Modularity of Protein Function: Chimeric Interleukin 1βs Containing Specific Protease Inhibitor Loops Retain Function of Both Molecules†

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ABSTRACT: Although it is widely recognized that many proteins contain discrete functional domains, it is less certain whether smaller, less obviously discrete, units of structure will retain their specific function when transplanted into a different context. The observation that the potent inflammatory cytokine human interleukin 1β has the same overall structure as soybean trypsin inhibitor (STI) (Kunitz) prompted us to replace a tight turn in the cytokine sequence with the large loop in soybean trypsin inhibitor that binds to the active site of trypsin. Wild-type interleukin 1β (IL–1β) is highly resistant to proteolysis, but the chimeric STI/IL is specifically cleaved by trypsin, apparently in the inserted loop. Other chimeric interleukins have also been constructed, by replacing the same tight turn with inhibitory loops from other protein protease inhibitors: turkey ovomucoid inhibitor (TOI), a chymotrypsin inhibitor, and α1-antitrypsin (AT), an elastase inhibitor. Although these loops come from proteins not related structurally to interleukin 1, they confer specific protease sensitivity or inhibition on the chimeric cytokine. The cytokine properties of these chimeric interleukins have also been evaluated. The chimeras formed from human IL–1β and all inhibitory loops tested bind to the interleukin 1 receptor with reasonable affinity. The typical cellular effects of IL-1, however, are not observed with all the recombinant proteins, thus confirming that receptor binding and signal transduction can be uncoupled. When these results are taken together with the results of site-directed mutagenesis of IL-1, reported in this paper and elsewhere, they allow the receptor and intracellular transduction sites on the protein to be mapped in detail.

Many proteins contain discrete functional domains, often segregated as unique folding units within a larger protein. In the case of smaller proteins, this segregation of function into domains is less clear. However, units of structure can have a specific functionality, an observation that is clearly demonstrated by the protein proteinase inhibitors, in which a single loop and very little else interacts with the target proteinase. The rest of the protein may be necessary for the integrity of that loop and it therefore becomes of interest to test the possibility of transplanting such a loop and its functionality into another protein context, thereby combining the functionality of the transplanted loop with the inherent functionality of the host protein. We have tested this hypothesis by moving a loop from a proteinase inhibitor into a cytokine in order to create a chimeric protein with both activities.

The cytokine human interleukin 1β (IL-1β)[1] is an immunomodulator which participates in the regulation of numerous immunological and inflammatory processes (Dinarello, 1984; Durum et al., 1990; Krakauer, 1986). The protein forms a receptor–ligand complex (Dower et al., 1986; Kilian et al., 1986) with a cell surface receptor, which then triggers physiological responses which are not yet well-understood at the molecular level.

The human protein is synthesized as a 31 000-Da precursor protein with low biological activity (Jobling et al., 1988; Hazuda et al., 1989) which is processed to the mature protein by cleavage of the N-terminal 116-residue segment (Thornberry et al., 1992; Cerretti et al., 1992). The X-ray diffraction structure of IL-1β has been described (Priestle et al., 1988; Finzel et al., 1989; Veerapandian et al., 1992). The structure is a 12-stranded β-barrel which bears a striking similarity to that of soybean trypsin inhibitor, (STI) (Priestle et al., 1988; Finzel et al., 1989; McLachlan, 1979), and human basic and acidic fibroblast growth factors (Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991).

Mutagenesis experiments involving individual amino acids (Chang et al., 1992; Gehrke et al., 1990; Young et al., 1990; Huang et al., 1987; Labriola-Tompkins et al., 1991) have mapped some of the residues which are involved in binding to the receptor. Deletion of amino acids from the mature protein leads to a decrease in biological activity (DeChiara et al., 1986; Huang et al., 1987; Jobling et al., 1988; Mosley et al., 1987; Lillquist et al., 1988), indicating that activity is linked to structural integrity of the mature protein. Chemical modification of arginine residues (Dinarello et al., 1982) has demonstrated that these residues are required for the inflam-

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[1] Abbreviations: STI, soybean trypsin inhibitor; IL-1, interleukin 1; TOI, turkey ovomucoid inhibitor; AT, α1-antitrypsin; TOI/IL, chimera consisting of the IL-1 framework with a loop from TOI inserted; STI/IL, chimera with a loop from STI inserted in IL-1; AT/IL, chimera with a loop from AT inserted in IL-1.

