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The N-terminal Hexapeptide Fragment of IGF II Stimulates Thymidine Incorporation into Fibroblasts

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Summary We synthesized the N-terminal hexapeptide fragment of IGF II to study potential binding to NMDA receptors in analogy to the N-terminal tripeptide of IGF I. The amino acid sequence of the hexapeptide is furthermore identical with the C-terminal sequence of the casiragua insulin B chain. The hexapeptide did not bind to the NMDA receptors, but was found to promote [^{3}H]-thymidine incorporation into fibroblasts at concentrations of 10^{-8} - 10^{-5} M in a dose-dependent manner. Since [^{125}I]-hexapeptide did not bind to IGF receptors, indirect competition studies using either labelled IGFs or insulin had to be used. The competition of hexapeptide at a concentration of 10^{-5} M with labelled IGF I or II was about equal to that of 10^{-9} M IGF I or II. IGF receptors were apparently up-regulated by the hexapeptide, as has also been described for insulin. When using casiragua insulin as labelled ligand, IGF II and casiragua insulin competed with equal potency, whereas the hexapeptide at 10^{-7} M caused an apparent up-regulation of the casiragua insulin binding sites. Our results that the hexapeptide stimulates [^{3}H]-thymidine incorporation and up-regulates IGF II and casiragua insulin binding sites may be connected to one or several of the following findings: the hystricomorph insulins - of which the casiragua insulin is a member - stimulate DNA synthesis to a greater extent than other insulins; the insulin and type 1 IGF receptor binding regions are localized predominantly in the C-terminal region of the insulin B chain ; and the "cooperative" site regulating the affinity of the insulin receptor is also located in the C-terminal region of the insulin B chain. Further experiments will be needed to clarify the exact mechanism.

Insulin-like growth factors (IGF) I and II are peptide hormones related to insulin (1). The role of IGF II in man is far from clear; in some species it appears to be a fetal and/or developmental growth factor.

Sara et al. (2) described a truncated brain IGF I lacking the N-terminal tripeptide GPE. This latter was suggested to be a neuropeptide and a ligand for the brain N-methyl-D-aspartate (NMDA) receptors. This same truncated IGF I has meanwhile been shown to also occur in bovine colostrum (3) and in porcine uterus (4).

In the N-terminal sequence, the first amino acid which both IGF I and II have in common is glutamate, i.e. the C-terminal amino acid of the IGF I N-terminal tripeptide GPE (Table I). If one assumes that the same enzymatic mechanism liberating the N-

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terminal tripeptide GPE is also operative in the case of IGF II, a hexapeptide with the sequence AYRPSE would be cleaved off. We have synthesized this hexapeptide to investigate its potential biological effects.

A computer search for alignment of the amino acid sequence of the hexapeptide revealed that the YRPSE sequence is the C-term of the B chain of casiragua insulin (Table I). The casiragua insulins diverged from typical mammalian insulins leading to higher growth promoting biological potencies (5) as compared to "classical" mammalian insulins. DeMeyts et al. (6) studied the receptor binding of different insulin molecules. By mapping the residues responsible for negative cooperativity he could localize them in the domain comprising the eight C-terminal residues of the B chain. There is consensus that this region - not only in insulin but also in IGF I - is responsible for high affinity binding to the type 1 receptor (7-9). The N-terminal hexapeptide sequence of IGF II may thus be considered of particular interest.

Material and Methods

Hexapeptide AYRPSE was synthesized by an automatic peptide synthesizer 430 A (Applied Biosystems) from BOC amino acids (courtesy of Drs. B. Gutte and St. Klauser). The peptide was cleaved from the resin by trifluoro-methane sulfonic acid according to Applied User Bulletin, issue 16 Sept. 1986. The hexapeptide was purified first by chromatography on Sephadex G 25 in 1 M acetic acid and subsequently by reverse phase chromatography on a semipreparative column (Brownlee Aquapore RP-300, 250 x 9 mm). Solvent A: 0.1 % trifluoroacetic acid (TFA) in H_2O; solvent B: 0.08% TFA/80% acetonitrile in H_2O; gradient: 0-20% B in 50 minutes. The synthesized peptide was quantified by amino acid analysis and the sequence verified by sequence analysis. Separately, a batch of this hexapeptide was synthesized by Bachem Chemicals, Switzerland.

