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STRUCTURE-ACTIVITY STUDY OF LVV-HEMORPHIN-7: ANGIOTENSIN AT_4 RECEPTOR LIGAND AND INHIBITOR OF INSULIN-REGULATED AMINOPEPTIDASE (IRAP)

Joohyung Lee*, Tomris Mustafa*, Sharon G. McDowall, Frederick A.O. Mendelsohn, Michelle Brennan, Rebecca A. Lew, Anthony L Albiston, and Siew Yeen Chai.

Howard Florey Institute (J.l., T.M., S.G.M., F.A.O.M. M.B. A.L.A. and S.Y.C.) and Department of Pharmacology (J.L.), University of Melbourne, Parkville, Victoria, 3010, Australia and Baker Heart Research Institute, Melbourne, Victoria 8008, Australia (R.A.L.)

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Address correspondence: Dr Siew Yeen Chai

Howard Florey Institute of Experimental Physiology and Medicine,

University of Melbourne, Parkville, Victoria, 3010, Australia

Tel: +61 3 8344 7332

Fax: +61 3 9348 1707

Email: sychai@hfi.unimelb.edu.au

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Abstract

The decapeptide LVV-hemorphin-7 binds with high affinity to the angiotensin IV (Ang IV) receptor (AT_4 receptor), eliciting a number of physiological effects, including cellular proliferation and memory enhancement. We have recently shown that the AT_4 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 analogues of LVV-hemorphin-7 were evaluated for their abilities to compete for ^{125}I-Ang IV binding in sheep adrenal and cerebellar membranes. Selected analogues were also analysed 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, whilst deletion of the first three N-terminal residues abolished binding. Mono-substitutions of Tyr^4 and Trp^6 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 analogues tested, except for Leu-Val-Val-Tyr, inhibit the cleavage of the synthetic substrate, leucine β-naphthylamide, by IRAP, with Ki values between 56 nM and 620 nM. We find that the Val^3 residue is crucial for LVV-hemorphin-7 binding to IRAP whilst the C-terminal domain appear to play a minor role. The current study highlights the minimal residues necessary for binding and inhibition of IRAP, and provide a basis to design peptidomimetic analogues 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; Wright *et al*., 1999), modulation of sodium uptake in the kidney (Hamilton *et al*., 2001) and vasodilatory effects (Haberl *et al*., 1991; Kramar *et al*., 1997; Kramar *et al*., 1998). These actions are mediated by a specific binding site that has been termed the AT_4 receptor. We previously isolated an alternative AT_4 ligand, LVV-hemorphin-7 (LVVYPWTQRF), from the sheep cerebral cortex using a multi-step procedure of reverse-phase and ion-exchange chromatography based on its ability to compete with [^{125}I]-Ang IV for the AT_4 receptor (Moeller *et al*., 1997). LVV-hemorphin-7 shares identical sequence to residues 30-39 of sheep β-globin and residues 32-41 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), while 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 (Lee et al. 2002, Submitted for publication).

We have recently identified the AT_4 receptor as the transmembrane enzyme insulin regulated aminopeptidase (IRAP) via mass spectral analysis of tryptic peptides generated from AT_4 receptor purified from bovine adrenal membranes (Albiston *et al*., 2001). Analysis of the biochemical and pharmacological properties of IRAP confirm that it is the AT_4 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 AT_4 ligands exert their effects (Albiston *et al*., 2001). For coherence we will describe the previously 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 characterised by a zinc binding motif $HEXXH(X)_{18}$-E and an exopeptidase motif GXMEN. IRAP is a type II membrane-spanning protein such that when at the plasma membrane the catalytic site is extra-cellular (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 disulphide loop, notably in oxytocin or vasopressin, but *in vitro* has also been demonstrated to cleave a range of peptides not containing disulphide loops (Matsumoto & Mori, 1998; Matsumoto *et al*., 2000). Our preliminary studies indicate that AT_4 ligands are not cleaved by IRAP(Lew et al submitted).

