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Insertion of Argos Sequences into the B-Loop of Epidermal Growth Factor Results in a Low-Affinity Ligand with Strong Agonistic Activity†

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ABSTRACT: Recently, it has been shown that the activation of the Drosophila EGF receptor (DER) by its natural ligand Spitz is inhibited by Argos [Schweitzer, R., et al. (1995) Nature 376, 699–702]. Argos and Spitz both have an EGF-like domain which in the case of Argos differs from that of Spitz and other EGF receptor agonists in that it has an extended B-loop of 20 amino acids instead of 10 amino acids which in addition contains an unusual cluster of charged residues. To investigate whether B-loop sequences are an important determinant for receptor activation and play a causal role in the antagonistic activity of Argos, three human (b)EGF mutants were constructed in which amino acids derived from the Argos B-loop were introduced. In one mutant (E3A4E/B10), replacement of four amino acids in the B-loop of hEGF (123, E24, D27, and K28) by the corresponding Argos residues neither altered the binding affinity of the growth factor for the hEGF receptor nor did it change its ability to induce a mitogenic response. Insertion of 2 additional Argos residues (E3A4E/B12) or extension of the B-loop by 10 amino acids (E3A4E/B20) resulted, however, in a significant loss of binding affinity. In spite of this, both E3A4E/B12 and E3A4E/B20 appeared to be strong agonists for the hEGF receptor with similar dose–response curves for mitogenic activity and MAPK activation as wild-type hEGF. These data show that several nonconservative substitutions in the hEGF B-loop are tolerated without affecting receptor binding or activation. Furthermore, they show that receptor binding and receptor signaling efficiency can be uncoupled which is a prerequisite for the development of receptor antagonists.

Epidermal growth factor (EGF) belongs to a family of structurally related growth factors which all exert their action by binding to the epidermal growth factor receptor (Carpenter & Wahl, 1991). Many tumor cells express this receptor, and also secrete members of this family of EGF-like molecules, thus creating the possibility of an autocrine growth factor cycle. In particular, the role of transforming growth factor α (TGF α) in the outgrowth of various tumors has been well established (Lee et al., 1995). Many studies have been performed on the structure–function relationship of EGF-like molecules, with the final aim of developing EGF-receptor antagonists, which can be used clinically to interfere with such autocrine processes (Carpenter & Wahl, 1991; Groenen et al., 1994; Prigent & Lemoine, 1992). 2D NMR studies have provided evidence that amino acids surrounding the second and sixth cysteine residues are in close contact with each other, possibly forming a nonlinear receptor binding pocket (Hommel et al., 1992). Among these are Y13, L15, H16, R41, E43, and L47 in hEGF. In addition, our previous data, using exchange mutants of hEGF and hTGFα, have shown that also R45 in hEGF belongs to the receptor binding domain (Van de Poll et al., 1995).

There is still discrepancy in the literature as to whether the B-loop of hEGF, the main β-sheet structure, located between the third and fourth cysteine, is directly involved in receptor binding or only of structural importance for determining the correct conformation required for binding. Nonconservative substitutions of I23 and A30 cause a significant reduction in binding affinity [see for a recent review Groenen et al. (1994)], but EGF molecules with truncated forms of the B-loop have been claimed to be biologically active (Taggart et al., 1993). Domain-exchange studies between EGF and TGFα have also not given definite conclusions in this respect (Kramer et al., 1994; Richter et al., 1995).