Crude IGF type 2 receptor preparation K562 erythroleukemic cells (5 x 10^8) (obtained from Dr. P. Groscurth) were pelleted (5 min, 385g), washed with cold PES buffer (10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.25 M sucrose), homogenized for 30 seconds on ice (Branson Sonifier Cell Disrupter B15), followed by centrifugation at 30000g for 40 min (4°C). The pellet was resuspended in 10 mM Tris HCl buffer (pH 7.4), centrifuged at 30000g for 30 min, and stored at -20°C in 1 mM Tris-HCl buffer/10% Triton-X. Protein concentrations were determined by the Coomassie blue method (Bio-Rad).

Radioreceptor Assay (RRA) a) with K 562 membranes: The assay was performed as described by Czech et al., (10) with ^{125}I-labelled IGF II (5x10^4 cpm), incubated overnight at 4°C with the IGF II receptor preparation. b) with lung fibroblasts and NIH 3T3 cells: The cells were seeded in 35 mm dishes at 60000 cells/ml, 1.5 ml/dish. After 24 h, the cultures were washed with PBS/10 mg/ml BSA and incubated for 90 minutes with 0.98 ml PBS/BSA, 10 µl labelled ligand and 10 µl substance to be tested at 15° C. The cells were washed 3 times with cold PBS/BSA on ice and extracted with 0.5 % SDS and the cpm counted. For the dissociation experiments, the cells were first incubated for 30 min with the labelled ligand.

Binding Protein Assays were carried out as described previously (11) and Radioimmuno Assays as described by Zapf et al. (12)

Thymidine Assay NIH 3T3 cells were synchronized for 20 h with 0.3% FCS. [Methyl-^{3}H]-thymidine, 0.5 µCi/ml [5 Ci/mmol (Amersham)] was added from 16-20 h and the cells processed as described previously (13).

Iodination a) Hexapeptide: 2 µg were iodinated according to the Chloramin T method (12). The [^{125}I]-labelled hexapeptide was purified first on Sephadex G 15 (Pharmacia, 50 cm x 0.8 cm), and then on Sephadex G 50 (50 x 0.8 cm), using 0.1 M ammonium acetate/0.1 % BSA (pH 7.4) as elution buffer. The specific activity was 25000 cpm/ng. b) Casiragua insulin was iodinated by the same method, but chromatographed on G 50.

Lung Fibroblasts were grown up to the 20th passage (obtained from Prof P. Groscurth who established cultures from human fetal lung tissue from legal abortions from the Universitäts-Frauenklinik Zürich.)
Synaptosomal Membranes were prepared by the method described by Foster et al. (14) with fresh bovine brain (50 g). The membrane preparation was suspended in 50 mM Hepes buffer (pH 7.4) and used the same day.
Binding Assay with Synaptosomal Preparation a) Hexapeptide: Aliquots of synaptosomal preparation (3-300 mg protein) were incubated with $5x10^4$ cpm ^{125}I-labelled hexapeptide, filtered on Whatman glass filters (GF/C) precipitated with TCA and counted. b) [^{3}H]-Glutamic acid: Different amounts of the synaptosomal preparation were incubated for 15 min at 37°C with $5x10^4$ cpm [^{3}H]-glutamic acid and various concentrations of hexapeptide or 10^{-2}M glutamic acid. The samples were then pelleted in an Eppendorf centrifuge and the radioactivity in the pellets counted.
Casiragua insulin was obtained from Dr. J. Zapf whom it was originally donated by the courtesy of Dr. O'Connor from the Wellcome Foundation, England.

Results and Discussion

We first tested the hypothesis that the hexapeptide could - analogously to GPE - also bind to synaptosomal NMDA receptors. When a synaptosomal membrane preparation was incubated with labelled glutamate, the specific binding of L[^{3}H]-glutamate was 169 ± 14 fmol/mg of protein, a value similar to that reported by Sharif and Roberts (15). However, hexapeptide in concentrations up to 10^{-5}M did not compete with labelled glutamate for binding (data not shown). These results are taken to indicate that the hexapeptide does not bind to synaptosomal NMDA receptors for glutamate.