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 (Krishnan *et al*., 1999; Sardinia *et al*., 1993; Sardinia *et al*., 1994). The presence of an amino-terminal valine, and more precisely, a primary α-amine in the L-amino acid conformation in position 1, appears to be important in the binding process. Glycine substitutions at positions 1, 2 or 3 of Ang IV greatly reduces affinity for IRAP, whereas substitutions at positions 4, 5 or 6 of Ang IV has little effect (Sardinia *et al*., 1993). Moreover, N-terminal elongation of Ang IV results in a marked reduction in affinity, whilst 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, while 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 analogues of LVV-hemorphin-7 were screened for their abilities to compete for ^{125}I-Ang IV binding in sheep adrenal and cerebellar membranes. Moreover, selected truncated LVV-hemorphin-7 analogues were also analysed for their ability to bind to and inhibit the recombinant form of human IRAP.

Materials and Methods

Synthesis and preparation of peptides. Truncated analogues of LVV-hemorphin-7 and Val-Tyr-Pro-motif extended peptides were synthesized by Mimotopes (Clayton, VIC. Australia). The N-terminally extended analogues and Ala-substituted analogues of LVV-hemorphin-7 were synthesised in the peptide laboratory at the Howard Florey Institute, 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^{o}C. The fluorescent substrate, Leu-β-naphthylamide (Leu-β-NA), its cleavage product β-naphthylamine, and other reagents were purchased from Sigma (Castle Hill, NSW, Australia).

Tissue samples. Sheep adrenal glands and cerebellum obtained from the abattoir were frozen in isopentane on dry ice at –40 ^{o}C and stored at –80 ^{o}C.

Expression of hIRAP in HEK-293T cells. HEK 293T cells were transiently transfected with either pCI-IRAP (a gift from M. Tsujimoto) or empty vector using LipofectAMINE transfection reagent (Invitrogen) 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, homogenised for 10 s and centrifuged at 600 *g* for 5 min at 4^{o}C to remove cellular debris. The supernatant was incubated for 20 min at 65 ^{o}C followed by centrifugation at 50,000*g* for 20 min at 20^{o}C. Membranes were resuspended in 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride buffer containing 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin, 100 μM phenanthroline & 0.1% bovine serum albumin.

Western blot analysis of IRAP in ovine adrenal and cerebellum membranes. Ovine adrenal and cerebellum crude membranes (200 μg of total protein) and separated on SDS-PAGE. The resolved proteins were transferred to a Protran® BA nitrocellulose membrane

(Schleicher and Schuell, Germany) and immunodetected using an in house rabbit anti-IRAP polyclonal antibody (Raised against the peptide 25-47 amino acids of human IRAP). The primary antibody was detected using horseradish peroxidase (HRP) conjugated sheep anti-rabbit secondary antibody (Chemicon International, USA) and ECL™ was used to detect conjugated HRP activity and was captured using a Luminescent Image Analyser LAS-1000 plus (FUJIFILM, Japan).

Binding Assays. A/ Competition Crude membranes (20 µg for transfected cells and 65 µg for ovine tissues) of protein were incubated with 0.5 µCi/ml of [^{125}I]-Ang IV and increasing concentrations (10^{-12} – 10^{-5} M) of unlabelled peptide, for 2 h at 37 °C. Bound and free radioligand was separated using the standard filtration method as described (Moeller *et al*., 1997). The radioligand binding data was analysed using the GraphPad Prism program (GraphPad Software Inc., San Diego, U.S.A.) to determine the IC_{50} for each analogue. LVV-hemorphin-7 or Ang IV was included for each set of experiments serving as controls.

B/ Saturation Binding studies were carried out by incubating transfected cell membranes (2 ug) in the presence of increasing concentrations (1-12,000 pM) of [^{125}I]-Ang IV and non specific binding determined in the presence of 10 uM of unlabelled Ang IV; K_d and B_{max} values obtained by Scatchard analysis. K_i values were obtained using the equation $IC_{50} = K_i(1 + [S]/K_d)$.

Enzyme inhibition assay. For enzyme activity assays, cell membranes were prepared as described above omitting the EDTA in the harvesting buffer. The membrane pellet was re-suspended in 20 mM HEPES, 255 mM sucrose, 100 mM NaCl, pH 7.4, with protease inhibitors (10 µg/ml aprotinin, 10 µM, leupeptin 1 µM pepstatin and 1 mM PMSF), snap frozen on dry ice and stored at -70 ^{o}C for up to 3 months.

Aliquots of crude membranes were thawed, centrifuged at 9000 *g* in a tabletop microfuge at 4 °C for 15 min, and the supernatant discarded. Membranes were resuspended in Tris-buffered saline (TBS: 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. Following solubilization, the membranes were pelleted by centrifugation as above, the supernatant stored at 4 °C and used in assays within 24 h.

