Inhibition of Cellular Proliferation by Peptide Analogues of Insulin-like Growth Factor 1

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ABSTRACT

The activation of the insulin-like growth factor I (IGF-1) receptor by its ligand plays a central role in the growth of most cell types. We have used the techniques of computational chemistry in order to design and synthesize several novel analogues of IGF-1. These analogues are able to inhibit the autophosphorylation of the IGF-1 receptor as well as the growth of several different cell types, including prostate carcinoma cells and SV40-transformed cells. Additionally, we have found that ε-amino acid analogues of these peptides are apparently resistant to the proteolytic degradation that occurs in the presence of whole sera. Consequently, these analogues seem to show great potential both as probes of the structure/function activities of the IGF-1 signalling pathway and as novel clinical strategies in controlling abnormal cellular growth.

INTRODUCTION

The activation of the IGF-1R by its ligand plays a central role in the proliferation of a wide variety of cell types, such as fibroblasts, hemopoietic cells, chondrocytes, osteoblasts, smooth muscle cells, and others (for reviews, see Refs. 1 and 2). Some hemopoietic cell lines grow in and require only IGF-1 for growth (3–5). Furthermore, the plasma levels of IGF-1 are critical in the development of the human body (6).

IGF-1 and/or its receptor are induced by a variety of growth regulatory agents, which include protooncogenes like c-myc (7, 8); growth factors like PDGF, EGF (9–12), and interleukin 2 (13); hormones like growth hormone (6); estrogens (14–16); and viral DNA oncogenes like the SV40 T-antigen.4 IGF-1, by itself, induces meiosis in Xenopus oocytes (17, 18) and regulates the levels of cdc2 mRNA in fibroblasts (19).

Recently, we have shown that an overexpressed IGF-1 receptor activated by its ligand is sufficient for growth of 3T3 fibroblasts in SFM, to the total exclusion of the receptors for the other two 3T3 cell growth factors, PDGF and EGF (20). On the contrary, EGF-mediated growth of cells overexpressing the EGF receptor still required a functional IGF-1 receptor (12). In view of the dramatic effect that the activation of the IGF-1 receptor has on cellular proliferation, we thought it desirable to produce peptide analogues of IGF-1 that, by competing with the ligand, would inhibit the activation of the receptor and, therefore, cellular proliferation. Under controlled conditions, such analogues might prove useful in treating abnormal cell growth.

MATERIALS AND METHODS

Cell Lines. BALB/c3T3 cells and WI-38 human diploid fibroblasts have been used and passaged in our laboratory for several years. p6 cells are BALB/c3T3 constitutively overexpressing the human IGF-1R RNA; they do not grow in SFM, but they grow vigorously with the sole addition of IGF-1 (20). 6a cells are derived from p6 cells; they carry a stably integrated SV40 T-antigen-coding gene, they are 100% T-positive, grow in SFM, and have the transformed phenotype. ΔU145 cells are an epithelial cell line from a metastatic prostate carcinoma (21); they were obtained from the American Tissue Culture Collection (ATCC HTB81) and grow well in SFM.

Unless otherwise stated, for growth curves, the cells were plated in 35-mm dishes in serum-supplemented medium at a concentration of 10^6 cells/cm². They were made quiescent in SFM for 48 h, then stimulated with growth factors, and counted 48 h later. All growth curves were done in duplicate or triplicate. The growth conditions are indicated in the legends of the figures. The growth factors were obtained from UBI (IGF-1) or Gibco (PDGF BB and EGF). The composition of the SFM has been described in a previous paper (20).

Autophosphorylation of IGF-1 and EGF Receptors. This was done essentially as described by Lammers et al. (22). Briefly, cell lysates were immunoprecipitated with the appropriate antibody (see below) to the respective receptors, followed by a Western blot and staining with a phosphotyrosine antibody (UBI) according to the protocol of the ECL kit manufacturer (Amersham, Arlington Heights, IL). The specific antibodies used were the anti IGF-1R antibody (from Oncogene Science), a mouse monoclonal IgG, that recognizes the α chain of the human IGF-1R, and the anti-EGF R antibody (from Oncogene Science), a mouse monoclonal IgG2a to the human EGF receptor. Phosphorylation of phospholipase C-1 by PDGF BB was determined as previously described (20), using an anti-bovine monoclonal antibody to phospholipase C-1 from UBI, which recognizes phosphorylated C-1 from several species, including mouse.

Design and Synthesis of Peptide Analogues. The peptides (Fig. 1) were designed by using a molecular model of the IGF-1 protein. The B and A domains of IGF-1 (residues 1–29 and 42–62) were homology modeled (23), based on the known crystallographic structure of insulin (24). The C and D domains (residues 30–41 and 63–70) were fitted to the insulin-based core structure. They were then minimized to convergence by using a standard conjugate gradient algorithm in a bulk aqueous environment. The programs used in these procedures are from the Biograf software package (Molecular Simulations/Polygen, Inc.) and the calculations were performed on a Silicon Graphics Power Series 4D-480 computer. After extensive minimization, extended (i.e., 100 ps) energy-dependent molecular motion simulations (dynamics calculations) were performed on the C and D domains while restraining motion of the insulin-based core structure. A stable low energy structure was obtained from the resulting trajectory file, minimized, and subjected to heating and cooling cycles. The insulin-like core of the IGF-1 remained stable under these conditions. However, the C and D domains displayed several different low energy structures.