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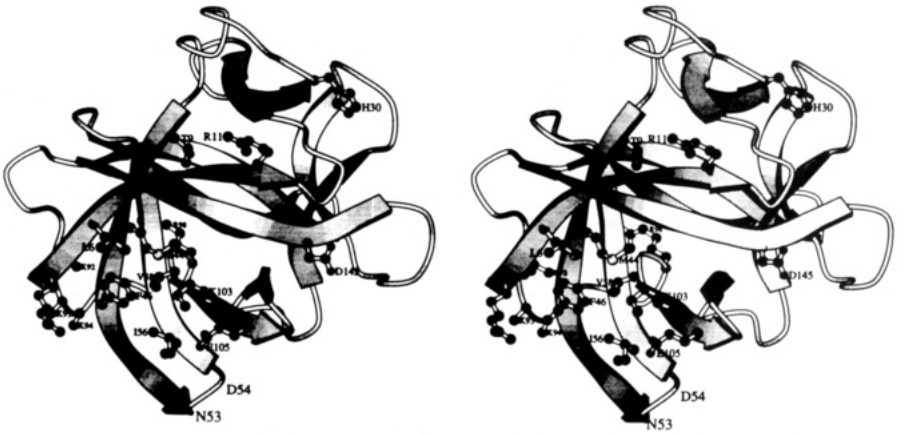

FIGURE 1: Structure of wild-type IL-1β. The region chosen for replacement is the loop designated N53–D54. The molecule was constructed using data from Finzel et al. (1989) and spatial coordinates available from the Protein Data Bank. The structure was displayed and manipulated using the AVS Program on the Stardent computer. The following residues have been implicated in the bioactivity and binding of IL-1β to its receptor: R4 (Huang et al., 1986); K103 and E105 (Chang et al., 1992); L6, M44, F46, I56, K92, K93, K94, and R98 (Labriola-Tompkins et al., 1991); and H30 (MacDonald et al., 1986). Three residues affect bioactivity without affecting binding: T9 (Young et al., 1990; Simon et al., 1993), R11 (Gehrke et al., 1990), and D145 (Ju et al., 1992). All of these residues cluster on one face of the molecule, implying that this region is the recognition site for the receptor.

matory response. Mapping the results of mutagenesis and chemical experiments onto a three-dimensional model (see Figure 1) shows that the region of IL-1β which interacts with the receptor is on one face of the molecule composed of residues coming from a region bounded by strands 1, 4, 5, 8, and 12.

The similarity in structure of a human cytokine to a plant protease inhibitor suggested to us that the protease binding region from STI could be identified with an analogous region of IL-1β. This region is the loop between strands 4 and 5 which is a tight turn in the IL-1β structure and an extended loop in the STI structure. Moving this loop from STI into IL-1β would determine the importance of the protein context in eliciting inhibitory behavior from a peptide strand which is normally a substrate in another context.

In addition, protease-inhibiting regions from other protein inhibitors could be used to construct hybrid mutant IL-1βs which contain inhibitory activity toward a protease and still elicit interesting cytokine bioactivity.

Proteinases are involved in the regulation of a great many physiological processes, including the digestion of food, the cascade system of blood clotting and complement, the activation of hormones, and the degradation of endogenous proteins within cells. In most cases, proteolysis is closely controlled in order to prevent tissue damage (Hörl, 1988). The most common mechanism of control in vivo involves specific proteinase inhibitors. These inhibitors are themselves proteins which form essentially irreversible, noncovalent 1:1 enzyme–inhibitor complexes, which resemble Michaelis complexes. Such a complex results in a low rate of hydrolysis, presumably because the energy barrier for hydrolysis is large and unfavorable (Laskowski & Kato, 1980; Read & James, 1986; Longstaff et al., 1990), or because product release after cleavage is extremely slow. The specificity of binding is very precise and is, in general, not found with inhibitors which are smaller molecules. The portion of the protein inhibitor which interacts with the proteinase is usually an external loop. In all proteinase inhibitors studied structurally so far, this loop is stabilized by intramolecular interactions which are thought to contribute to the reduction in rate of cleavage.

