Analysis of mutations in the putative nuclear localization sequence of interleukin-1 β

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Previous studies have shown that, after receptor-mediated endocytosis, interleukin-1 α (IL1 α) and interleukin-1 β (IL1 β) are translocated to the nucleus, where they appear to accumulate. It has been suggested that nuclear translocation may be involved in the biological responsiveness of target cells to IL1 stimulation. The human IL1 β molecule contains a seven-amino-acid sequence (-Pro²⁰⁸-Lys-Lys-Lys-Met-Glu-Lys-) that shows some sequence identity with the nuclear localization sequence of the simian-virus-40 large T-antigen. The effects of point mutations within this putative nuclear localization sequence on IL1 β binding, receptor-mediated endocytosis and biological activity have been characterized. Mutants M49 (Lys²¹⁰ → Ala), M50 (Lys²¹¹ → Ala) and M51 (Pro²⁰⁸ → Ala) all retained the ability to bind to the IL1 receptor, albeit with lower affinity than the wild-type molecules. However, mutants M49, M50 and M51 showed greater biological potency than wild-type IL1 α or IL1 β , as measured by the induction of IL2 secretion. However, receptor-mediated endocytosis and nuclear accumulation of M50 were comparable with those in the wild-type. These observations suggest that the putative nuclear localization sequence may play an important role in the generation of biological responses to IL1 stimulation, even though it may not influence internalization of the ligand.

INTRODUCTION

The traffic of macromolecules into and out of the nucleus is an essential process for cellular function and requires appropriate sorting mechanisms to ensure targeting to the correct destination. The translocation of proteins from the cytosol into the nucleus appears to depend, at least in part, upon the recognition of a specific nuclear localization sequence (NLS). These NLSs have been identified in a variety of proteins from viruses to mammals and are characteristically short stretches of basic amino acid residues [1–3]. Perhaps the best-studied NLS is that of the simian-virus-40 (SV40) T-antigen, which has the sequence:

-Pro-Lys-Lys-Lys-Arg-Lys-Val-

There is evidence that some polypeptide hormones, including interleukin-1 (IL1), are translocated to the nucleus after receptor-mediated endocytosis [4]. The predicted amino acid sequences of IL1 [5–7], and the IL1 receptor [8] contain basic regions which are reminiscent of the SV40 T-antigen NLS motif. The biological significance of nuclear transport and the importance of the putative NLS are still not clear; however, there are indications that it may play an important role in the generation of appropriate biological responses [9–11]. In order to investigate the functional significance of the putative NLS in IL1 β , we have prepared a series of point mutations in this region of the protein and have measured both the receptor-binding characteristics, receptor-mediated endocytosis and biological activities of the modified forms of the recombinant molecule.

MATERIALS AND METHODS

Materials

Na¹²⁵I, ¹²⁵I-labelled IL1 β and ¹²⁵I-labelled Bolton and Hunter

reagent were purchased from Amersham (Amersham, Bucks., U.K.), Millilitre plates from Millipore (Bedford, MA, U.S.A.), and PD10 columns from Pharmacia (Milton Keynes, Bucks., U.K.). Myoclonal foetal-calf serum (FCS) and RPMI 1640 were purchased from Gibco, Uxbridge, Middx. All other reagents, of the highest purity available, were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Cell culture

EL4 6.1 and CTLL cell lines were routinely grown in suspension culture in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μ g/ml), 2 mM-glutamine, and 20 μ M-2-mercaptoethanol (2-ME). CTLL cells were additionally supplemented with interleukin-2 (IL2; 2 units/ml).

Preparation of recombinant-derived IL1

Point mutations were introduced into the human IL1 β molecule by site-directed mutagenesis as described previously, and wild-type IL1 α , IL1 β and site-directed mutants were all purified as described previously [12–14]. The concentration of all IL1 preparations was measured by both colorimetric methods and by determination of the A_{280} as previously described [12–14].