In recent years, various studies have been performed on the characterization of EGF-like molecules in Drosophila. Spitz, a 26 kDa transmembrane protein containing an EGF-like motif in the extracellular domain (Rutledge et al., 1992), was found to be a potent activator of the Drosophila homologue of the mammalian EGF receptor (DER), and to play a central role in cell fate decisions during embryonic development (Livneh et al., 1985; Schweitzer et al., 1995a). A second EGF-like molecule, Argos, has been identified as a primary regulator of cell fate in the Drosophila eye (Freeman et al., 1992). Recently, it has been shown that Argos prevents Spitz-induced signal transduction by DER, possibly by competing with Spitz for binding to the receptor (Schweitzer et al., 1995b; Golemo et al., 1996). Both proteins share the characteristic spacing of conserved cysteine residues present in EGF-like growth factors, but most strikingly, Argos differs from all other EGF-like molecules in that the B-loop contains 20 amino acids instead of the usual 10 amino acids in EGF receptor agonists. In addition, this sequence in Argos contains an unusual cluster of basic amino acids opposite to acidic amino acids (Freeman et al., 1995).
In the present study, we have investigated the role of the hEGF B-loop in receptor binding and activation using three hEGF B-loop mutants in which the Argos B-loop characteristics of aberrant length and charge distribution were introduced. The results show that the hEGF B-loop can be modulated to a high extent without loss of biological activity. Furthermore, it shows that it is possible to uncouple biological activity from binding affinity which is a prerequisite for the development of receptor antagonists.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs.** The synthetic gene for human EGF was obtained from British Biotechnology (Oxford, United Kingdom). Constructs encoding the hEGF B-loop mutants E3A4E/B20, E3A4E/B12, and E3A4E/B10 (Figure 1) were made by cloning the gene coding for hEGF at the third cysteine codon with NsiI and at the fourth cysteine codon with SpHl. The gap was then filled in using double-stranded oligonucleotides. The genes were linked at the 5'-end to the sequence coding for the recognition sequence of the proteolytic enzyme factor X (Ile-Glu-Gly-Arg; see Nagai and Thogersen (1987)), and the FX/growth factor constructs were cloned into the expression vector pEZZ18 (Pharmacia, Uppsala, Sweden) 3' of the sequence coding for two synthetic protein A-derived IgG binding domains (so-called Z domains) as described previously (Van de Poll et al., 1995; Kramer et al., 1994).

**Expression and Purification of Wild-Type and Mutant Growth Factors.** Wild-type hEGF and the hEGF mutants were expressed and secreted as ZZ/FX/growth factor fusion proteins in the periplasmic space of Escherichia coli KS474, a degP protease-deficient mutant [a generous gift from Drs. K. L. Strauch and J. Beckwith, Harvard University; see Strauch et al. (1989)]. The isolation of periplasmic proteins and the purification of recombinant growth factors were performed as described by Nilsson and Abrahamson (1990) and Van de Poll et al. (1995). Briefly, this involved the binding of fusion proteins to IgG-Sepharose, removal of the protein A sequence by digestion with factor Xe followed by a second run on IgG-Sepharose, and a final purification step using HPLC on a C18 reverse phase column with a linear gradient of CH3CN in 0.1% trifluoroacetic acid.

**Analysis of Mutant Growth Factors by SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.** Fusion proteins were analyzed on a 12.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose (0.45 μm), and the Western blots were probed with a rat anti-goat antibody linked to horseradish peroxidase to detect protein A sequences. Enzyme activity was detected by incubation with tetramethylbenzidine/sodium dioctylsulfosuccinate/H2O2 in phosphate/citrate buffer (pH 5).

Purified growth factors were analyzed on a 16.5% T/6% C tricine SDS-polyacrylamide gel in the presence of 2% β-mercaptoethanol as a reducing agent (Schagger & Von Jagow, 1987). The proteins were transferred to nitrocellulose (0.1 μm), and the Western blots were incubated at room temperature with a polyclonal antibody (Ab-3) raised against recombinant hEGF (Oncogene Science Inc., Cambridge, MA). Previous studies have shown that this antibody recognizes a hEGF/hTGFα chimera in which the hEGF B-loop had been replaced by hTGFα (Van de Poll, unpublished results). Probed proteins were detected by a goat anti-rabbit antibody linked to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL, Boehringer, Mannheim).

**Quantification of Mutant (Fusion) Proteins.** The amount of ZZ/FX/growth factor fusion protein present in the unpurified periplasm was estimated by measuring the total amount of IgG binding activity. This was done in a competitive enzyme-linked immunosorbent assay using protein A as a standard and biotin-labeled protein A as a competitor [both obtained from Sigma Chemical Co., St. Louis, MO; see Van Zoelen et al. (1993)]. The amount of growth factor obtained after the final purification by RP-HPLC was calculated from the peak area (absorption at 229 nm) using natural murine (m)EGF (Bioproducts for Science Inc., Indianapolis, IN) analyzed under the same conditions as a standard.