The control of elastase activity in the lung is, in part, due to the complexation of the proteinase with a specific inhibitor, α_1–antitrypsin (AT). The loop which contains the recognition site of this inhibitor for elastase has been moved into interleukin in order to create a chimeric protein which will retain some of the properties of the proteins from which it was made (i.e., proteinase inhibition and interleukin receptor recognition).

EXPERIMENTAL PROCEDURES

Materials. DNA-modifying enzymes were obtained from Bethesda Research Laboratories, New England BioLabs, or U.S. Biochemical Corp. and used as suggested by the suppliers. The Sequenase kit for DNA sequencing is a product of U.S. Biochemical Corp. Radioactive compounds were from Amersham. S-Sepharose, DEAE-Sephacel, and Sephadex G-50 were obtained from Pharmacia. The engineered gene for IL–1β was obtained from Beckman. The gene is cloned into the *Bam*HI site in the polylinker region of pUC19. The expression plasmid pMGIL 1β was the generous gift of SmithKline Beecham. It contains the 17-kbp mature human IL-1β gene from cDNA under the control of the λP_L heat-inducible promoter (Meyers et al., 1987). Oligonucleotides were purchased from the DNA synthesis facility at Brandeis University. Expression was carried out in AR58 cells using LB or enriched medium containing ampicillin.

Proteolytic enzymes (elastase, trypsin, and chymotrypsin) were purchased from Sigma. Elastase was from porcine pancreas, 60 units/mg of protein; trypsin was from porcine pancreas, 16 000 BAEE units/mg of protein; and chymotrypsin was from bovine pancreas, 55 units/mg of protein. Substrates for trypsin (N^{α}-benzoyl-DL-arginine-*p*-nitroanilide), chymotrypsin [*N*-succinyl-(L-Ala)$_2$-Pro-Phe-*p*-nitroanilide], and elastase [*N*-succinyl-(L-Ala)$_3$-*p*-nitroanilide] were obtained from Calbiochem.

Methods. Oligonucleotides were purified by TLC in a solvent system of 1-propanol/NH_4OH/H_2O (55:35:10) and phosphorylated using T4 polynucleotide kinase. The Beckman plasmid (pUC19 containing the gene for IL–1β) was treated with *Stu*I and *Sty*I. The resulting 2.7-kbp fragment was purified by PAGE, extracted with phenol, and precipitated with ethanol. This was ligated with the oligonucleotides coding for the peptide segment, containing the loop so as to replace residues 50–53 of wild-type interleukin (see Table I). The resulting mutant plasmids were used to transform competent JM-101 cells, and the transformed cells were cultived in LB medium containing 100 μg/mL ampicillin at 37 °C.

Colony-purified DNAs were analyzed by restriction analysis, taking advantage of new restriction sites in the insert. Both strands of DNA were sequenced (Tabor & Richardson,

Table I: Protein and Gene Sequences for Wild-Type IL-1β and Insertion Mutants[a]