The synthetic analogues were designed to incorporate the amino acid sequence of the C and D domains of IGF-1. An attempt was made to maintain the distance geometries and torsional properties of the initial dihedral angles of the domains as they "bud-off" of the hydrophobic (insulin-like) protein core. Experimental evidence as well as our theoretical calculations indicate strong conformational flexibility of these domains. In order to maximize the overlap between the conformational repertoire of the native protein with that of the synthetic analogues, we have cycled the peptides via an artificially introduced disulfide bridge. Computer simulations of the restrained synthetic analogues indicated that they should adopt a folding pattern similar to that imparted by the native structure.
The first peptide synthesized represents the looped-out region of the D domain, residues 61–69:

Native sequence: \(^{\text{60}}\text{M-Y-C-A-P-L-K-P-A-K-S-A}^{70}\) (JB1)


The distance from Met-60 to Ala-70 spans ~6.0 Å as measured in our molecular model. This distance and geometry can be maintained by the use of a disulfide bridge. The cysteine at position 62 was replaced with alanine in order to avoid an inappropriate disulfide linkage. The peptide was synthesized by using L-amino acids and standard solid phase peptide synthesis.

Because the peptide described above has a well-exposed trypsin cleavage site, the L-amino acids of the peptide were substituted with D-amino acids. Since most enzymes involved in degradation recognize a tetrahedral α-carbon, the D-amino acids were utilized in order to avoid enzyme recognition and subsequent cleavage. Our computer studies indicated that the same folded presentation of the peptide is accomplished by reversing the amino acid sequence, using D-amino acids. The following D-amino acid peptide was synthesized (using standard solid phase techniques):


To control for sequence and conformational specificity of these peptides a scrambled version of the L-amino acid analogue was synthesized.


Finally, we synthesized a peptide from the looped-out region of the C domain, residues 29–37:

Native sequence: \(^{\text{29}}\text{T-G-Y-G-S-S-R-R}^{37}\) (JB2)

Synthesized: \(\text{C-G-T-G-Y-G-S-S-R-R-C(D-CYS)}\)

In order to maintain flexibility, torsional properties, and distance, a Cys-Gly was placed on the amino-terminus of the peptide and a Cys (D) was placed on the carboxy-terminus.

RESULTS

IGF-1 Peptide Analogues. Nuclear magnetic resonance studies indicate that the core of IGF-1 is strikingly similar to insulin (25). In this light, it is interesting to note that in addition to binding its own type I receptor, IGF-1 also binds the insulin receptor, albeit with much lower affinity (26). The most striking structural differences between IGF-1 and insulin occur because of the inclusion of the C and D domains in the IGF-1 structure. Both the C and D domains (resides 30–41 and 63–70) were poorly resolved in the nuclear magnetic resonance structures due to their intrinsic motility. We have modeled the poorly resolved domains of the human IGF-1 (for general details regarding the building of this molecular model, see Ref. 23 and "Methods" section). In this model, the C and D domains appear as flexible "flaps" which flank the receptor-binding cleft motif which is conserved in both insulin and IGF-1 (residues 21–24; Refs. 27 and 28). These flaps seem to be directly involved in the specific binding to the type 1 receptor. Consistent with this notion, it was observed that deletion of the D domain of IGF-1 increased the affinity of the mutant IGF-1 for binding to the insulin receptor, while decreasing its affinity for the type 1 receptor (27). Furthermore, some or all of the residues within the C domain, which flank the conserved binding cleft in IGF-1 but not in insulins, appear to be required for distinguishing between the type 1 and insulin receptors (28, 29). Thus, the C and D domains were used as templates in our synthetic des-

5 Unpublished data.

Inhibition of IGF-1R Autophosphorylation. We first tested these peptides for their ability to inhibit the autophosphorylation of the IGF-1R by its ligand. For this purpose, we used p6 cells (20), that are 3T3 cells constitutively overexpressing IGF-1R RNA; these cells have about 10 times more IGF-1 binding sites than 3T3 cells and, therefore, are preferable for detecting receptor autophosphorylation. Fig. 2 shows the result of an experiment in which the JB1 (D domain) peptide was used as the analogue. Lane 1 shows the negative control, no IGF-1 added. Lanes 2 and 3 show that both insulin (20 μg/ml) and IGF-1 (20 ng/ml), 10 min before lysis, induce autophosphorylation of the IGF-1R. The JB1 peptide inhibits IGF-1R autophosphorylation in a concentration-dependent manner (compare Lane 3 with Lanes 4 and 5). Fig. 2 also shows that the phosphorylation of the EGF-R by EGF and of phospholipase Cγ-1 by PDGF are not inhibited by the peptide analogue, indicating that in these cells the IGF-1 peptide analogue does not interfere with the receptors of the other two growth factors that are sufficient for optimal growth of 3T3 cells.