Iodination of IL1

IL1 α was iodinated by the method of Salacinski *et al.* [15]. Briefly, 9.25 μ g of recombinant-derived human IL1 α [in phosphate-buffered saline (20 mM-sodium phosphate/150 mM-NaCl), pH 7.5] were incubated with 37 MBq of Na¹²⁵I (diluted 1:1 in 0.5 M-phosphate buffer, pH 7), in a glass tube coated with 0.5 μ g of Iodogen. The reaction was allowed to proceed on ice for 30 min, then the incubation mixture was desalted on a PD10 column (equilibrated with phosphate-buffered saline and 0.2%

Abbreviations used: IL1 β etc., interleukin-1 β etc.; NLS, nuclear localization sequence; FCS, foetal-calf serum; 2-ME, 2-mercaptoethanol; EC₅₀, concentration causing half-maximal effect; FGF, fibroblast growth factor; bFGF, basic FGF.

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BSA). Fractions (500 μ l each) were collected, and the appropriate fractions pooled. In all cases, the incorporation of 125 I was in the range of 60–80 %, and the final products exhibited a specific radioactivity of 450–2000 Ci/mmol. The preparation of 125 I-IL1 α was found to be at least 90 % bindable, as assessed by the method of Chin *et al.* [16]. The biological activity of 125 I-IL1 α was tested in the EL4/CTLL IL2 production assay, and was found to be identical with that of the native unlabelled protein.

M50 was radioiodinated with the di-iodo derivative of the Bolton and Hunter reagent according to the manufacturer's instructions, giving a specific radioactivity of approx. 300 Ci/mmol.

Binding studies

EL4 cells were resuspended in binding medium (RPMI 1640 supplemented with 25 mM-Hepes, 5 % myocloned FCS and 0.5 % NaN_3), and 50 μ l aliquots were placed in wells of a 96-well 0.2 μ m-pore-size Millititre filtration plate (Millipore). 125 I-IL1 α and IL1 α were added at the concentrations indicated in the Figure legends. After incubation to equilibrium for 4 h at 37 °C, the unbound 125 I-IL1 α was separated from the cells by vacuum filtration, followed by four washes with washing medium (RPMI 1640 supplemented with 25 mM-Hepes/0.5 % NaN_3). The filtration plates were dried for 20 min at 80 °C, and the filtration membranes were subsequently punched out and counted for radioactivity in an LKB Clinigamma counter.

Endocytosis studies

Receptor-mediated endocytosis and nuclear accumulation of 125 I-labelled IL1 α , IL1 β and M50 were quantified as previously described [17]. Radiolabelled ligand was allowed to bind to EL4 cells overnight at 4 °C, in the presence or absence of a 100-fold molar excess of unlabelled ligand, to determine non-specific binding. After binding, cells were washed extensively with ice-cold medium to remove unbound ligand, and the total radioactivity associated with the cells was determined. The cells were rapidly warmed to 37 °C and incubated for various lengths of time. At timed intervals, aliquots of cells were removed and washed either with ice-cold acid buffer (0.15 M-NaCl/10 mM-sodium acetate, pH 3.5) or with medium containing 2 % Triton X-100. Acid washing removed any ligand remaining on the cell surface, and consequently radioactivity remaining in the cell pellet after acid washing was taken to represent internalized ligand. Radioactivity remaining associated with the cell pellet after Triton washing was taken to represent nuclear accumulation, as described previously [17]. In order to compare the different forms of IL1, internalization and nuclear accumulation were calculated as the percentage of the total cell-associated radioactivity at zero time.

