17.2 and 19.0 nM, respectively) (Table 2). Subsequent addition of a Thr residue (Val-Tyr-Pro-Trp-Thr) increased the affinity by a further 10-fold (IC<sub>50</sub> = 1.9 and 1.1 nM, respectively), with no further increase with addition of the last three residues, Gln, Arg, and Phe. Addition of both Leu and Val to the N terminus of Val-Tyr-Pro (Leu-Val-Tyr-Pro) increased the

TABLE 4

K<sub>i</sub> values obtained for enzyme inhibition and competition binding of LVV-hemorphin-7 analogs with recombinant human IRAP

Competition binding studies on [125]<sup>I</sup>-Ang IV binding to crude membranes in the presence of LVV-H<sub>7</sub> analogs was performed as described under Materials and Methods. IRAP enzymatic activity was determined by the hydrolysis of the synthetic substrate, Leu-β-NA in the presence or absence of LVV-H<sub>7</sub> analogs (n = 3 for each peptide).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Enzyme Inhibition K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
<th>Competition Binding K&lt;sub&gt;i&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe</td>
<td>56</td>
<td>1.0</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Trp-Thr</td>
<td>112</td>
<td>11</td>
</tr>
<tr>
<td>LVV-hemorphin-7</td>
<td>196</td>
<td>55</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr-Pro-Trp-Thr</td>
<td>560</td>
<td>73</td>
</tr>
<tr>
<td>Val-Tyr-Pro</td>
<td>620</td>
<td>3100</td>
</tr>
<tr>
<td>Leu-Val-Val-Tyr</td>
<td>N.D.</td>
<td>3400</td>
</tr>
</tbody>
</table>

N.D., not detected.
Affinity to 46 nM in both adrenal and cerebellar membranes, respectively (Table 2).

Alanine-Substitution of LVV-Hemorphin-7. To determine the importance of specific residues at defined positions, residues 4 to 7 from LVV-hemorphin-7 were monosubstituted with alanine. Substitution of the Tyr⁴ and Trp⁶ residues with Ala resulted in a 10-fold decrease in affinity with respect to the parent peptide (P < 0.05) (Table 3). However, the replacement of the Pro⁵ and Thr⁷ residues had little effect on the peptide's affinity for the IRAP.

Analysis of LVV-Hemorphin-7 Analogs with Recombinant Human IRAP. Saturation binding studies using ¹²⁵I-Ang IV indicate that the IRAP-transfected cells contain a high-affinity Ang IV binding site with $K_d = 1.8$ nM and $B_{max} = 5000$ fmol/mg. Competition binding studies using recombinant human IRAP and selected LVV-hemorphin-7 analogs demonstrated the same rank order of affinity as obtained for the ovine tissues (Fig. 2). IRAP $K_i$ values for the peptides were calculated (Table 4) and ranged between 1 and 3500 nM. The $K_i$ (human IRAP) and $IC_{50}$ (sheep IRAP) values for Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe are nearly the same (1.0 nM), whereas for the other truncated LVV-hemorphin-7 peptides the $K_i$ (human IRAP) values obtained are higher than the $IC_{50}$ (sheep IRAP) values (Tables 1 and 2). Therefore, although the rank order of the affinity of the selected LVV-hemorphin-7 analogs is the same for human IRAP and ovine IRAP, the relative affinities differ. This may reflect species-specific differences in the affinity of the different peptides for IRAP. All of the LVV-hemorphin-7 analogs tested, except for Leu-Val-Val-Tyr, inhibit the cleavage of the synthetic substrate Leu-β-NA, by IRAP, with $K_i$ values between 56 and 620 nM (Fig. 3; Table 4).

Discussion

The current study aims to delineate the structural requirements for LVV-hemorphin-7 binding to IRAP and thus to extend the knowledge on the ligand-enzyme interaction in this system. This was achieved by initially screening a series of N- and C-terminally modified analogs of the decapptide for their ability to competitively inhibit the binding of ¹²⁵I-Ang IV to sheep adrenal and cerebellar membranes. IRAP in the central nervous system is approximately 10% smaller compared with IRAP from peripheral tissues (Keller et al., 1995; Zhang et al., 1998). It has been suggested that in part this difference in size may be due to differential glycosylation. In this study, we demonstrated that in ovine tissues the same variation occurs, adrenal IRAP is 165 kDa and cerebellum IRAP is 145 kDa. The results from this study did not identify selectivity for either central or peripheral IRAP binding sites.

The results from the N-terminal deletion studies indicate that the Val³ residue of LVV-hemorphin-7 is crucial for interaction with IRAP, because deletion of this residue completely abolishes binding to IRAP. Along these lines, Garreau et al. (1998) investigated the ability of LVV-hemorphin-7 and related peptides to inhibit ¹²⁵I-Ang IV binding in collecting duct principal cell membranes. They reported that the most potent competitors were LVV-hemorphin-7 and VV-hemorphin-7 (1.3 nM), whereas hemorphin-7 (YPWTQRF) failed to compete for ¹²⁵I-Ang IV binding sites. Similarly, deletion or substitution of the Val¹ residue from Ang IV significantly reduced its affinity for IRAP (Sardinia et al., 1993). Sardinia et al. (1994) suggested that the hydrophobic nature of the Val residue is necessary for Ang IV to bind to IRAP with high affinity. Indeed, a hydrophobic amino acid, Ile, in position 1 increased affinity, whereas the charged amino acid Glu decreased affinity for IRAP (Sardinia et al., 1994).