1. Original sequence

replaced sequence

50 60

F V Q G E E S N D K I P V A L G L K

TTC GTC CAA GGT GAA GAG TCT AAC GAC AAG ATC CCA GTT GCA TTA GGC CTG AAA

AAGCAG GTT CCA CTT CTC AGA TTG CTG TTC TAG GGT CAA CGT AAT CCG GAC TTT

StyI StuI

2. STI loop sequence

STI loop

F V Q G P Y R I R F D K I P V A L G

(1)CAA GGT CCA TAC CGT ATA CGC TTC(3)GAC AAG ATA CCG GTT GCA TTA GG

(2)CA GGT ATG GCA TAT GCG AAG CTG T(4)TCTATGGC CAA CCT AAT CC

3. TOI loop sequence

TOI loop

F V Q G P A C T L E Y R D K I P V A L G

(1)CAA GGT CCA GCA TGC ACT TTA GAG TAT CGC(3)GAC AAG ATA CCG GTT GCA TTA GG

(2)CAGGT CGT ACG TGA AAT CTC ATA GCG CTGT(4)TCTAT GGC CAA CCT AAT CC

4. AT loop sequence

AT loop

F V Q G E A I P M S I P P E D K I P V A L G

(1)CAA GGT GAA GCA ATC CCG ATG AGT ATA CCG CCA GAG(3)GAC AAG ATA CCG GTT GCA TTA GG

(2)CA CTT CGT TAG GGC TAC TCA TAT GGC GGT CTC CTGT(4)TCTAT GGC CAA CCT AAT CC

[a] Plasmid DNA from *Sty*I to *Stu*I sites was replaced in each case by the oligonucleotides shown as numbered in sequences 2, 3, and 4.

1987) and were shown to contain the mutated sequences. Transformation and expression of the mutant constructs has been described previously (Wolfson et al., 1991). Purification of proteins was achieved with a modification of the method of Meyers et al. (1987) for purification of recombinant wild-type IL-1β (Wolfson et al., 1991).

Enzyme Assays. Elastase activity was assayed by a modification of the method of Bieth et al. (1974). A stock solution (25 mg/mL) of the enzyme was prepared in 10^{-3} M HCl and kept on ice. Serial dilutions of substrate [*N*-succinyl-(L-Ala)$_3$-*p*-nitroanilide] (2, 4, or 10 mM) were made in 0.1 M potassium phosphate, pH 7.5. The buffer contained 0.005% (w/v) Triton X-100, to reduce adherence of proteins to the cuvette (Empie & Laskowski, 1982). In some cases, the buffers also included 14 mM mercaptoethanol (Travis et al., 1985). The reaction was carried out in 1-mL disposable plastic cuvettes at room temperature. Ten microliters of a diluted enzyme solution was added to 0.5 mL of buffer or buffer plus an inhibitor. The reaction was initiated by the addition of 0.5 mL of substrate solution. The increase in absorbance at 410 nm, reflecting the release of *p*-nitroaniline from the artificial substrate, was monitored and determined to be linear for at least 5 min.

Chymotrypsin and trypsin activities were assayed in the same way, except that the original solutions of enzyme were 10 mg/mL and the stock substrate concentrations were 0.5 mM (*N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide) and 1 mM (N^{α}-benzoyl-D,L-Arg-*p*-nitroanilide).

Biological Activity Assays. Binding to Il-1 receptors was determined according to the method of Dower et al. (1985) using fibroblasts. Induction of IL-2 in response to IL-1 was determined by the method of Simon et al. (1985) using cultured murine thymomas (EL4). Induction of prostaglandin synthesis in muscle and liver cells was done by the method of Xiao and Levine (1986).

Cleavage of the Mutant Proteins with Proteases. The mutant proteins and wild-type IL-1β were exposed to the proteases trypsin, chymotrypsin, and elastase. Incubations were carried out in a total volume of 5–10 μL, at 25 °C for 10 min. One of the chimeric proteins, or IL-1β, ~5 μg in 0.1 M phosphate buffer, was added to 3 μL of phosphate buffer alone or to a solution of protease, ~0.025 μg in the same buffer. The reaction was terminated by the addition of an equal volume of SDS running buffer, and the solution was boiled for 2 min. Samples were then subjected to SDS–polyacrylamide gel electrophoresis.

RESULTS

The chimeric proteins constructed from IL-1β and loops chosen from protease inhibitors were tested for inhibitory activity against several proteases. Only the mutant containing the elastase-binding loop from AT displayed appreciable inhibition toward its target enzyme (Wolfson et al., 1991).

All of the chimeras were incubated with a variety of proteolytic enzymes to determine susceptibility to proteolysis. Each was cleaved at what appeared to be a single position, with some specificity. Cleavage in all cases gave rise to a fragment with an apparent molecular weight of approximately 14 500, as determined by SDS–polyacrylamide gel electrophoresis. This fragment is consistent with the product expected if cleavage occurs at the specificity site engineered into IL-1β.

