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Immunology

Determination of the minimum polypeptide lengths of the functionally active sites of human interleukins 1α and 1β

(*in vitro* expression/*in vitro* mutagenesis/receptor assay)

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ABSTRACT **Interleukin 1 (IL-1) is a two-member family of proteins (IL-1α and IL-1β) that mediates a diverse series of immune and inflammatory responses. These two proteins have only 26% amino acid homology yet bind to the same receptor. It is of importance to define the active sites of these molecules in order to understand their receptor interactions and the mechanisms involved in their multiple biological functions. We report here the localization of the biologically active portions within the initial polypeptide translation products. An *in vitro* transcription and translation system was used to generate specific fragments of each of the IL-1 molecules, which then were assayed for receptor binding capability and biological activity. Using this system, we have demonstrated that core sequences of 147 amino acids for IL-1β (numbers 120–266) and 140 amino acids for IL-1α (numbers 128–267) must be left intact to retain full biological activity and further that the biological activities of the IL-1 polypeptides parallel their receptor binding capabilities.**

Human interleukin 1 (IL-1), a polypeptide hormone produced by activated macrophages and other cell types, modulates a wide variety of immune and inflammatory responses by its ability to regulate proliferation, maturation, or functional activity of a broad spectrum of cell types (1–3). These activities of IL-1 include murine thymocyte stimulation *in vitro*, interleukin 2 (IL-2) production, acute-phase protein production by hepatocytes, bone resorption, fever, prostaglandin E_2 production, and chemotactic effects on leukocytes. Protein purification from various cell sources has yielded IL-1 biological activity in polypeptides ranging in molecular mass from 2 to 75 kDa, with the predominant species at 17.3 kDa (2). Isoelectric points for proteins containing IL-1 activity vary from 4.0 to 8.0, with two predominant species at pI values 5.0 and 7.0 (4–6).

cDNA cloning of IL-1 from human monocytes has shown that IL-1 biological activities are not due to a single molecular entity but to a class of at least two distinct polypeptides termed IL-1α and IL-1β (7). Although these two proteins have only 26% amino acid homology, both forms of IL-1 bind to the same cell surface receptor (8) and, where tested, their biological activities appear to be nearly identical (9).

Both IL-1α and IL-1β are initially synthesized as 31-kDa intracellular precursors (of 271 and 269 amino acids, respectively) that are subsequently found as mature proteins of 17 kDa in monocyte supernates (5, 7). Amino acid sequencing of the mature forms of IL-1 have defined amino acid 117 as the amino terminus of IL-1β (7, 10, 11) and 119 as the amino terminus of at least one form of IL-1α (5). The amino terminus of the mature form of murine IL-1α has been located at amino acid 115, which corresponds to amino acid 113 of the human form (7, 12). The carboxyl terminus of mature IL-1β has been defined at amino acid 269 (7), while that of mature IL-1α must be at or close to amino acid 271.

The mechanism by which IL-1 or the IL-1 receptor complex mediates such a vast range of biological responses is largely unknown. It will be of importance to define the active site(s) of the IL-1 molecule in order to understand its interaction with the IL-1 receptor and to determine if the multiple biological functions are due to multiple structural domains of IL-1.

To localize more precisely the active regions of the IL-1 molecules, we constructed a series of truncated forms of both IL-1α and IL-1β by removing amino acids from both the amino-terminal and carboxyl-terminal ends until we had defined for both molecules the smallest polypeptide capable of binding to the receptor and producing a biological response. We describe here the use of an *in vitro* expression system to generate truncated forms of both IL-1α and IL-1β. These truncated forms were then assayed for both their ability to stimulate IL-2 production (by the murine EL-4-6.1-C10 cell line) and their ability to bind to the IL-1 receptor.