IRAP activity was monitored by the increase in fluorescence following cleavage of leucine β-naphthylamide (Leu-β-NA). Assays were performed in black 96-well microtiter plates: each well contained 2 µg hIRAP-HEK 293T solubilized membrane protein, 25 µM Leu-β-NA, and the peptide of interest in a final volume of 200 µL TBS. Reactions proceeded at 37°C for 30 min in a thermostatted *f*Max fluorescence microplate reader (Molecular Devices, Sunnyvale, CA), prior to reading the fluorescence ($\lambda_{excitation}$ = 320 nm, $\lambda_{emission}$ = 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 (Lew et al. Submitted).

Statistics. The IC_{50} value for each peptide was determined and expressed as the mean ± SEM (Graphpad Prism, Graphpad Prism Software Inc., San Diego, U.S.A.). Statistical differences between IC_{50} values for the various peptides were determined by one-way analysis of variance (ANOVA) (Graphpad Prism). Where there was a significant effect between LVV-hemorphin-7 and the modified peptide ($P < 0.05$) on ANOVA, Bonferroni's *post hoc* test was used to determine the significance of difference between the two peptides.

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 weight of ovine adrenal IRAP is165 kDa and ovine cerebellum IRAP is 145 kDa (Figure 1). However there were no statistically significant differences in the IC_{50} values obtained, between the cerebellum and adrenal IRAP, for any of the peptides tested (Tables 1 to 4).

C-terminal deletions of LVV-hemorphin-7. Deletions of the C-terminal residues Phe^{10}, Arg^{9}, Gln^{8}, and Thr^{7} (Leu^{1}-Val^{2}-Val^{3}-Tyr^{4}-Pro^{5}-Trp^{6}) 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^{9} residue in cerebellar membranes. Subsequent removal of the Trp^{6} and Pro^{5} (Leu^{1}-Val^{2}-Val^{3}-Tyr^{4}) resulted in rightward shifts in the competition binding curves for both adrenal and cerebellar IRAP (5-10-fold for Leu^{1}-Val^{2}-Val^{3}-Tyr^{4}-Pro^{5} and 23-50-fold for Leu^{1}-Val^{2}-Val^{3}-Tyr^{4}, relative to LVV-hemorphin-7) ($P < 0.01$) (Table 1).

N-terminal deletions of LVV-hemorphin-7. Deletion of the Leu^{1} residue of LVV-hemorphin-7 (Val^{2}-Val^{3}-Tyr^{4}-Pro^{5}-Trp^{6}-Thr^{7}-Gln^{8}-Arg^{9}-Phe^{10}) did not significantly affect its affinity for IRAP (Table 1). Serial deletion of the Val^{2} residue (Val^{3}-Tyr^{4}-Pro^{5}-Trp^{6}-Thr^{7}-Gln^{8}-Arg^{9}-Phe^{10}) 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^{3} 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_{50} = 0.48 μM) (Sardinia *et al*., 1993). We therefore investigated the effect of sequential C-terminal or N-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 ^{125}I-Ang IV binding at (>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_{50} = 17.2 nM 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_{50} = 1.9 nM 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-Val-Tyr-Pro) increased the 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 mono-substituted with alanine. Substitution of the Tyr^4 and Trp^6 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 Pro^5 and Thr^7 residues had little effect on the peptide's affinity for the IRAP.

Analysis of LVV-hemorphin-7 analogues with recombinant human IRAP. Saturation binding studies using [^{125}I]-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}= 5,000 fmol/mg. Competition binding studies using recombinant, human IRAP and selected LVV-hemorphin-7 analogues demonstrated the same rank order of affinity as obtained for the ovine tissues (Figure 2). IRAP K_i values for the peptides were calculated (Table 4) and ranged between 1 and 3,500 nM.. The Ki (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 Ki (human IRAP) values obtained are higher than the IC_{50} (sheep IRAP) values (Table 1 and 2). Therefore although the rank order of the affinity of the selected LVV-hemorphin-7 analogues 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 analogues 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 nM and 620 nM (Figure 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 analogues of the decapeptide for their ability to competitively inhibit the binding of ^{125}I-Ang IV to sheep adrenal and cerebellar membranes. IRAP in the central nervous system is approximately 10% smaller in size compared to 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 150 kDa in size. 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 Val^3 residue of LVV-hemorphin-7 is crucial for interaction with IRAP, as 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 ^{125}I-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 ^{125}I-Ang IV binding sites. Similarly, deletion or substitution of the Val^1 residue from Ang IV significantly reduced its affinity for IRAP (Sardinia *et al*., 1993). Sardinia and colleagues (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).