[125I]-mEGF Binding Competition Assay. The ability of the fusion proteins and the purified growth factors to bind to the hEGF receptor was measured in an [125I]-mEGF binding competition assay on confluent layers of NIH-3T3 fibroblasts transfected with the hEGF receptor (HER-14 cells, 4.0 × 10⁵ receptors/cell) as described previously (Van de Poll et al., 1995). Shortly, serial dilutions of lyophilized periplasm or purified growth factors were made in serum-containing Dulbecco's modified Eagle's medium (DMEM), buffered at pH 7.7 with 15 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], and 100 μL was added to the cells together with 0.1 ng/well [125I]-mEGF. After incubation for 2 h at room temperature, cells were washed twice with ice-cold phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) and once with ice-cold PBS. The cells were incubated subsequently in 1% Triton X-100 for 1 h at room temperature prior to γ-counting. Experiments were performed in triplicate using natural mEGF as a standard and repeated twice.

**Mitogenic Assay.** HER-14 cells were seeded in gelatinized 24-well dishes at a density of 6.0 × 10⁴ cells/well in 1 mL of DMEM containing 10% newborn calf serum (NCS). After 24 h of incubation, the medium was replaced by 0.9 mL of DMEM/Ham's F12 medium (1:1) supplemented with 30 mM Na2SeO3, 10 μg/mL human transferrin, and 0.5% BSA. After an additional 48 h of incubation, serial dilutions of lyophilized periplasm or purified growth factor were added in 0.1 mL of DMEM containing 50 mM BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 6.8]. Eight hours later, 0.5 μCi of [3H]thymidine was added in 0.1 mL of Ham's F12 medium. Incorporation of the tracer into cellular DNA was determined 24 h after growth factor addition. For this, cells were washed twice with PBS and subsequently fixed with methanol at room temperature. After 15 min, the methanol was aspirated, and the cells were lysed in 1 mL of 0.2 N NaOH for 30 min at 37 °C as described (Van Zoelen et al., 1985). Radioactivity was determined by liquid scintillation counting. Experiments were performed in duplicate and repeated twice.

**Mitogen-Activated Protein Kinase (MAPK) Assay.** HER-14 cells were grown to confluence in 6-well dishes (9.8 cm²) and subsequently incubated for 48 h in 950 μL of DMEM supplemented with 0.1% BSA. Serial dilutions of recombinant growth factors were added in 50 μL of DMEM.
Western blotting showed a major band migrating at ~20 kDa in the wild-type hEGF and the E3A4E/B10 and E3A4E/B12 excreted into the periplasmic space of E. coli. Individual amino acids in the B-loop are indicated: boxes, hEGF-derived amino acids; boxes with dark arrow, Argos-derived amino acids; circles, conserved third and fourth cysteine residues.

buffered at pH 6.8 with 50 mM BES and incubated for 10 min at 37 °C. Then the medium was aspirated, and the cells were washed once with ice-cold PBS and rapidly lysed in 300 μL of sample buffer (10% glycerol, 60 mM Tris-HCl, pH 6.8, 2% SDS, 0.3 M β-mercaptoethanol, and bromophenol blue). The lysate was sheared and boiled for 10 min. Aliquots of 50 μL were analyzed by gel electrophoresis on a 12.5% SDS—polyacrylamide gel. Proteins were transferred to nitrocellulose, and the blots were probed with a polyclonal antibody directed against MAPK (Burgering et al., 1993). Probed proteins were detected by a goat anti-rabbit antibody linked to horseradish peroxidase and visualized by ECL. The amount of phosphorylated MAPK was expressed as a percentage of total MAPK.

### RESULTS

**hEGF B-Loop Mutants.** The EGF-like motif of Argos contains 20 amino acids between the third and fourth cysteine residue whereas this so-called B-loop sequence in hEGF consists of only 10 amino acids. To evaluate the effect of a B-loop extension on receptor binding and activation, 10 residues derived from the Argos B-loop were inserted between A25 and L26 in hEGF. The resulting hEGF B-loop mutant thus contained a B-loop of 20 amino acids similar to Argos and was designated E3A4E/B20 (Figure 1).