MATERIALS AND METHODS

Construction of Bacteriophage SP6 Expression Vectors. Phage SP6 vectors SP64 and SP65 (13) were supplied by Promega Biotec (Madison, WI). Appropriate restriction fragments for IL-1α and IL-1β were from cDNA clones previously described (7). The construction of expression vectors for the full-length forms IL-1β-(1–269) and IL-1α-(1–271), designated β1-269 and α1-271, respectively, and for the mature forms IL-1β-(117–269) and IL-1α-(113–271), designated β117-269 and α113-271, respectively, have been described elsewhere (14). Most of the amino-terminally truncated forms of IL-1β were constructed by three fragment ligations using the *Nco* I/*Pst* I vector fragment from the expression plasmid for full-length IL-1β-(1–269), synthetic oligonucleotide duplexes with *Nco* I and *Hpa* II compatible ends that specified a translational initiation codon followed by codons for appropriate amino acids, and a *Hpa* II/*Pst* I fragment encoding amino acids 127–269 of IL-1β from the expression plasmid for mature IL-1β-(117–269). The β71-269 plasmid was constructed by digesting β1-269 with *Nco* I (which cuts at amino acids 1 and 71) and religating. Carboxyl-terminal truncations were based on the construction of β117-259. For this construct, the *Hph* I site located near amino acid 259 was blunted and ligated to a blunted *Xba* I site in the polylinker of the SP65 vector. This created a silent base change in the codon for Ile-259, followed by a stop codon. This base change also created an *Eco*RV site that would, after cleavage, leave a blunt end after the codon for amino acid 258. Synthetic oligonucleotides were then made that extended from the *Eco*RV site and specified codons for amino acids up to the appropriate terminus, followed by a stop codon and

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Abbreviation: IL-1 and -2, interleukins 1 and 2.

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nucleotide sequences recreating the polylinker region of SP65 up to the *Pst* I site. These oligonucleotides were ligated into an *Eco*RV/*Pst* I vector fragment isolated from the β117-259 construct.

Constructs for IL-1α amino-terminal truncations were made by a mechanism analogous to the IL-1β constructs. Oligonucleotides were synthesized with an *Nco* I site at the initiator methionine and with codons specifying the desired amino terminus, followed by appropriate amino acids up to the *Eco*RI site near amino acid 134. These oligonucleotides along with the *Eco*RI/*Bam*HI structural region of IL-1α specifying amino acids 134–271 were ligated to the *Nco* I/*Bam*HI vector fragment of the full-length IL-1α expression plasmid (14). Constructs α113-271 and α115-271 were made using oligonucleotides specifying amino acids up to the *Alu* I site at amino acid 117. These were ligated to the *Alu* I/*Bam*HI structural region and the *Nco* I/*Bam*HI vector fragment as described above. The α61-271 construct was formed by using synthetic oligonucleotides to create an in-frame initiation codon, which was ligated to a blunted *Hin*dIII site at amino acid 64 in the full-length form (α1-271). Plasmids for the carboxyl-terminal truncations of IL-1α were made by synthesizing oligonucleotides from the *Sau*96I site near amino acid 257 incorporating codons for the proper amino acids until the desired carboxyl terminus was reached. A stop codon was added with polylinker sequences extending to the *Bam*HI site of SP65. These oligonucleotides were ligated to the *Nco* I/*Sau*96I fragment of mature IL-1α specifying amino acids 113–257 and the *Nco* I/*Bam*HI vector as above. Constructs are designated by either an α or β to indicate either IL-1α or IL-1β sequences followed by the amino acids in that construct numbered as previously described (7).

***In Vitro* Transcription and Translation.** DNA was prepared by a standard alkaline lysis procedure (15), followed by purification over 0.5-ml Sephadex G-50 spun columns to remove small molecular mass contaminants. RNA transcription was done as described (Promega Biotec Riboprobe technical literature) with the modifications described previously (14). Synthesized RNA was translated without further modification in a rabbit reticulocyte lysate translation system (16, 17). After translation, lysates were spun through Sephadex G-50 columns to remove small molecular mass contaminants responsible for high backgrounds in the receptor binding assay.