The hexapeptide was assayed for its ability to induce DNA synthesis as measured by [^{3}H]-thymidine incorporation in synchronized cultures of NIH 3T3 cells or human lung fibroblasts. As shown in Figure 1, the hexapeptide clearly stimulated [^{3}H]-thymidine incorporation in a dose dependent manner. In lung fibroblast cultures, the result was virtually the same and maximal stimulation with 10^{-5}M hexapeptide was 25% of the

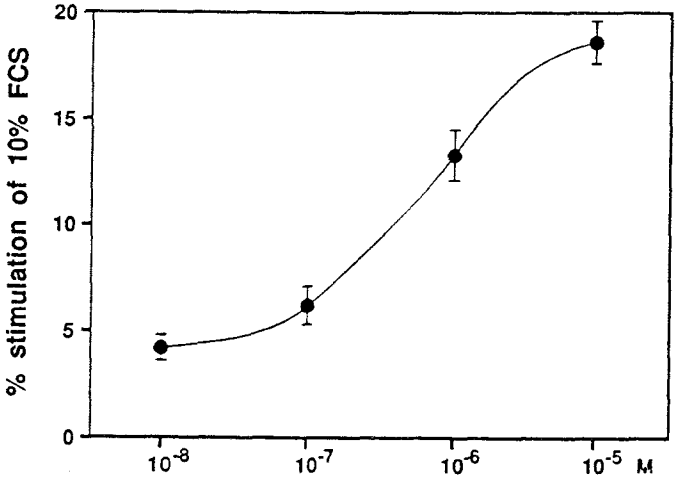

Fig. 1.
Stimulation of [3]H-Thymidine Incorporation into NIH 3T3 Cells
The cells were stimulated with the indicated concentration of hexapeptide and treated as described. The results are the mean from 6 separate experiments ± SE.

stimulation obtained by 10% FCS. To ascertain that the stimulation of DNA synthesis was specific for the hexapeptide sequence AYRPSE, two other unrelated hexapeptides were assayed: one with a terminal glutamate (GRDKYE), the other one without (TRGRSS). Furthermore, a mixture of the free amino acids of the hexapeptide was also tested. None of these 3 control preparations showed any stimulation above background (data not shown).

Of obvious concern was the possibility that the increase in [^{3}H]-thymidine incorporation was an artifact due to some unidentified growth factor contaminating the synthetic hexapeptide batch. Although the hexapeptide eluted as a single peak on HPLC, a trace amount of a potent growth factor could have escaped detection. Therefore, we had another hexapeptide preparation synthesized by a commercial company. Assaying aliquots of both batches of hexapeptide in the same [^{3}H]-thymidine assay and in a receptor binding assay yielded identical results, thus excluding a contaminant.

Hexapeptide, once radiolabelled with ^{125}I at the tyrosine residue, showed no binding activity above background, neither to cultured cells, nor to type 2 receptors or to synaptosomal membranes (data not shown). The only way we could therefore study receptor interactions was by measuring a potential competition of unlabelled hexapeptide for binding with various labelled ligands.

Several such competition assays were carried out. When labelled IGF II was incubated with preparations of IGF binding proteins or IGF II polyclonal antibodies in the presence of hexapeptide, the results clearly showed that hexapeptide did not compete for binding with IGF II in either of these preparations (data not shown).

The question of whether IGF II excerts its mitogenic effect via the type 1 or 2 receptor is complex (16). Earlier and particularly recent evidence clearly implicates the type 1 receptor in the action of IGF II (17). Attempting to answer the question through which receptor the hexapeptide exerts its effect on [^{3}H]-thymidine incorporation, competition experiments were done with type 2 membrane receptor preparation of K 562 cells which bind preferentially IGF II (18). In our membrane preparation the maximal binding of labelled IGF I was 10 fmol/mg protein and 300 fmol/mg for IGF II. When the extent of the competition of hexapeptide (10^{-6}M) with labelled IGF I and II was measured, binding of labelled IGF I and II was reduced to 81% and 69% respectively.

Subsequently, cultures of fibroblasts were incubated simultaneously with the labelled ligands and increasing concentrations of the indicated unlabelled ligand or hexapeptide for 90 min at 15°. The displacement curve for labelled IGF I is shown in Fig. 2 and illustrates that the hexapeptide affected the binding of labelled IGF I. Very similar curves were obtained for labelled IGF II (not shown). The extent of the increased binding or apparent "up-regulation" at 10^{-8} M variied considerably in different experiments. There are various reports on this phenomenon: Insulin induced increased binding of IGF II to intact adipocytes (19,20); and increased the number of IGF receptors (21). In rats the injection of insulin has been described to lead to a doubling in the amount of type 2 receptors circulating in serum (22). Also, an apparent up-regulation of IGF I receptors by