IL2 production assay

EL4 6.1 cells were plated out on a 96-well plate at a cell density of 10^5 cells/well, and incubated with the indicated concentrations of IL1 α , IL1 β or the mutants M49, M50 and M51. The final volume was 200 μ l/well. After 24 h, 100 μ l aliquots of supernatant were removed, taking care not to disturb the EL4 6.1 cells, and assayed for IL2 content. IL2 was measured by the proliferation of an IL2-dependent cell line, CTLL. On the day of passage, CTLL cells were starved of IL2 for 24 h. The following day, 10^4 cells were added to a 96-well plate, containing 100 μ l of test supernatant or 100 μ l of IL2 at known concentrations. The cells were incubated at 37 °C for 24 h, before being pulsed with [3 H]thymidine (1 μ Ci/well). The cells were

incubated for a further 24 h before harvesting on to filter mats using a Scatron semi-automatic harvester. The filter mats were dried for 20 min at 80 °C, and the [3 H]thymidine incorporated into the cells was counted in an LKB 1205 Betaplate liquid-scintillation counter.

RESULTS

The predicted amino acid sequence of human IL1 β [5–7] contains a polybasic region which shows some sequence identity with the nuclear localization sequence (NLS) of the SV40 T-antigen. In the present study, three point mutations were introduced into this putative NLS of IL1 β by site-directed mutagenesis (Table 1). These mutant forms of IL1 β were used to determine the importance of this putative NLS for IL1 binding, receptor-mediated endocytosis and biological responsiveness.

Our first aim was to determine whether the point mutations modified the ability of IL1 β to bind to the IL1 receptor. The binding of wild-type and mutant forms of IL1 to the type I IL1 receptor was determined by competition binding studies on EL4 cells. For these studies, azide-treated EL4 cells were incubated with 66 pM- 125 I-IL1 α to equilibrium for 4 h at 37 °C in the presence of increasing concentrations of unlabelled IL1 α , IL1 β and the three mutants M49, M50 and M51. The relative receptor-binding affinity of each form of IL1 was determined by the concentration of unlabelled IL1 required to inhibit binding of 125 I-IL1 α by 50 % (defined as the K_i). As Fig. 1 shows, unlabelled IL1 α , IL1 β and the mutants M49, M50 and M51 competed for 125 I-IL1 α binding to its receptor in a dose-dependent manner, although the K_i differed for each form of IL1. The relative affinity for binding to the IL1 receptor was estimated for each form of IL1 by calculating the ratio of their respective K_i values to the K_i of IL1 α . Analysis of the binding data by this means revealed that the various forms of IL1 could be ranked in terms of their affinity for receptor binding as follows:

$$\text{IL1}\alpha > \text{M50} > \text{IL1}\beta > \text{M49} > \text{M51} \text{ (Table 2)}$$

In order to quantify the biological activity of IL1 α , IL1 β , M49, M50 and M51, the induction of IL2 secretion from EL4 cells after stimulation with each form of IL1 was determined. IL2 secretion was quantified by measurement of the proliferation of CTLL cells after their stimulation with EL4 conditioned supernatant. A comparison of the ability of IL1 α , IL1 β , M49, M50 and M51 to stimulate IL2 release from EL4 cells is shown in Fig. 2. Both IL1 α and IL1 β were equipotent in inducing IL2 secretion; the half-maximal concentration (EC_{50}) of IL1 required to stimulate IL2 secretion was 458 fM for IL1 α and 475 fM for IL1 β . The three site-directed mutants, M49, M50 and M51, were all capable of inducing IL2 secretion and, indeed, each mutant was more potent in this respect than wild-type IL1 α . The relative potency of the wild-type and mutant IL1s was calculated as the ratio of their respective EC_{50} value to the EC_{50} of IL1 α . These relative potencies were calculated for each form of IL1 and are shown in

Table 1. Putative NLSs for human IL1 β from Pro²⁰⁸ to Lys²¹⁴

Protein	Putative NLS	Mutation
SV40 antigen	Pro-Lys-Lys-Lys-Arg-Lys-Val	–
IL1 β	Pro-Lys-Lys-Lys-Met-Glu-Lys	–
M49	–	Pro-Lys- <i>Ala</i> -Lys-Met-Glu-Lys
M50	–	Pro-Lys-Lys- <i>Ala</i> -Met-Glu-Lys
M51	–	<i>Ala</i> -Lys-Lys-Lys-Met-Glu-Lys