Protein Product Analysis. Aliquots (2 μl) of ^{35}S-labeled reticulocyte translation products were precipitated with trichloroacetic acid; from the amount of labeled methionine incorporated, the concentration of synthesized protein was determined. This approach can be justified in two ways. Analysis by size separation on a 15–20% exponential polyacrylamide gel (18) showed that >80% of the loaded counts were in the appropriately sized band. Furthermore, independent analysis of translation products by radioimmunoassay gave equivalent protein concentrations (14).

Receptor Binding Assay. *In vitro* translated IL-1 proteins were tested for their ability to compete with radioiodinated recombinant IL-1α for binding to receptors on EL-4 6.1 C10 cells as described (14), except that purified translation products were used (as noted above).

Biological Assay. Biological activity was assayed by the capacity of IL-1 to stimulate IL-2 release from EL-4 6.1 C10 cells (19). The assay was conducted using protocols described previously (14, 20).

RESULTS

Synthesis and Translation of IL-1 RNAs. We have reported (14) an *in vitro* system that can be used to express biologically active IL-1 molecules. Insertion of the IL-1 structural gene downstream of the SP6 RNA polymerase promotor in the

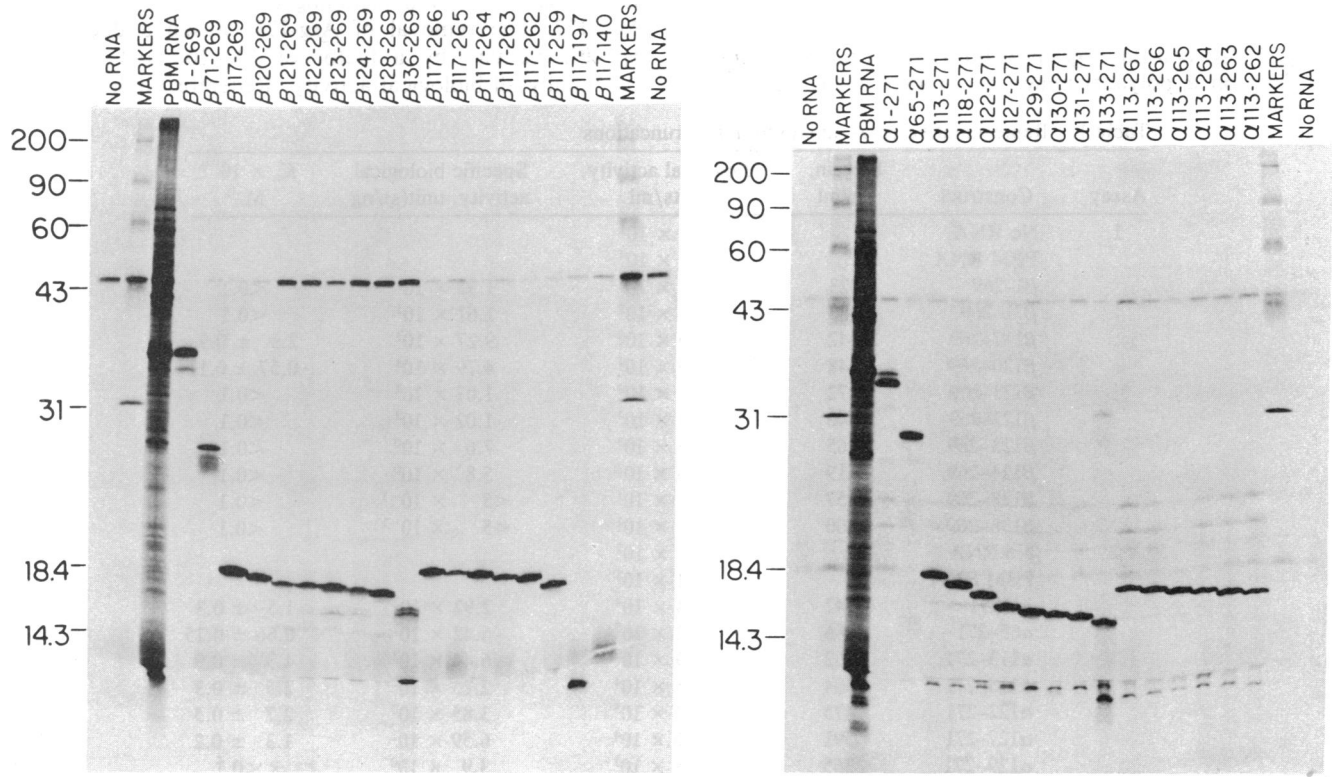

FIG. 1. NaDodSO$_4$/polyacrylamide gel analysis of *in vitro* translated IL-1 peptides. NaDodSO$_4$/polyacrylamide gel electrophoresis was performed as described. Each lane represents translated products from the construct indicated above. PBM RNA was purified from human peripheral blood monocytes stimulated with lipopolysaccharide as described (7). (*Left*) Translation products of the IL-1β truncation series. (*Right*) Translation products of the IL-1α truncation series. Indicated molecular mass of standard proteins are in kilodaltons.

SP65 vector allows synthesis of large amounts of pure IL-1 RNA. With no further processing of the RNA, translations are performed in a rabbit reticulocyte translation system, yielding between 10 and 40 ng of protein per μg of RNA (unpublished data).

Cloning of the IL-1 full-length cDNAs into the SP65 vector required the use of synthetic oligonucleotides, the exact sequence of which has been published (14). In the case of IL-1β, the oligonucleotide was initiated with an *Eco*RI site compatible with the polylinker region of SP65. This was followed by the natural sequence for IL-1β starting about 20 base pairs upstream of the initiation codon. The oligonucleotide was then extended to recreate the *Sst* I site at approximately amino acid 8. This oligonucleotide, with the *Sst* I/*Pst* I structural fragment of IL-1β, was inserted into the *Eco*RI/*Pst* I vector fragment of SP65. The synthetic oligonucleotide for IL-1α was analogous to the IL-1β oligonucleotide, extending from an *Eco*RI site upstream of the initiation codon to a *Bal* I site at amino acid 2 