Another characteristic of the Argos B-loop is the presence of a cluster of basic amino acids opposite to acidic amino acids. Therefore, two mutants were constructed in which four amino acids of the hEGF B-loop (I23, E24, D27, and K28) were replaced by respectively four and six residues derived from the Argos B-loop. Individual amino acids in the B-loop are indicated: boxes, hEGF-derived amino acids; boxes with dark arrow, Argos-derived amino acids; circles, conserved third and fourth cysteine residues.

Identification of E3A4E/B20. Analysis of purified recombinant growth factors on SDS—polyacrylamide gel under reducing conditions showed that both wild-type hEGF and E3A4E/B20 migrated as a single band at the expected molecular mass of ~6 and 7.5 kDa, respectively (Figure 3). The molecular weight of E3A4E/B20 was confirmed by mass spectrometry (carried out in collaboration with Dr. M. O'Connor-McCourt and Dr. Y. Konishi, Biotechnology Research Institute, Montreal). Analysis of the combined mutant product identified a major product (67%) with an average compound mass of 7518 Da corresponding to the predicted MW of E3A4E/B20, and a minor product (33%) with an average compound mass of 7535 Da. The exact nature of the latter compound was not established, but the
higher molecular weight could be the result of a chemical modification, e.g., oxidation of M21, which is known to occur during storage and does not affect the biological activity of wild-type EGF (Carpenter & Wahl, 1991).

It is concluded that the main component of the combined mutant product is a single-chain protein with the expected characteristics of E3A4E/B20.

**Binding Affinity of Mutant Growth Factors for the hEGF Receptor.** The binding activity of the mutant growth factors was measured in a [125I]-mEGF binding competition assay on confluent layers of HER-14 cells. RP-HPLC-purified E3A4E/B20 was compared in this assay with purified recombinant wild-type hEGF, whereas the activity of the E3A4E/B10 and E3A4E/B12 fusion proteins was compared with wild-type hEGF fusion protein expressed in a parallel culture. Exchange of the hEGF B-loop residues I23, E24, D27, and K28 for four Argos-derived amino acids, KRDE, as in E3A4E/B10 did not significantly reduce the binding affinity of hEGF for its receptor (Figure 4A). The amount of E3A4E/B10 fusion protein required for 50% displacement of [125I]-mEGF (IC50) was 20 ± 1 pmol/mL, and the IC50 calculated for hEGF fusion protein was 22 ± 2 pmol/mL. However, extension of the B-loop by only two amino acids in the E3A4E/B12 mutant caused already a 7-fold reduction in EGF receptor binding affinity (Figure 4B; IC50 of 149 ± 0 pmol/mL). The affinity of E3A4E/B20, which has a B-loop of 20 amino acids as in Argos, was likewise reduced, being only ~5% of wild-type growth factor (Figure 4B; IC50 of 53 ± 10 pmol/mL compared to 3.3 ± 0.3 pmol/mL for wild-type hEGF).

**Mitogenicity of Mutant Growth Factors.** The E3A4E/B10 and E3A4E/B12 fusion proteins (Figure 5A) and HPLC-purified E3A4E/B20 (Figure 5B) were tested for their ability to stimulate the incorporation of [3H]thymidine into the cellular DNA of quiescent HER-14 cells. The mitogenic activity of the mutant fusion proteins was compared with hEGF fusion protein (ZZ/FX/hEGF) as well as with purified recombinant hEGF. Furthermore, it was verified that no dose-dependent increase in [3H]thymidine incorporation occurred after incubation with control periplasm and that control periplasm did not alter the mitogenic response of HER-14 cells to purified recombinant hEGF.