Fig. 1. Competitive inhibition of ^{125}I -IL1 α binding to EL4 cells

EL4 cells were incubated to equilibrium with 66 pM- ^{125}I -IL1 α in the presence of increasing concentrations of unlabelled IL1 α (○), IL1 β (●) or M51 (□) (a) or unlabelled IL1 α (○), M49 (▲) or M50 (■) (b). The concentration of unlabelled IL1 required to achieve 50% inhibition of ^{125}I -IL1 α binding was determined from these competition curves (K_i). Each point represents the mean of triplicate determinations and is a representative of two independent experiments. The experimentally determined K_i for each agonist was compared with the K_i for IL1 α to calculate a 'relative K_i ' (see Table 2).

Table 2. On the basis of these calculations the different forms of IL1 can be ranked in order of biological potency as follows:

$$\text{M50} > \text{M49} > \text{M51} > \text{IL1}\alpha = \text{IL1}\beta$$

The data presented reveal differences in both receptor binding and biological potency between the wild-type and mutant forms of IL1. The mutants bound to the IL1 receptor with lower affinity than IL1 α ; however, all three mutants had greater biological potency when compared with the wild type. The concept of efficacy describes the ability of different agonists to initiate the same biological response when occupying different proportions of receptors. We estimated a value for efficacy for

Fig. 2. IL1 induction of IL2 secretion by EL4 cells

EL4 cells were incubated for 24 h at 37 °C with the indicated concentrations of IL1 α (○) or M51 (□) (a) or with IL1 α (○), IL1 β (●), M49 (▲) and M50 (■) (b). The concentration of IL1 required to induce a half-maximal biological response was determined from these dose-response curves (EC_{50}). Each point represents the mean $\pm$ S.E.M. of quadruplicate determinations and is representative of two independent experiments. The experimentally determined EC_{50} for each agonist was compared with the EC_{50} for IL1 α to calculate a 'relative EC_{50} ' (see Table 2).

each form of IL1 as the ratio of relative K_i /relative EC_{50} (Table 2). The different forms of IL1 could consequently be ranked in order of relative efficacy as follows:

$$\text{M49} > \text{M50} > \text{M51} > \text{IL1}\beta > \text{IL1}\alpha$$

The data presented demonstrate that mutations in the putative NLS of IL1 β have profound effects on the biological potency of the molecule. We wished to determine whether these changes could be correlated to an alteration in the normal intracellular

Table 2. Comparison of the relative affinities (K_i), relative biological potency (EC_{50}) and efficacies of IL1 α , IL1 β , M49, M50 and M51

The values of K_i and EC_{50} for each agonist was calculated using the ALLFIT program [44]. The relative K_i and EC_{50} for each agonist was calculated by normalizing the values for IL1 α to unity. The ratio of K_i/EC_{50} was taken as a measure of efficacy.

IL1/agonist	Relative K_i	Relative EC_{50}	Relative efficacy
IL1 α	1	1	1
IL1 β	6	1.04	5.8
M49	20	0.082	244.0
M50	2	0.015	133.0
M51	34	0.36	94.4

routing of IL1. We have previously demonstrated that, after binding of IL1 α to its receptor, it undergoes internalization and accumulates in a detergent-insoluble intracellular compartment in an energy- and temperature-dependent fashion [17]. By using the same protocol we show in the present study that both IL1 β and M50 are internalized and accumulate in a Triton-insoluble fraction (Fig. 3). Moreover, internalization of IL1 β and M50 is quantitatively and kinetically indistinguishable from that of IL1 α .