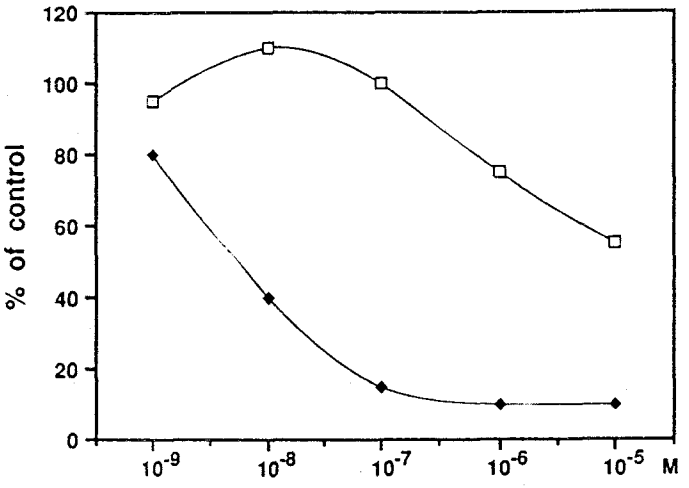

Fig. 2.
Binding of [125]I IGF I to lung fibroblasts
Labelled IGF I (280,000 cpm) was added with unlabelled ligand for 90 min at 15° and the cells processed as described. The maximal binding was approx. 1%. Open square: hexapeptide, filled square:IGF I.

IGF II has been described by Werther et al. (23). Thus, it appears that insulin and the hexapeptide can regulate IGF receptors.

Another possibility which we investigated was the involvement of an unrelated receptor which is influenced by the hexapeptide, since an interaction of IGF I with the PDGF receptor had been described (24). However, when the hexapeptide in concentrations up to 0.2 mM was included in a radioreceptor competition assay for A and B receptors of PDGF, it did not compete for binding with labelled PDGF (Heldin and Haselbacher, unpublished results).

Hystricomorph insulins at high concentrations were shown to stimulate DNA synthesis to a greater level than other mammalian insulins or IGFs (25). As illustrated in Table I, the hexapeptide sequence is the same as the C-terminal pentapeptide sequence of casiragua B chain insulin. To find out whether the hexapeptide effects the dissociation of labelled IGF I or II as various analogues of insulin do (6), cultures of fibroblasts were first incubated at 15° for 60 min with either labelled IGF I or II. The influence of hexapeptide and

Table I

Sequence Comparisons

	N-term									C-term					
B-chain	-2	-1	1	2	3	4	5	6	7	8	24	25	26	27	28	29
IGF I				Gly	Pro	Glu	Thr	Leu	Cys	Gly ...	Phe	Tyr	Phe	Asp	Lys	Pro
IGF II	Ala	*Tyr*	*Arg*	*Pro*	*Ser*	*Glu*	Thr	Leu	Cys	Gly ...	Phe	Tyr	Phe	Ser	Arg	Pro
Hexapeptide	Ala	*Tyr*	*Arg*	*Pro*	*Ser*	*Glu*										
Casiragua insulin			Tyr	Val	Gly	Gln	Arg	Leu	Cys	Gly ...	Phe	*Tyr*	*Arg*	*Pro*	*Ser*	*Glu*
Human insulin			Phe	Val	Asp	Gln	His	Leu	Cys	Gly ...	Phe	Tyr	Thr	Pro	Lys	Thr