As IRAP is an aminopeptidase, it is less surprising that the C-terminal residues do not appear 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 ($T^7Q^8R^9F^{10}$) do not significantly affect the peptide'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.

In order to determine the influence of amino acid side chains on the ligand-enzyme interaction, a selected group of residues ($Tyr^4Pro^5Trp^6Thr^7$) from LVV-hemorphin-7 were substituted with alanine. Substitution of either Tyr^4 or Trp^6 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^2 residue in Ang IV is important for IRAP binding, due to its hydrophobic nature and planar geometry (Krishnan *et al*., 1999).

Sardinia and colleagues (1993) demonstrated that the tripeptide VYI 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 LVVYPWTQRF sequence, to IRAP. The VYP peptide displayed poor affinity for IRAP in both cerebellar or 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-terminal 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^1Val^2 residues to the N-terminus of VYP peptide

also improve binding significantly. Thus, in the absence of the C-terminal residues, the Leu^1Val^2 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 (AT_4 receptor) is that they are performed in the presence of chelating agents (phenanthroline and/or EDTA) whereas *in vivo* IRAP, a zinc metalloproteinase, 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 AT_4 ligands to IRAP in a biologically relevant context. AT_4 ligands, including LVV-hemorphin-7, are not substrates of IRAP, as 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 hours) (Lew et al unpublished observations). LVV-hemorphin-7 and the five selected truncated analogues inhibited the aminopeptidase activity of IRAP in the same rank order as obtained for competition binding. However the K_i values obtained for the truncated LVV-hemorphin-7 analogues using the enzyme inhibition and the competition binding assays differ markedly. The Ki values obtained from the enzyme inhibition assay were up to 100 fold lower compared to values obtained from the competition binding assay. Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe the most potent peptide in both the enzyme inhibition and the competition binding assays had Ki values of 56 nM 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 analogues.

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 disulphide loop, notably in oxytocin, vasopressin and somatostatin (Herbst *et al*., 1997),. N-terminal cysteine residues appear to be the preferential targets for the enzyme, however, other peptides that possess N-terminal cysteine residues and intramolecular disulphide 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, neurokinin A and neuromedin B (Herbst *et al*., 1997), which possess a range of N-terminal residues. In contrast to AT_4 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^3 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 appear to play an important role in determining ligand's affinity for IRAP. The results from the current study indicate that the minimal sequence required for high affinity binding and inhibition of IRAP is VYPWT. Modification of this truncated analogue of LVV-hemorphin-7, using systematic cyclization and bicyclization, may yield specific, potent inhibitor(s) of IRAP.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Western blot analysis of IRAP in crude membrane preparations of ovine cerebral (cer) and adrenal (adr) tissues. Crude membrane preparations (200 μg), prepared as described in materials and methods, were submitted to SDS/PAGE, blotted on to nylon membrane and immonodetected with the anti-IRAP antibody and developed with ECL chemiluminiscence.

Figure 2. Competition binding of [^{125}I]-Ang IV binding to HEK293Tcells transfected with PCI-IRAP. Crude membranes were prepared, as described in materials and methods, and inhibition of [^{125}I]-Ang IV binding to IRAP by peptides ■ LVV-H7, ▲ LVVYPWT, VYPWTQRF, ▼ VYPWT, ◆ LVVY, ● VYP performed.. Values are the mean ± of three experiments performed in duplicate. B/Bo x 100 = % of available binding sites occupied.

Figure 3. Inhibition of IRAP catalytic activity by LVV-H7 analogues. Crude membranes were prepared from HEK293T cells transfected with pCI-IRAP. IRAP enzymatic activity was determined by the hydrolysis of the synthetic substrate, Leu-β-NA. The substrate was added to solubilised membrane protein (2 μg) in the presence or absence of LVV-H7 analogues (symbols as for fig 2A) at the indicated concentrations and the fluorescence monitored for 30 mins. Values are the mean ± of three experiments performed in duplicate.