of the IL-1α gene. In this case, however, the sequence immediately upstream of the initiation codon was changed to conform with the consensus translational initiation sequence as published by M. Kozak (21). This sequence allowed much higher translation rates than that of the natural IL-1α gene. The translational initiation sequence of the IL-1β gene conforms closely with the consensus sequence, and high translation rates were achieved without modification.

Analysis of *in Vitro*-Translated Protein Products. Because the IL-1 RNAs generated by SP6 polymerase were translated in an *in vitro* translation system where no processing of the polypeptide occurs, the presence of an initiator methionine is unavoidable. In some instances the methionine used as an initiator was already contained in the IL-1 sequence (α1-271, α127-271, β1-269, and β71-269). In other cases there is a Met-Gly sequence on the amino terminus of the IL-1 construct (α65-271 and α118-271). All other constructs contain only a methionine on the amino terminus of the indicated polypeptide.

Fig. 1 *Left* is a fluorogram of the IL-1β truncation series analyzed on a polyacrylamide gel. Lanes 4–13 show ^{35}S-labeled translation products from amino-terminal truncations, while lanes 14–21 show the products of carboxyl-terminal deletions.

Fig. 1 *Right* shows the translation products for the IL-1α deletion series, where lanes 4–13 represent amino-terminal deletions, and lanes 14–19 show the protein products of the carboxyl-terminal deletions.

Biological and Receptor Binding Activities. Each series of truncations were assayed between four and seven times, and although the absolute amount of biological activity varied from assay to assay, the relative activity of the constructs within each assay was consistent. The data presented in Table 1 (also see Fig. 2) are representative of the amino-terminal truncations. The full-length translation product of IL-1β (β1-269) contained no significant biological activity and no detectable receptor binding activity, consistent with earlier reports (7, 14). Removal of 70 amino acids from the amino-terminal end of the full-length protein (β71-269) also produced a polypeptide that was inactive in both assays. However, the removal of 116 amino acids (β117-269) from the amino terminus to produce the mature form of IL-1β resulted in a protein giving full activity in both the biological and receptor binding assays. The further removal of three amino acids from this amino terminus reduced activity by a factor of 4–5, but the removal of Arg-120 reduced the specific activity by a factor of >100 from that of the mature form. Removal of the next 2 or 3 amino acids completely abolished biological activity. The receptor binding constants (K_a) paralleled the biological activity, with detectable binding only in the mature form and the β120-269 form of IL-1β. The removal of Arg-120 abolished receptor binding activity.