Figure 5A,B shows that all three mutants are agonists of the human EGF receptor with similar dose—response curves for the induction of mitogenic activity as wild-type EGF. The protein concentration required to give 50% stimulation of [3H]thymidine incorporation (EC50) was estimated to be 0.23 ± 0.15 and 0.25 ± 0.08 for the E3A4E/B10 and E3A4E/B12 fusion protein, respectively, and the EC50 of purified E3A4E/B20 was calculated to be 0.08 ± 0.02 compared to 0.15 ± 0.02 for purified recombinant hEGF. Thus, in spite of the fact that E3A4E/B20 and E3A4E/B12 display a strongly reduced ability to bind the human EGF receptor, they are as potent as wild-type hEGF in generating a mitogenic response in quiescent HER-14 cells.

**Mitogen-Activated Protein Kinase (MAPK) Activation.** To investigate whether the observed divergence between receptor
binding affinity and mitogenicity is due to an uncoupling of binding ability and signaling efficiency, E3A4E/B20 was tested for its ability to induce MAPK phosphorylation in HER-14 cells. Figure 6 shows that, in spite of its very low binding ability, E3A4E/B20 is also a relatively potent activator of MAPK, being only 2—3 times less active than wild-type hEGF. This indicates that also MAPK activation by E3A4E/B20 is not a reflection of the equilibrium binding constant of the mutant growth factor.

**DISCUSSION**

The EGF-motif of the DER antagonist Argos is characterized by the presence of an unusual B-loop sequence consisting of 20 amino acids as opposed to 10 residues in known EGF receptor agonists. In addition, this sequence in Argos contains a cluster of positively charged amino acids opposite to negatively charged residues (Freeman et al., 1992). To investigate the effect of an enlargement of the B-loop and/or the introduction of charged side chains in hEGF on receptor binding and activation, three hEGF mutants were constructed containing aberrant B-loop sequences derived from the DER antagonist Argos.

The simultaneous mutation of three amino acids, I23K, E24R, and K28E, as in the E3A4E/B10 mutant neither affected the affinity of the growth factor for the hEGF receptor nor lowered its ability to stimulate the proliferation of quiescent HER-14 cells. Additional pairwise insertion of a lysine and a valine residue (E3A4E/B12) or an extension of the hEGF B-loop by 10 amino acids derived from Argos (E3A4E/B20) resulted in a significant loss of binding affinity, however, without a concomitant decrease in mitogenic potency. All three mutants appeared to be as mitogenic as wild-type hEGF.

Ever since the first report by Komoriya et al. (1984) that a synthetic linear peptide corresponding to the B-loop sequence of murine EGF is biologically active when added at high concentrations, the B-loop ß-sheet has been the subject of extensive research. Point mutation studies have frequently indicated I23 as an important determinant for high-affinity receptor binding (reviewed in Groenen et al., 1994). Only the nonpolar residues valine and leucine can replace I23 without significant loss of binding affinity, and a lysine residue at this position, as in E3A4E/B10, was thus expected to lower the affinity. Our observation that E3A4E/B10 has a similar high affinity as wild-type hEGF for the hEGF receptor suggests that specific pairwise mutations in the B-loop may have less impact on receptor binding than single-
point mutations and that the effect of the I23K mutation may be more or less compensated for by the K28E mutation.

Another prerequisite for high-affinity binding seems to be the correct length of the B-loop. Pairwise insertion of only 2 amino acids in the E3A4E/B12 mutant already resulted in a 7-fold reduction in binding affinity while further extension of the B-loop to 20 amino acids in E3A4E/B20 caused an additional 2-fold reduction. Whether the reduced affinity is due to the introduction of specific amino acids or whether any enlargement of the B-loop sequence, even if it is only by two amino acids, is not tolerated is at present not known, but the fact that most of the mammalian EGF receptor agonists, such as EGF, TGFα, amphiregulin, heparin binding EGF, betacellulin, and epiregulin, have B-loop sequences consisting of only 10 amino acids (Groenen et al., 1994) is in favor of the latter explanation. Furthermore, the poxvirus family members Shope fibroma growth factor and myxoma growth factor with 13 residues located between the third and fourth cysteine residues have a strongly reduced affinity compared to hEGF (Lin et al., 1988, 1991). In addition, we have found that a shortening of the B-loop by pairwise deletion of four or six amino acids also causes a strong reduction in binding affinity and biological activity (M. L. M. van de Poll, unpublished results).