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Peptides	Sheep Adrenal IC_{50} (nM)	Sheep Cerebellum IC_{50} (nM)
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (LVV-hemorphin-7)	17.6 ± 6.2	5.0 ± 0.7
C-terminal deleted peptides		
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg	6.8 ± 1.0	10.7 ± 1.1
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln	34.0 ± 5.7	23.2 ± 2.1
Leu-Val-Val-Tyr-Pro-Trp-Thr	8.2 ± 2.3	3.9 ± 1.9
Leu-Val-Val-Tyr-Pro-Trp	13.3 ± 0.58	5.9 ± 1.9
Leu-Val-Val-Tyr-Pro	46.1 ± 13.8	46.0 ± 7.1 **
Leu-Val-Val-Tyr	185.9 ± 82.3 ***	189.6 ± 60.4 ***
N-terminal deleted peptides		
Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	14.7 ± 3.2	35 ± 6.1
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	0.98 ± 0.3 *	0.50 ± 0.1 *
Tyr-Pro-Trp-Thr-Gln-Arg-Phe	ND (> μM)	ND (> μM)

Table 1 Binding affinities (IC_{50}) of truncated analogues of LVV-hemorphin-7 for IRAP. Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of N-terminal or C-terminal truncated peptides were performed as described in materials and methods. Values are the mean ± of three experiments performed in duplicate.

ND = Not detectable

* ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) Significantly different from LVV-hemorphin-7

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Peptides	Sheep Adrenal IC_{50} (nM)	Sheep Cerebellum IC_{50} (nM)
VAL-TYR-PRO-extended peptides		
Val-Tyr-Pro	ND (> μM)	ND (> μM)
Val-Tyr-Pro-Trp	17.2 ± 4.5 *	19.0 ± 3.6 *
Val-Tyr-Pro-Trp-Thr	1.9 ± 0.9	1.1 ± 0.1
Val-Tyr-Pro-Trp-Thr-Gln	1.2 ± 0.2	1.0 ± 0.1
Val-Tyr-Pro-Trp-Thr-Gln-Arg	4.1 ± 0.5	2.0 ± 0.7
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	0.98 ± 0.3	0.5 ± 0.1
Leu-Val-Val-Tyr-Pro	46.1 ± 13.8 *	46.1 ± 7.1 *
Val-Tyr-Ile-His-Pro-Phe (Ang IV)	2.9 ± 0.8	1.9 ± 0.6

Table 2 Binding affinities (IC_{50}) of Val-Tyr-Pro-extended analogues for IRAP. Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of Val-Tyr-Pro-extended analogues were performed as described in materials and methods (n = 3 for each peptide).

(ND = Not detectable)

* Significantly different from Ang IV ($P < 0.05$)

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Peptides	Sheep Adrenal IC_{50} (nM)	Sheep Cerebellum IC_{50} (nM)
Alanine-substituted peptides		
Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	26 ± 7.2	18.6 ± 6.5
Leu-Val-Val-**Ala**-Pro-Trp-Thr-Gln-Arg-Phe	236.4 ± 22.4 *	221.7 ± 61.5 *
Leu-Val-Val-Tyr-**Ala**-Trp-Thr-Gln-Arg-Phe	55.7 ± 15.1	57 ± 21.3
Leu-Val-Val-Tyr-Pro-**Ala**-Thr-Gln-Arg-Phe	273.9 ± 61.1 *	140 ± 12.3 *
Leu-Val-Val-Tyr-Pro-Trp-**Ala**-Gln-Arg-Phe	19.9 ± 7.7	9.4 ± 3.2

Table 3 Binding affinities (IC_{50}) of alanine-substituted LVV-hemorphin-7 analogues for IRAP. Competition binding studies on [^{125}I]-Ang IV binding to sheep adrenal or cerebellar membranes in the presence of increasing concentrations of alanine-substituted analogues were performed as described in materials and methods (n = 3 for each peptide).

(ND = Not detectable)