In contrast to the full-length IL-1β polypeptide, the full length form of IL-1α (α1-271) has significant biological activity. High specific activities (within 10-fold of the mature form) were maintained through the amino-terminal truncation series up to and including the construct initiating at Met-127. The removal of Arg-128 caused a decrease in specific activity by a factor of 100–200 and a loss of essentially all biological activity. Receptor binding activity

Table 1. Representative data—amino-terminal truncations

Assay	Construct	Protein, ng/ml	Biological activity, units/ml	Specific biological activity, unit(s)/ng	$K_a \times 10^{-9}$, M^{-1}
1	No RNA		$<5 \times 10^1$		
	PBM RNA		1.42×10^4		
	β1–269	323	4.31×10^3	1.33×10^1	<0.1
	β71–269	368	5.94×10^3	1.61×10^1	<0.1
	β117–269	442	4.10×10^6	9.27×10^3	2.9 ± 0.3
	β120–269	248	1.19×10^6	4.79×10^3	0.57 ± 0.11
	β121–269	272	2.91×10^4	1.07×10^2	<0.1
	β122–269	140	1.43×10^4	1.02×10^2	<0.1
	β123–269	165	1.26×10^3	7.6×10^0	<0.1
	β124–269	519	3.00×10^3	5.8×10^0	<0.1
	β128–269	57	$<5 \times 10^1$	$<5 \times 10^{-1}$	<0.1
	β136–269	100	$<5 \times 10^1$	$<5 \times 10^{-1}$	<0.1
2	No RNA		$<2 \times 10^1$		
	PBM RNA		7.61×10^3		
	α1–271	142	4.14×10^4	2.92×10^2	1.5 ± 0.3
	α65–271	486	3.12×10^5	6.42×10^2	0.66 ± 0.15
	α113–271	262	1.69×10^5	6.44×10^2	4.3 ± 0.9
	α118–271	304	6.85×10^4	2.25×10^2	2.5 ± 0.3
	α122–271	475	1.83×10^5	3.85×10^2	2.7 ± 0.5
	α127–271	591	3.90×10^4	6.59×10^1	1.3 ± 0.2
	α129–271	365	1.43×10^3	3.9×10^0	<0.1
	α130–271	386	1.28×10^3	3.3×10^0	<0.1
	α131–271	143	2×10^1	1×10^{-1}	<0.1
	α133–271	463	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1

PBM, peripheral blood monocytes.

Table 2. Representative data—carboxyl-terminal truncations

Assay	Construct	Protein, ng/ml	Biological activity, units/ml	Specific biological activity, unit(s)/ng	$K_a \times 10^{-9}$, M^{-1}
3	No RNA		$<2 \times 10^1$		
	PBM RNA		1.51×10^3		
	β117–269	183	8.58×10^4	4.69×10^2	2.9 ± 0.3
	β117–266	319	1.16×10^5	3.62×10^2	1.5 ± 0.2
	β117–265	108	3.27×10^3	3.02×10^1	0.67 ± 0.11
	β117–264	177	2.38×10^3	1.35×10^1	0.96 ± 0.26
	β117–263	185	4.28×10^2	2.32×10^0	0.41 ± 0.9
	β117–262	165	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
	β117–259	62	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
	β117–197	136	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
	β117–140	9.3	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
4	No RNA		$<2 \times 10^1$		
	PBM RNA		8.25×10^3		
	α113–271	262	1.51×10^5	5.17×10^2	4.3 ± 0.09
	α113–267	185	5.28×10^4	2.35×10^2	0.6 ± 0.12
	α113–266	241	2.76×10^4	8.31×10^1	1.0 ± 0.1
	α113–265	353	8.57×10^2	1.4×10^0	0.25 ± 0.03
	α113–264	605	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
	α113–265	332	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1
	α113–262	225	$<2 \times 10^1$	$<1 \times 10^{-1}$	<0.1

paralleled biological activity, with binding detectable until Arg-128 was removed.