Because IRAP is an aminopeptidase, it is less surprising that the C-terminal residues do not seem to play an important role in the determination of the ligands affinity for IRAP. Deletion of the last four C-terminal residues of LVV-hemorphin-7 (T⁷Q⁸R⁹F¹⁰) do not significantly affect the pep-
tide's affinity for IRAP. In line with this, substitutions, deletions, or extensions of the C-terminal residues of Ang IV had little to modest effects on IRAP binding (Sardinia et al., 1993). These results support the notion that the N-terminal residues primarily determine the affinity of a ligand for IRAP.

To determine the influence of amino acid side chains on the ligand-enzyme interaction, a selected group of residues (Tyr-Pro-Trp-Thr) from LVV-hemorphin-7 were substituted with alanine. Substitution of either Tyr or Trp with alanine results in a significant decrease in affinity, suggesting that these aromatic amino acids play a role in determining affinity for IRAP. The Tyr residue in Ang IV is important for IRAP binding, due to its hydrophobic nature and planar geometry (Krishnan et al., 1999).

Sardinia et al. (1993) demonstrated that the tripeptide YVI is the minimum requirement for Ang IV binding to IRAP. Ang IV and LVV-hemorphin-7 sequence both share the sequence VY in the N terminus. This led us to investigate the binding of the tripeptide VYP, derived from the LVVYP-WTQR sequence, to IRAP. The VYP peptide displayed poor affinity for IRAP in both cerebellar and adrenal membranes. However, the addition of the hydrophobic amino acid Trp to the C terminus of VYP (VYPW) improves the affinity significantly. Taken together, the presence of a hydrophobic amino acid at the C-terminus end of VYP may be important for high-affinity binding. In support of this, a hydrophobic amino acid is required at position three of Ang IV to achieve high-affinity binding (Krishnan et al., 1999). Alternatively, the addition of the Leu-Val residues to the N terminus of VYP peptide also improves binding significantly. Thus, in the absence of the C-terminal residues, the Leu-Val amino acids may be important for binding to IRAP, possibly by altering the tertiary conformation of the VYP peptide to maximize peptide-enzyme interaction.

A limitation of competition binding studies to delineate the structural requirements for high-affinity binding to IRAP (ATR receptor) is that they are performed in the presence of chelating agents (phenanthroline and/or EDTA), whereas in vivo IRAP, a zinc metalloprotease, is present with a bound zinc. Therefore, the enzyme inhibition assay is a useful system to assess the structural requirements for high-affinity binding of AT4 ligands to IRAP in a biologically relevant context. AT4 ligands, including LVV-hemorphin-7, are not substrates of IRAP, because we have recently demonstrated that HEK293T cells transfected with IRAP do not degrade these peptides beyond the level observed for mock-transfected cells (<15% over 4 h) (R. A. Lew, T. Mustafa, S. Y. Chai, and A. L. Albiston, unpublished data). LVV-hemorphin-7 and the five selected truncated analogs inhibited the aminopeptidase activity of IRAP in the same rank order as obtained for competition binding. However, the Ki values obtained for the truncated LVV-hemorphin-7 analogs using the enzyme inhibition and the competition binding assays differed markedly. The Ki values obtained from the enzyme inhibition assay were up to 100-fold lower compared with values obtained from the competition binding assay. Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe is the most potent peptide in both the enzyme inhibition and the competition binding assays had Ki values of 56 and 1 nM determined from the respective assays. The differences in the Ki values obtained from the two assays is likely to be due to the presence or absence of zinc bound to IRAP altering the affinity of the LVV-hemorphin-7 analogs.

Beyond the prerequisite for a free N terminus, the requirements for substrate binding to IRAP are difficult to define. The enzyme has previously been defined as specifically cleaving the N-terminal amino acid CysXaa-, in which the half-cystine residue is involved in a disulfide loop, notably in oxytocin, vasopressin, and somatostatin (Herbst et al., 1997). N-Terminal cysteine residues seem to be the preferential targets for the enzyme; however, other peptides that possess N-terminal cysteine residues and intramolecular disulfide bonds, such as calcitonin and endothelin, are not cleaved by the enzyme. Other peptides that are readily cleaved by IRAP include Lys-bradykinin, met-enkephalin, dynorphin A, neuropeptide A, and neuromedin B (Herbst et al., 1997), which possesses a range of N-terminal residues. In contrast to AT4 ligands the affinities of such substrates are in the mid-micromolar range, as is common with peptidases.

In conclusion, we have demonstrated that the Val residue is crucial for LVV-hemorphin-7 binding to IRAP. This observation is in keeping with the suggestion that a hydrophobic amino acid is required at the N terminus for high-affinity binding to IRAP. In contrast, the C-terminal domain of LVV-hemorphin-7 does not seem to play an important role in determining ligands affinity for IRAP. The results from the current study indicate that the minimal sequence required for high-affinity binding and inhibition of IRAP is VYWP. Modification of this truncated analog of LVV-hemorphin-7, using systematic cyclization and bicyclization, may yield specific, potent inhibitor(s) of IRAP.

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References


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