DISCUSSION

Interleukin 1 (IL1) is a family of polypeptides that elicit a wide range of physiological responses of central importance to immune regulation and inflammation [18]. The cloning and sequencing of both IL1 α and IL1 β demonstrate considerable differences in their primary structures [5–7]; however, the available evidence indicates that they are capable of binding to the same receptor [19]. After binding to the IL1 receptor, the IL1–receptor complex is endocytosed [20–26]. Biochemical studies on the pre-B-cell line 7OZ/3 and on osteoblastic cells have indicated that, after endocytosis, most of the ligand and receptor are subject to lysosomal degradation [26,27]. However, other workers have shown that the endocytosed IL1 is accumulated within the cell in a largely intact form, and there is little release of intact material

into the cell supernatants [17,21,23,25]. On the basis of autoradiographical and binding studies, the IL1 appears to be translocated to the nucleus [11,17,23,25,28], although the precise intracellular routing of IL1 to the nucleus remains unclear. There is evidence that it is the IL1–IL1-receptor complex which is translocated to the nucleus and that the sorting information for this traffic is contained within the IL1 receptor itself [11]. However, binding studies have demonstrated that 125 I-IL1 α can bind specifically to purified nuclear preparations, suggesting that IL1 binding sites are present in the nucleus and consequently that the IL1 receptor may not be required for nuclear translocation [17]. The biological role of endocytosis and nuclear localization of IL1 is still unknown. Several groups have demonstrated that subclones of EL4 cells that are defective in endocytosis of IL1 are unable to respond to IL1 stimulation and have suggested that internalization of the ligand is a prerequisite for the IL1 induction of IL2 secretion [9–11]. Those studies provide evidence that endocytosis, and perhaps nuclear targeting, of IL1 are involved in the signal-transduction pathway for this cytokine.

Studies on protein import into the nucleus have revealed that the information required for targeting may be encoded in a short polybasic amino acid sequence [1–3]. The best-studied nuclear localization signal (NLS) is that of the SV40 large T-antigen. The seven-amino-acid sequence -Pro-Lys-Lys-Lys-Arg-Lys-Val- within this protein is both necessary and sufficient for its nuclear localization. The importance of this sequence is confirmed by the finding of similar sequences on other viral and non-viral proteins such as steroid-hormone receptors [29] and nucleoplasmin [30], which are also targeted to the nucleus. In support of this finding, BSA can be targeted to the nucleus when cross-linked to the SV40 targeting sequence [31]. Recent evidence suggests that the polybasic targeting sequence is recognized by a polyacidic sequence contained within a receptor localized in the nuclear pore [32].

In addition to IL1, several polypeptide hormones, including the fibroblast-growth-factor (FGF) family, have been shown to be translocated to the nucleus. In the case of acidic FGF, a potential NLS has been identified [33], and removal of this sequence results in a loss of mitogenic activity in cell-proliferation assays. The FGF family is similar to IL1 in that these proteins lack leader sequences and are synthesized on free ribosomes in the cytosol [34]. It has been revealed that the use of alternative translation start codons generates *N*-terminal variants of basic

Fig. 3. Internalization of IL1 α , IL1 β and M50

125 I-labelled preparations of IL1 α , IL1 β and M50 were bound to EL4 cells to equilibrium at 4 °C. After binding, cells were extensively washed with ice-cold medium to remove unbound ligand. Cells were rapidly warmed to 37 °C, and at timed intervals aliquots were removed and washed four times, either with ice-cold acid buffer to remove surface-bound ligand or with buffer containing 2% Triton X-100. Accumulation of radioactivity in cells after acid washing was taken as a measure of receptor-mediated endocytosis (●), and accumulation of radioactivity in the Triton-insoluble fraction was taken to represent nuclear transport (■). Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled ligand. The results represent specific binding determined in quadruplicate and are expressed as percentage of total surface-bound radioactivity at zero time (results are means $\pm$ S.E.M.).