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

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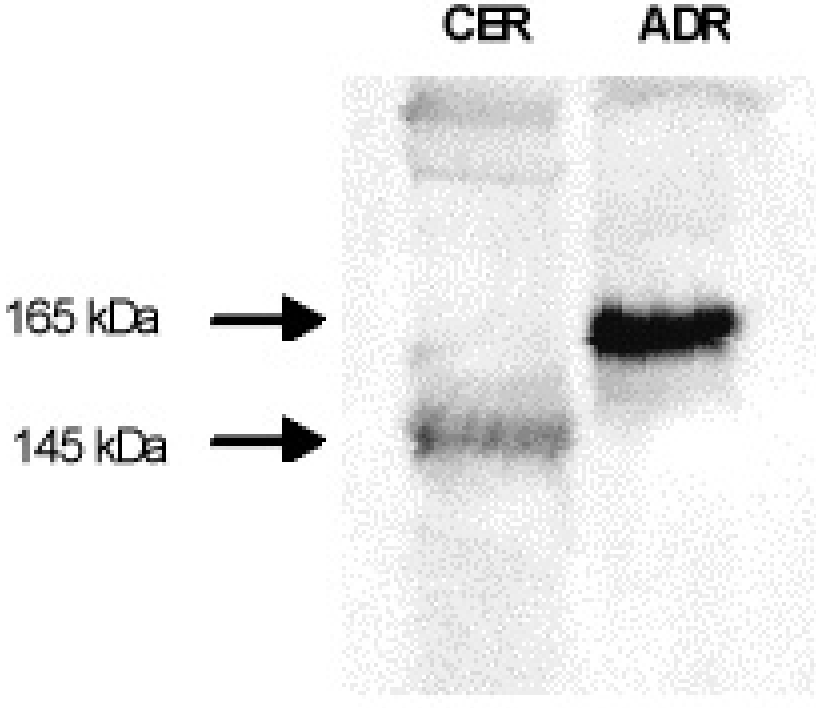
Peptide	Enzyme Inhibition K_i (nM)	Competition binding K_i (nM)
Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	56	1.0
Val-Tyr-Pro-Trp-Thr	112	11
LVV-hemorphin-7	196	55
Leu-Val-Val-Tyr-Pro-Trp-Thr	560	73
Val-Tyr-Pro	620	3,100
Leu-Val-Val-Tyr	ND	3,400