IGF-1 Peptide Analogues Inhibit Cellular Proliferation. This inhibition of autophosphorylation is reflected in the inhibition of cellular proliferation caused by the IGF-1 peptide analogues on various cell types. Fig. 3 shows the effect of analogues JB1 (D domain), JB2 (C domain), and JB3 (D-amino acid analogue of JB1) on the proliferation of p6 cells, stimulated by IGF-1 (20 ng/ml). The inhibition is concentration-dependent and is almost complete at the highest concentration; JB1, JB2, and JB3 are all effective and the combination of JB1 and JB2 is slightly synergistic. Two other findings should be noted in Fig. 3. p6 cells incubated with the JB1 peptide and without growth factors remain stationary, indicating that, at these concentrations, there is no appreciable toxicity; the scrambled peptide, JB4, with the same amino acid composition of JB1, but in random sequence, has no effect on IGF-1-mediated growth.

We have tested these peptide analogues on other cell types known to require IGF-1 for growth. Fig. 4 shows data on 3 cell lines: BALB/c3T3 stimulated with PDGF, EGF, and IGF-1, W1-38 human diploid fibroblasts stimulated with 10% fetal calf serum, and one prostatic carcinoma cell, DU-145, of epithelial origin and exponentially growing. All these cells were inhibited by the peptide analogues. Other cell types (not shown) that were tested include: one T-lymphocyte cell line, one line of smooth muscle cells, and two other prostatic carcinoma cell lines. All cell lines tested were inhibited by the IGF-1 peptide analogues. Thus far, we have only found two cell lines that did not respond to the inhibitory effect of IGF-1 peptide analogues: a Jurkat cell line (see below) and, surprisingly, T98G cells, which derive from a human glioma (data not shown).

Fig. 5 shows that the JB1 peptide effectively inhibits the growth of 3T3 cells transformed by the SV40 large T-antigen. This cell line, p6A cells, were generated by cotransfecting the T-antigen-coding gene with a selectable marker (resistance to hygromycin) into p6 cells (20). These cells are transformed, grow in SFM, and have a constitutively autophosphorylated IGF-1R (30). The IGF-1 analogue JB1 inhibits the growth of these cells in a concentration-dependent manner (Fig. 5), and also inhibits the constitutive autophosphorylation of the IGF-1R (not shown).
DISCUSSION

Molecular modeling has allowed us to design IGF-1 peptide analogues that are capable of inhibiting the proliferation of several types of cells, either exponentially growing or stimulated after quiescence. The broad spectrum of inhibition by these peptides is not surprising, since, as mentioned in the Introduction, the activation of the IGF-1R by its ligand is a required step for cellular proliferation in many types of cells (1, 2). Interestingly, of the various cell lines we tested, only two...
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were not inhibited by the IGF-1 peptide analogues: one was a Jurkat cell line, and Lee et al. (31) have reported that Jurkat cells have a very low number of IGF-1Rs and a high number of insulin receptors. The other cell line, T98G cells, instead, do have IGF-1 receptors, but they also produce large amounts of IGF-1.5

All 3 peptides reported in this paper are effective in inhibiting cellular proliferation in a concentration-dependent manner (see Fig. 3 for p6 cells and Fig. 5 for pA6 cells). The fourth peptide (which differs from JB1 only in absolute sequence order) was used as a control; it gives a very modest inhibition (compare bar 8 with bar 2 in Fig. 3, and also the curves in Fig. 5), at 1 μg/ml, much less than with the active peptides.

The peptides also inhibit the autophosphorylation of the IGF-1R by IGF-1, and again in a concentration-dependent manner. The inhibition is specific, at least in terms of the growth factors that are required for the growth of 3T3 and WI-38 cells (32-34). The autophosphorylation of the EGFR by EGF and of phospholipase Cγ-1 by PDGF BB are not inhibited by the JB peptides.

There are several ways in which these IGF-1 peptide analogues can be useful: (a) to investigate the role of IGF-1 in the growth of different cell types. They represent a simple, quick and highly specific method to determine if a given cell line or cell type has an obligatory IGF-1 requirement for growth; (b) to distinguish and separate events that are specifically due to the activation of the IGF-1 receptor from those due to other growth factors (or serum) in cells requiring multiple growth factors for optimal proliferation; (c) to study at a molecular level the mechanism(s) by which IGF-1 binds to and activates its receptor; and (d) last but not least, these IGF-1 analogues have potential practical applications as topical or targeted inhibitors of cell growth. Their solubility, low molecular weight, low toxicity, and high specificity for IGF-1 makes them particularly attractive. Macaulay (35) in a recent review has marshalled the available evidence that IGF-1 and its receptor are often overexpressed in human tumors, and could be an appropriate target for therapeutic interventions. However, these analogues could also be useful in nonneoplastic alterations of normal growth such as smooth muscle hyperplasia in asthma or smooth muscle-induced restenosis of blood vessels in angioplasty.

REFERENCES


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