Although E3A4E/B12 and E3A4E/B20 displayed strongly reduced binding affinity for the hEGF receptor, they were as potent as wild-type hEGF in generating a mitogenic response in cells expressing the hEGF receptor. Uncoupling of binding affinity and signaling efficiency has so far been described for only one EGF mutant, mEGF L47V, and it was suggested that the relative high mitogenic potency of this mutant was associated with a resistance to receptor-mediated degradation (Walker et al., 1990). E3A4E/B20, however, is not likely to be more resistant to degradation than wild-type hEGF since both proteins share the same C-terminal region. For most of the other EGF analogues studied to date, receptor binding affinity and biological activity seem to be strongly correlated.

It has been well established that cells can contain both high- and low-affinity EGF receptors, and it has been put forward that cellular responses are mainly mediated by the high-affinity receptor sites (Defize et al., 1989; Walker et al., 1990). Since these high-affinity receptors have a relatively low abundance, it is theoretically possible that E3A4E/B20 has similar affinity as hEGF for the high-affinity receptors, resulting in similar mitogenic activity, and only differs from hEGF by its strongly reduced affinity for the low-affinity receptors. It has been shown that low-affinity receptors can be extracted from the cells by Triton X-100, since they are not linked to the cytoskeleton (Van Bergen en Henegouwen et al., 1988; Berkers et al., 1990). Using this approach, we observed that the remaining 5% high-affinity receptors still had a 20-fold lower affinity for E3A4E/B20 than for hEGF, indicating that the difference between binding affinity and mitogenic potential of E3A4E/B20 cannot be explained in this way.

In recent years, it has also become clear that the EGF receptor is a member of a multigene family, and that EGF-like growth factors can induce receptor heterodimers of which particularly those with erbB2 appear to generate the most potent mitogenic signals (Qian et al., 1994; Graus-Porta et al., 1995; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996). The HER-14 cell line used in our studies expresses a low level of endogeneous erbB2. It could be argued therefore that E3A4E/B20 only differs from hEGF in its ability to bind EGF receptor homodimers but can induce EGF receptor/erbB2 heterodimers with similar potency as hEGF resulting in a similar mitogenic response. Preliminary results using IL-3-dependent myeloid cells transfected with only erbB1, however, show that heterodimerization is not required for the high mitogenic activity of E3A4E/B20 (A. E. G. Lenferink and Y. Yarden, unpublished observation).

Furthermore, we found that the relatively high mitogenic potency of E3A4E/B20 was accompanied by a relatively high ability to activate MAPK. Because maximal MAPK activation is achieved already 5–10 min after growth factor addition, the rate of phosphorylation of this second messenger most likely reflects the time required for the ligand to interact with the receptor and is not related to the equilibrium binding constant. Recent BIAcore experiments with several EGF/TGFα chimeras suggest that a high ability to activate MAPK indeed correlates with a high association rate constant (A. E. G. Lenferink and M. O’Connor-McCourt, unpublished results). The fact that E3A4E/B20 is as potent as wild-type hEGF in the MAPK assay might indicate that its association rate constant is similar to hEGF and that the low affinity might be due to a higher koff. We are currently investigating this. If the association rate rather than the equilibrium binding constant appears to be an important determinant for mitogenic activity, this would pose a new concept on how receptor activation occurs and how a biological response is generated.

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REFERENCES


Page 2594. The paragraph below did not appear in the published paper.

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Page 7429. In Figure 6, the x-axis should read growth factor (fmol/mL).

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Page 8221 and throughout the article. The myoglobin mutants contain deletions or substitutions in residues 52—56 and not residues 51—55. Thus, D-helix mutants of sperm whale myoglobin have (1) deletion of Glu^52^-Ala-Glu-Met-Lys^56, Mb(D^52^-56); (2) replacement of these residues with Ala^52^-Ala-Ala-Ala-Ala^56, Mb(Ala52^-56); or (3) replacement of these residues with Ala^52^-Ala-Ala-Met^55^-Ala^56, Mb(Ala^52^-54Met^55^Ala^56). These mutations have been confirmed by both high-resolution crystallography and sequencing of the original genes.

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