When TOI/IL was incubated with chymotrypsin, approximately 50% of the protein remained intact (with a molecular weight of 17 500) and approximately 50% was represented by the band at 14 500. A minor band, with molecular weight below the limits of the standard, could occasionally be discerned. The same pattern was observed for TOI/IL when it was incubated with elastase. After incubation of TOI/IL with trypsin, only the original band at a molecular weight of 17 500 was seen.

When STI/IL was incubated with either elastase or chymotrypsin, most of the protein (>75%) remained intact, with the remainder appearing at a molecular weight of 14 500 and a smaller fragment. Incubation of STI/IL with trypsin led to complete cleavage of the protein; bands appeared at the 14 500 and lower molecular weight positions in approximately equal amounts.

As previously reported (Wolfson et al., 1991), AT/IL at these concentrations was cleaved completely by chymotrypsin and elastase, generating the fragment at 14 500 molecular weight. At higher concentrations of AT/IL, cleavage was

Table II: Bioactivity of Wild-Type Interleukin 1β and Insertion Mutants

sample	% wild-type activity: lymphocyte stimulation[a] (units/mg)	% wild-type activity: PGI_2 production[b] [ng/(4 × 10^5 cells)]	K_I of binding to EL4 cells[c] (pM)
IL-1β	100	100	25
STI/IL	37	nd	13
TOI/IL	6.5	2.4	48
AT/IL	82	23	13

[a] Mutants and wild-type interleukin 1β were assayed for production of IL-2 in EL-4 cells by dilution, as described in Simon et al. (1985). Specific activity of wild-type IL-1β in this assay was 5 × 10^8 units/mg (Meyers et al., 1987). Lower limit of detection is 0.5 unit/mL. [b] Varying concentrations of mutants and wild-type interleukin 1β were assayed for amplification of PGI_2 production by rat liver cells (the C-9 cell line) in the presence of a constant quantity of vasopressin (10 nM). PGI_2 was measured by radioimmunoassay of its stable hydrolytic product, 6-keto-$PGF_{1\alpha}$ (Levine, 1986). Whereas 1.5 pM wild-type IL-1β increased vasopressin's PGI_2 production 2-fold, 6.4 and 63 pM AT/IL and TOI/IL, respectively (interpolated values), were required for equivalent amplification. Vasopressin (10 nM) produced 0.14 ng of PGI_2/(4 × 10^5 cells). [c] Binding was measured as the ability of mutants to compete for the IL-1 receptor on EL-4 cells, as determined via a competition radioreceptor assay using ^{125}I-human IL-1β (Lillquist et al., 1988). The inhibition constants are valid to a factor of 2.

not complete. Incubation for longer times (up to 48 h) or with chymotrypsin and elastase together did not generate any additional fragments. AT/IL was resistant to cleavage by trypsin. Wild-type IL-1β was not at all susceptible to proteolysis by these enzymes under the conditions used.

Cytokine activities for these chimeras are summarized in Table II. Activity was determined in two systems. The comitogenic IL-2 induction assay determines the ability of the chimeras to stimulate production of IL-2 in cultured murine thymomas (EL4 cells) (Simon et al., 1985). The presence of IL-2 causes growth of the cultured CTL20 T-cell line. Both STI/IL and AT/IL displayed considerable lymphokine-like activity. The STI/IL is approximately $^1/_3$ as active as wild-type IL-1β on a units per milligram basis; AT/IL has close to full activity. The second assay looks for stimulation of prostaglandin production in the presence of vasopressin in rat liver cells (Xiao & Levine, 1986). All chimeras tested had reduced activity in this assay. Finally, binding to IL/1 receptors in EL-4 cells was measured via a competitive radioreceptor assay using ^{125}I-human IL-1β (Lillquist et al., 1988). This latter assay suggests that the binding of IL-1s has been impaired very little by the introduction of loops between strands 4 and 5.

DISCUSSION

It is apparent from the activities measured that some of these insertions are compatible with IL-1 activity, whereas others completely abolish activity subsequent to receptor binding. The maintenance or loss of activity is not related to the size of the inserted loop; the largest loop replacement was for AT/IL, and this is the mutant which maintains the highest biological activity, close to wild type in the lymphocyte proliferation assay. Bioactivity is also not obviously correlated to the overall charge on the protein. AT/IL, with the highest bioactivity, is isoelectric with wild-type IL-1β, but STI-IL, which also displayed appreciable activity, has a larger positive charge than either AT/IL or TOI/IL, and TOI/IL showed very low IL-1 activity in either assay.