Data for the carboxyl-terminal truncations are presented in Table 2 and Fig. 2. Removal of three amino acids from the carboxyl terminus of IL-1β had little effect on the specific activity (>10-fold). Removal of the next four amino acids sequentially reduced the biological activity of the resulting protein to less than detectable levels. For the IL-1α series, four amino acids could be removed without a significant loss of biological activity, but the sequential loss of the next three amino acids abolished any activity. Receptor binding ability parallels biological activity at the carboxyl-terminal end, with receptor binding ability being lost over the same amino acid deletions as the biological activity.

DISCUSSION

Through the use of *in vitro* transcription and translation systems, we have generated various polypeptide forms of IL-1α and IL-1β. With this system we have determined the minimum polypeptide length of IL-1α and IL-1β required for biological activity and have shown that the IL-1 receptor

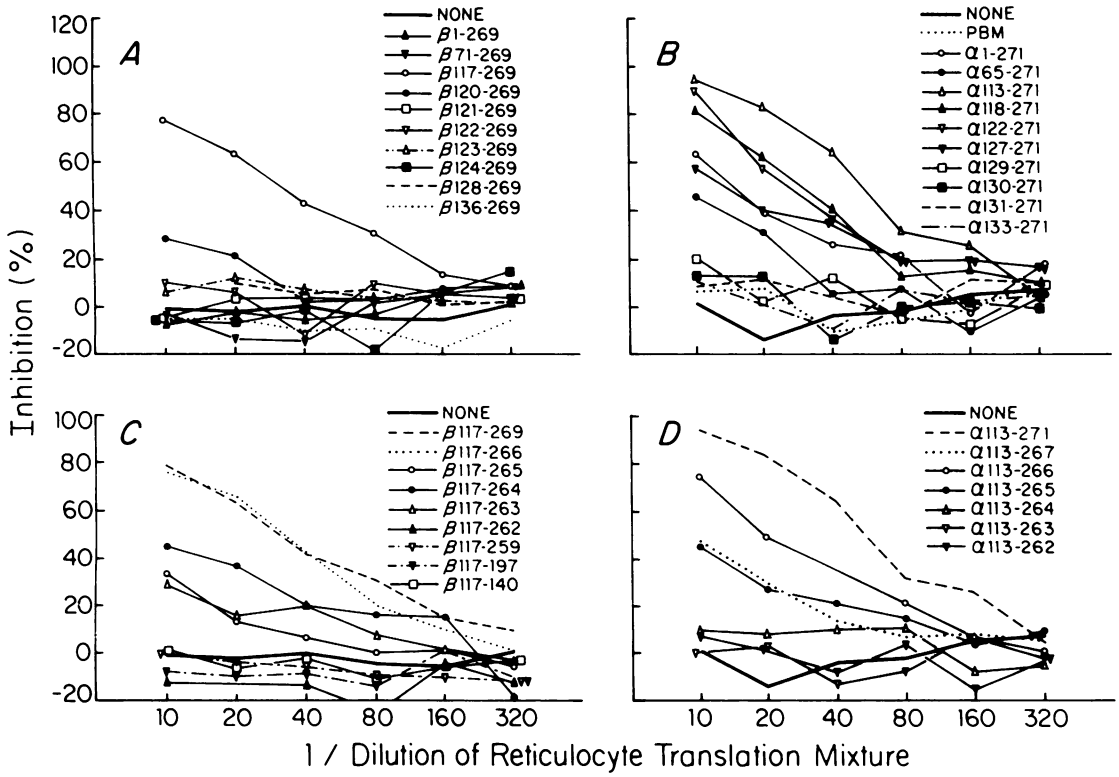

FIG. 2. Inhibition of ^{125}I-labeled IL-1α binding to EL-4 6.1 C10 cells at 8°C by *in vitro* translated polypeptides. Assays were performed as described (20). Control curves were generated by using either a translation mixture in which no RNA or PBM RNA was included. (*A*) IL-1β amino-terminal truncations. (*B*) IL-1α amino-terminal truncations. (*C*) IL-1β carboxyl-terminal truncations. (*D*) IL-1α carboxyl-terminal truncations.

