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Receptor-mediated endocytosis and nuclear transport of human interleukin 1α

Stephanie GRENFELL,* Nicholas SMITHERS,‡ Karen MILLER† and Roberto SOLARI‡§

*Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, †Department of Biochemistry, Imperial College, London SW7 2AZ, and ‡Department of Cellular Science, Glaxo Group Research Ltd., Greenford Road, Greenford, Middx. UB6 0HE, U.K.

In this study we demonstrate that ^{125}I-labelled interleukin (IL) 1α binds specifically to its receptor on the surface of EL4 6.1 cells and is subsequently endocytosed and translocated from the cell membrane to the nucleus, where it progressively accumulates. Two-dimensional polyacrylamide-gel electrophoresis revealed that the internalized ^{125}I-IL1α associated with the nucleus was intact, with negligible breakdown products present. Specific and saturable binding of ^{125}I-IL1α was demonstrated on purified nuclei isolated from these cells. Binding of the radiolabelled ligand showed similar kinetics to that of the plasma-membrane receptor, and was inhibited by both unlabelled IL1α and IL1β. Equilibrium binding studies on isolated nuclei revealed a single high-affinity binding site, with a K_d of 17 ± 2 pM, and 79 ± 12 binding sites per nucleus. These studies demonstrate that receptor-mediated endocytosis of IL1 results in its accumulation in the nucleus, and this mechanism may play an important role in mediating some of the actions of IL1.

INTRODUCTION

Interleukin 1 (IL1) is a family of polypeptides which elicits a wide range of physiological responses of central importance to immune regulation and inflammation [1]. The cloning and sequencing of both IL1α and IL1β demonstrate considerable differences in their primary structures [2–4]; however, the available evidence indicates that they are capable of binding to the same receptor [5]. Despite cloning of the murine receptor [6] and considerable efforts in recent years to elucidate the transmembrane signalling pathways, the biochemical mechanisms for the actions of IL1 have remained obscure. There is much conflicting evidence concerning the acute intracellular events following the binding of IL1 to its receptor. IL1 has been reported to induce both phosphatidylinositol 4,5-bisphosphate [7,8] and phosphatidylcholine hydrolysis, with subsequent generation of diacylglycerol [9], although neither increasing intracellular Ca^{2+} nor protein kinase C activation is seen [7,10]. Studies on the cyclic AMP response to IL1 stimulation also appear contradictory [11–13], and have not led to a clear consensus on the effector mechanism for IL1 signal transduction. Whatever the signalling pathway may be, there is considerable evidence showing that IL1 affects the transcription of specific genes in a number of cell types [14–19]. Whether this is the result of second-messenger generation after receptor–ligand binding or some later event is not known. In order to examine the response of target cells to IL1, we have chosen to investigate the fate of the ligand after binding to its receptor at the cell surface.

A number of studies have demonstrated receptor-mediated endocytosis of IL1 in a variety of cell types [20–22], and, based on autoradiography studies, it has been suggested that IL1 may be transported to the nucleus of EL4 cells and diploid fibroblasts [20,21]. However, little attention has been focused on the potential role of the internalized IL1–IL1-receptor complex, particularly as a possible mediator of biological signals. In this study we have investigated the fate of the IL1 endocytosed by EL4 cells, and we show that IL1 is transported to the nucleus, where it binds to specific receptors.

MATERIALS AND METHODS

Cells

EL4 6.1 cells were maintained in continuous suspension culture in RPMI 1640 medium containing 10% (v/v) myoclone fetal-calf serum, 100 μg of penicillin/ml, 100 μg of streptomycin/ml, 2 mM-glutamine and 200 μM-2-mercaptoethanol. This cell line was grown in standard tissue-culture flasks in a humidified air/CO_2 (19:1) incubator at 37 °C.

IL1

Recombinant human IL1α and IL1β were kindly given by Alan Shaw (Glaxo Institute for Molecular Biology), and the former was iodinated by the method of Salenski *et al.* [23]. Briefly, 9.25 μg of recombinant human IL1α (in Dulbecco's phosphate-buffered saline, pH 7.5) was incubated with 37 MBq of Na^{125}I (diluted 1:1 in 0.5 M-phosphate buffer, pH 7), in a glass tube coated with 0.5 μg of Iodogen. The reaction was allowed to proceed on ice for 30 min, and then the mixture was desalted on a PD10 column (equilibrated with Dulbecco's phosphate-buffered saline and 0.2% bovine serum albumin); 500 μl fractions were collected, and the appropriate fractions pooled. In all cases the incorporation of ^{125}I was in the range 60–80%, and the final products exhibited a specific radioactivity of 450–2000 Ci/mmol.