In spite of the fact that little biological activity was observed with TOI/IL and that STI/IL and AT/IL had activities somewhat lower than wild type, all of these proteins could bind to the cell surface receptor for IL-1 with affinity close to that of wild type. The calculated K_Is differed from that of wild type by a factor of approximately 2, which is within the experimental error of the assay.

Clore et al. (1991) have recently reviewed the effect of a large number of mutations on IL-1 activity and receptor binding. There is some indication that there are separate sites for receptor binding and subsequent events required for biological activity. Gehrke et al. (1990) (R11G), and Young et al. (1990) and Simon et al. (1993) (T9G), have described mutants of IL-1β which differ from wild type by a single amino acid substitution. These mutants display binding affinity close to wild type but have greatly reduced biological activity. Similarly, a naturally occurring antagonist for IL-1β has been found (Eisenberg et al., 1990; Hannum et al., 1990) which binds to receptor without triggering the usual IL-1 response. Our data suggest that even large changes in structure can be tolerated by the receptor in terms of ligand binding but that signal transduction and subsequent activity is more sensitive to alterations.

The region of IL-1β which we have subjected to mutagenesis has not previously been directly implicated in biological activity or receptor binding (Lillquist et al., 1988; Clore et al., 1991; Gehrke et al., 1990; Huang et al., 1987; MacDonald et al., 1986), although Priestle et al. (1989) have identified the loop between β sheets 4 and 5 as one of the solvent-accessible regions in IL-1β which is conserved in IL-1s across four species. Labriola-Tompkins et al. (1991) have identified a binding cleft for receptor which borders the region we have modified. In the case of the hybrids AT/IL and STI/IL, while variations in activity are observed when the loop is modified, a relatively large loop was inserted with little effect on bioactivity. [In the case of TOI/IL, it is possible that inclusion of a Cys residue in the loop resulted in dimerization or other alterations in structure. This possibility is suggested by the circular dichroism (CD) spectra of the mutants relative to wild-type IL-1β. The profile for AT/IL was identical to that of the wild type (Wolfson et al., 1991), whereas that of TOI/IL was clearly changed (data not shown).] Our data are, therefore, in agreement with a large body of evidence that this loop (residues 50–53) is adjacent to the region involved in receptor binding but does not seem to be directly involved. Even though the bioactivity of TOI/IL is considerably lower than that of the others, we cannot distinguish a direct effect of the loop from an effect due to an unpaired cysteine residue. The largest loop is in AT/IL, which did not appear to have a major effect on receptor binding.

Hynes et al. (1989) have shown that it is possible for β-turns to be transferred from one protein context to another, with little loss of ("host") activity for the resulting hybrid. Even more strikingly, Toma et al. (1991) have inserted a calcium-binding loop from thermolysin into the neutral protease from *Bacillus subtilis* and have observed both types of activity in the resulting hybrid. For each of these studies, the inserted region came from a protein structurally similar to the host. For this reason, it was not unexpected that the hybrid containing the loop from STI would have close to full IL-1 activity. The loops substituted from TOI and AT, however, were not expected to have any structural homology to the replaced regions of IL-1β. It is, therefore, surprising that the AT/IL mutant would retain significant biological activity in both bioassay systems examined. The fact that any biological activity was observed with these mutants suggests that the hybrid proteins are folding properly into an IL-1-type structure.

The results presented here, together with our previous findings that the mutant AT/IL can inhibit elastase (Wolfson et al., 1991), suggest that it is possible to design hybrid proteins by using IL-1β as a scaffold. Although the proteases targeted

by these inhibitors are secreted proteins, loops from inhibitors for other, intracellular proteases could equally well be inserted. These hybrids could be recognized by IL-1 receptors on cell membranes and taken up by cells, thereby creating a delivery system for active sites of proteins which could not otherwise enter target cells.

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