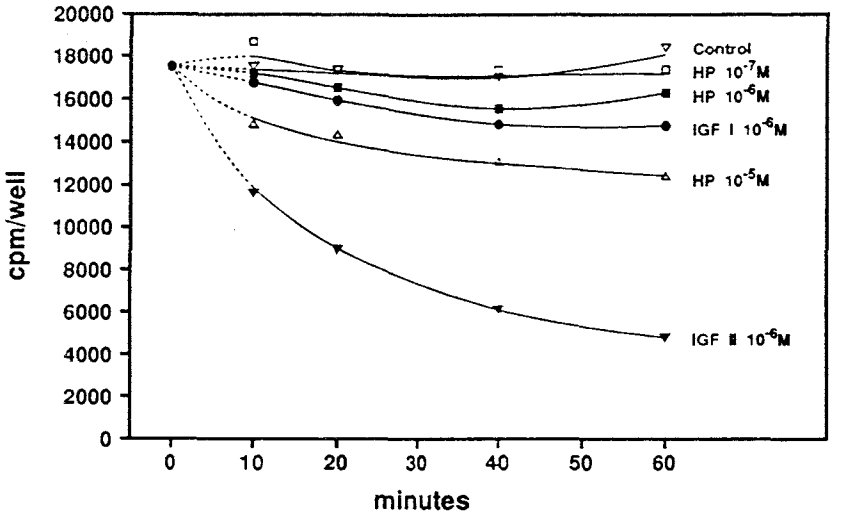

Fig. 3.
Dissociation of [125]II IGF II from lung fibroblasts
The cells grown in 24 well culture plates were incubated with IGF II (80,000 cpm) for 90 min at 15°, washed with PBS/BSA on ice and reincubated at 15° with the indicated unlabelled substances for the time periods indicated. The results are expressed as % bound at time zero.

unlabelled IGF I or II on the dissociation of the bound labelled ligands was then measured after a second incubation at 15° for various times intervals. Fig. 3 illustrates the dissociation pattern of labelled IGF II. The data are expressed as % binding at time zero, i.e. after the first incubation with the labelled ligand. The controls (no addition) show that at 15° the amount of bound labelled IGF II stayed constant for 60 min. Addition of

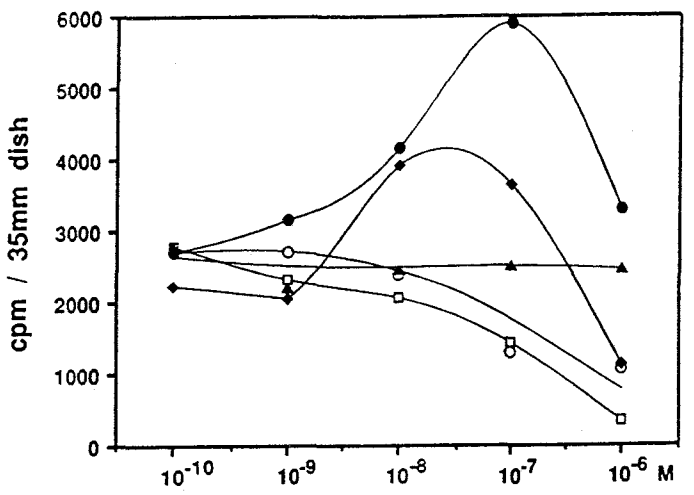

Fig. 4.
Binding of [125]I I casiragua insulin to lung fibroblasts
The labelled insulin was added with the unlabelled substances for 90 min at 15°. The results have been averaged from 2 separate experiments except where hexapeptide and casiragua insulin were combined. The maximal binding was 2%. Open square: casiragua insulin; filled square: casiragua insulin plus hexapeptide half the indicated molarity each; open circle: IGF II; filled circle: hexapeptide; filled triangle: IGF I.

unlabelled IGF II displaced the bound ligand; as hexapeptide at 10^{-5}M. If there are indeed "up-regulated" receptors, they would then have a "masking" effect, i.e. there would in fact be more competition than is really apparent. The hexapeptide also affected the dissociation of labelled IGF I in a similar way (not shown).

For competition studies with ^{125}I-labelled casiragua insulin, fibroblast cultures were incubated with the labelled casiragua insulin and increasing concentrations of either hexapeptide, IGF I from serum or rIGF II. Furthermore, a combination of casiragua insulin plus hexapeptide (half the indicated molarity of each) was also included. The results illustrated in Fig. 4 show that IGF I did not compete with labelled casiragua insulin at all, neither did whale insulin (data not shown). IGF II displaced the labelled bound insulin as well as casiragua insulin. Hexapeptide, however, caused a pronounced apparent "up-regulation" of the binding sites for casiragua insulin. When hexapeptide was combined with unlabelled casiragua insulin, the binding of the labelled ligand was decreased to about one half. The hexapeptide thus clearly affects the binding of casiragua insulin. Our findings that the hexapeptide competes with IGFs for binding and up-regulates binding sites for IGF and casiragua insulin leads us to consider the involvement of the so-called "common receptor" described by Hintz et al (26) and Casella et al. (27). This unique receptor appears to bind IGF I and II and insulin, though it may be an unusual type 1 receptor as described by Misra et al. (28).

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