Table 4 K_i values obtained for enzyme inhibition and competition binding of LVV-hemorphin-7 analogues with recombinant human IRAP. ND = Not detected. Competition binding studies on [^{125}I]-Ang IV binding to crude membranes in the presence of LVV-H7 analogues was performed as described in 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-H7 analogues (n = 3 for each peptide).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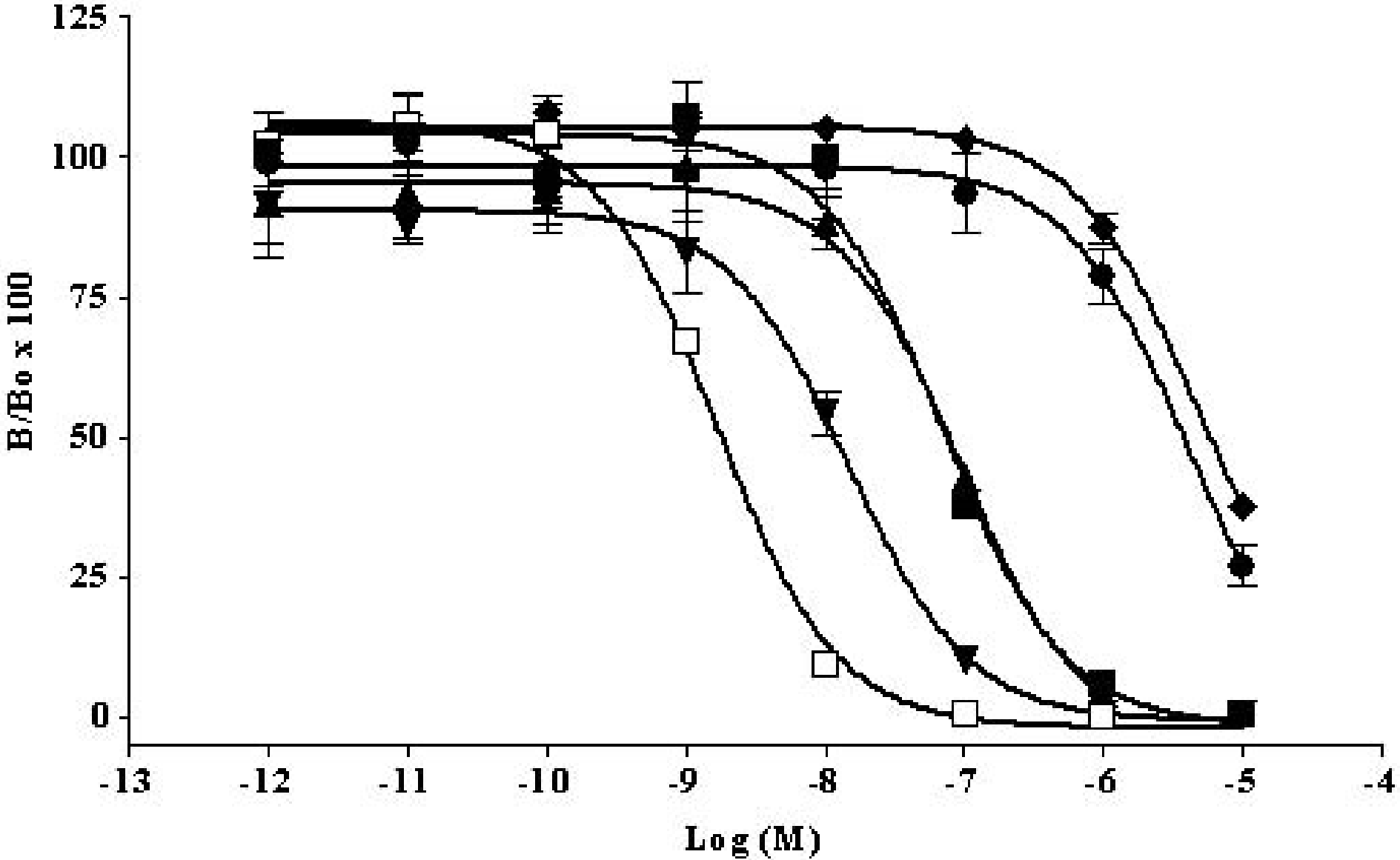
145 kDa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75
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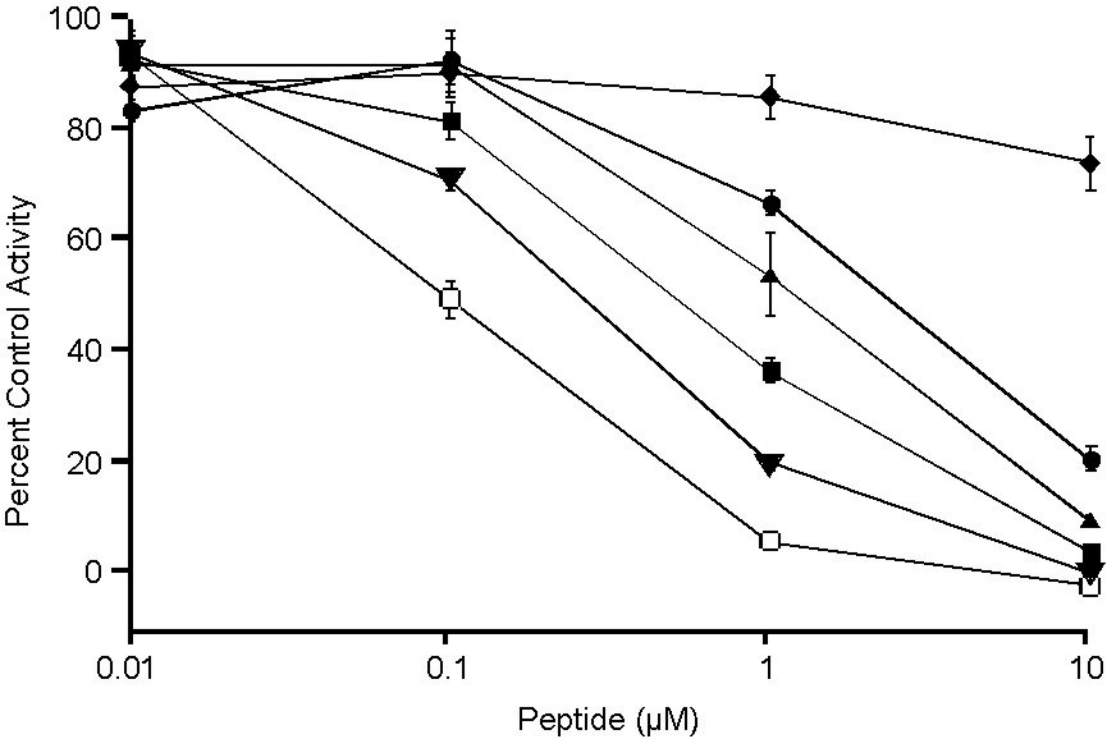
Log (M)


100
80
60
40
20
0
Percent Control Activity
0.01
0.1
1
10
Peptide (μM)