binding capabilities of the polypeptides parallel this activity. However, it should be noted that the sensitivity of the biological assay allows detection of IL-1 polypeptides with >0.01% of the activity of mature IL-1, yet the receptor binding assay detects only those polypeptides with >2% of maximal activity. This system has significant advantages over those used by other groups to assess the minimum size of IL-1 (22, 23). Specifically we are able to generate much higher levels of IL-1 activity than reported for COS cell transfections (23) without background IL-1 biological activity. Furthermore, each IL-1 polypeptide can be visualized on a polyacrylamide gel and can be quantitated. Additionally, the IL-1 polypeptides can be assayed without further purification.

The initial transcript of the IL-1β gene is biologically inactive and shows no receptor binding. However, after cleavage of the amino terminal 116 amino acids, full biological activity is attained. This activation occurs with the removal of a maximum of 46 amino acids proximal to the mature start site at amino acid 117. The maximum size of biologically active IL-1β was not determined. Biological activity was markedly decreased with the removal of the arginine at position 120.

Carboxyl-terminal truncations of IL-1β showed that the largely hydrophobic region near the carboxyl terminus of the mature protein is required for biological activity and receptor binding. Double truncations (β120-265 and β120-266) gave biological activities comparable to those of the parallel single truncations (unpublished data). Attempts to reconstitute biological activity by mixing an amino-terminally truncated nonactive polypeptide (β136-269) with a series of carboxyl-terminally truncated nonactive polypeptides (β117-140, β117-197, and β117-259) were unsuccessful, indicating that both regions must be present on the same polypeptide to retain biological activity (data not shown).

Truncations of IL-1α showed similar requirements for biological activity as did truncations of IL-1β. Activity was maintained in amino-terminal deletions until the arginine at position 128 was removed; at the carboxyl terminus, the hydrophobic region must be retained for biological activity and receptor binding.

Therefore, the minimum polypeptide size for retention of full biological and receptor binding activities for IL-1β consists of 147 amino acids extending from 120 to 266. The minimum size for IL-1α is 140 amino acids, representing amino acids 128 through 267. Both minimal IL-1 forms require an arginine at the amino terminus. It is striking that a basic amino acid is found at homologous positions in most IL-1 molecules yet characterized, namely Lys-130 in murine IL-1α (12) and Arg-128 in rabbit IL-1α (24). An exception is Gln-117 in bovine IL-1β, although lysine appears at positions 120 and 122 (unpublished results). DeChiara *et al.* (22) reported that murine IL-1α starting at Leu-131 retained full biological activity, but the construction used to express this IL-1 analog fused a small peptide upstream of Leu-131 and, thus, reintroduced an arginine codon at the equivalent of amino acid 129. Thus, it remains possible that a basic amino acid around this position in IL-1 is essential for biological activity. This provides a structural explanation for earlier observations from chemical modification studies that at least one arginine is required for IL-1 biological activity (25).

Our data is in disagreement with previous reports of biologically active subfragments of IL-1 having molecular weights of 2–7 kDa (23, 26, 27). The paper of Rosenwasser *et al.* (23) in which deletion mutants of IL-1β were transfected into COS cells described extremely low and variable levels of IL-1 produced by the cells. In no case were the biological activities characterized at the molecular level. It remains possible that smaller peptide fragments retain some biological activity below the level of detection in our assay. However, such peptides must have a specific activity less than that of the mature form by a factor of at least 10,000. From the data presented here, the smallest polypeptide capable of eliciting maximal biological response is 147 amino acids for IL-1β and 140 amino acids for IL-1α.

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