FGF (bFGF), some of which contain a putative NLS and are targeted to the nucleus in a cell-cycle-specific fashion [35–37]. Consequently, nuclear translocation must be considered in terms of whether the growth factor or cytokine is produced within the cell or whether it is exogenously supplied and enters the cell by receptor-mediated endocytosis.

The IL1 receptor, pro-IL1 α and IL1 β contain polybasic sequences which are similar to the SV40 T-antigen NLS. Given that IL1 appears to be translocated to the nucleus we wished to test whether these sequences may be important for the biological activity of IL1. Although IL1 α is endocytosed and translocated to the nucleus [11,17], the putative NLS resides in the *N*-terminal pre-piece of pro-IL1 α and, consequently, cannot be implicated in the nuclear targeting of exogenously supplied IL1 α . This NLS may, however, function in targeting endogenous pro-IL1 α to the nucleus of IL1-producing cells in a similar fashion to bFGF. On the other hand, IL1 β does contain a putative NLS, and in the present study we have made use of three point mutations within this putative NLS of IL1 β to establish its significance for the biological activity of IL1 towards target cells. Two of the mutations replaced lysine with alanine residues, and in the other mutation alanine replaced proline. This region of IL1 β is in a loop between two β -strands, and the peptide bond preceding this proline residue has been shown to be in the *cis* orientation [38].

The data presented reveal that all three mutations were able to bind to the IL1 receptor on EL4 cells. Similar to the results of other studies with site-directed mutants of IL1, the mutant proteins had altered affinity for the IL1 receptor when compared with the wild-type protein [38–42]. Measurement of the biological activity of the mutants revealed an unexpected finding. The three mutants all showed greater biological activity than wild-type IL1 α or IL1 β , and, given that they had a lower affinity for the receptor, this represents a remarkable increase in efficacy over the wild-type proteins. This demonstrates that receptor affinity and biological potency are not necessarily coupled. These findings suggest that the regions of the IL1 molecule responsible for receptor binding and signal transduction may in part be distinct, and that this polybasic loop region may play an important role in receptor activation by IL1 β . Previously IL1 β mutants (Pro¹¹⁸ $\rightarrow$ Met¹¹⁸) have been described with a greater biological potency than the wild-type; however, this enhancement was mirrored by enhanced receptor-binding affinity [40,41]. Other mutants (Arg¹²⁷ $\rightarrow$ Gly¹²⁷) have also shown uncoupling of receptor binding and biological responses; however, in that case receptor affinity was decreased by 25%, whereas biological potency was diminished 100-fold [42]. This ability to uncouple receptor binding and biological activity has also been shown by site-directed mutagenesis studies in other polypeptide hormones such as acidic FGF [43].

There are several lines of evidence which suggest that receptor-mediated endocytosis of IL1, and perhaps its transport to the nucleus, is required for the generation of a biological response to IL1 stimulation [9–11]. One of the site-directed mutants we made, M50, showed an approx. 70-fold greater biological activity than wild-type IL1 α or IL1 β , while retaining a receptor-binding affinity equivalent to that of the wild-type agonists. Consequently we quantified the receptor-mediated endocytosis and accumulation of M50 in a Triton-insoluble intracellular compartment to determine whether the increased potency of this ligand could be correlated with changes in its internalization. Our data revealed that the internalization of M50 was both quantitatively and kinetically indistinguishable from that of the wild-type, so demonstrating that the enhanced potency was not the result of modified internalization or nuclear targeting. This suggests that there is no clear correlation between biological responsiveness to IL1 stimulation and the efficiency of endocytosis and nuclear

accumulation. Previous studies have shown that IL1 is translocated to the nucleus still bound to its receptor, which may itself possess an NLS [11]. Our findings suggest that the putative NLS in IL1 β does not act independently of the receptor and, indeed, may not represent a true nuclear localization signal at all. Moreover, the finding that the biological potency of IL1 β was not related to its internalization and nuclear accumulation, suggests that nuclear transport may not play a significant role in IL1 signal transduction.

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