Abbreviations used: IL1, interleukin 1; PAGE, polyacrylamide-gel electrophoresis.

§ To whom correspondence and requests for reprints should be addressed.

Isolation of nuclei

Nuclei were isolated by modification of the methods of Goldfine [24] and Tata [25]. Cells were initially washed twice in ice-cold Tris buffer (0.15 M-NaCl/10 mM-Tris/HCl, pH 7.5). The washed cells were centrifuged (600 *g* for 5 min, 4 °C), and the cell pellet was resuspended in 3 ml of 1.5 mM-$MgCl_2$. After 5 min on ice, the suspension was homogenized with 20 strokes of a tight-fitting Dounce homogenizer, and centrifuged (600 *g* for 10 min, 4 °C). The supernatant was discarded (S1) and the pellet (P1) was resuspended in ice-cold 0.25 M-sucrose/10 mM-$MgCl_2$/20 mM-Tris/HCl, pH 7.5, overlaid on a 2 M-sucrose cushion in the same buffer, and centrifuged at 80000 *g* for 1 h at 4 °C, with no brake (SW41 Ti rotor). The overlay, interface and 2 M-sucrose cushion were carefully removed, and the pellet was resuspended in RPMI 1640 medium and washed twice. This nuclear fraction was called P2. The P2 pellet was subsequently washed with medium containing 2% Triton X-100. This Triton-washed nuclear fraction was called P3.

Binding studies

EL4 cells were suspended (1×10^7/ml) in binding medium (RPMI 1640 containing 5% fetal-calf serum and 0.5% NaN_3), and 50 μl samples were placed in wells of a 96-well Millititre filtration plate. ^{125}I-IL1α was added at the concentrations indicated in the Figure legends. Non-specific binding was determined by the inclusion of a 100-fold molar excess of unlabelled IL1α. After various time points, the unbound ^{125}I-IL1α was separated from the cells by vacuum filtration, followed by four washes with washing medium (RPMI 1640 containing 0.5% NaN_3). After 20 min at 80 °C, the filtration membranes were punched out and counted for radioactivity in a LKB Clinigamma spectrometer.

Internalized and nuclear-bound ^{125}I-IL1α were measured in NaN_3-free binding medium. ^{125}I-IL1α was allowed to bind to EL4 cells overnight at 4 °C. After washing to remove the unbound ligand, the cells were further incubated at 4 °C, 16 °C or 37 °C for various lengths of time. Internalized ^{125}I-IL1α was taken to be the ^{125}I-IL1α that remained associated with the cells after washing four times with acid buffer (0.15 M-NaCl/0.01 M-sodium acetate, pH 3.5). Nuclear-bound ^{125}I-IL1α was determined by washing the cell pellet with medium containing 2% Triton X-100, followed by counting of the radioactivity associated with the detergent-insoluble pellet.

The internalization of ^{125}I-IL1α after the removal of the cell-surface receptors was determined by incubating cells in the absence and presence of 1 mg of trypsin/ml at 37 °C. The trypsin activity was terminated after 20 min by the addition of medium containing 5% fetal-calf serum, and the cells were washed twice. The cells were resuspended in binding media containing 0.5 mM-cycloheximide and 0.3 nM-^{125}I-IL1α, and transferred to 37 °C. Total cell-associated and nuclear binding was determined as above.

Binding of ^{125}I-IL1α to the P2 fraction of isolated nuclei was performed in 96-well plates as described for binding to whole cells. The P2 fraction used for these studies was prepared as described above and plated out at 10^6 nuclei per well. Binding was terminated by vacuum filtration, followed by two washes with washing medium and two washes with washing medium containing 2% Triton X-100.

Analytical techniques

Two-dimensional polyacrylamide-gel electrophoresis (PAGE) was performed as described by O'Farrell [26]. Protein was determined by the method of Bradford [27], and DNA by using the Hoechst stain 33258 as described by Mates *et al.* [28].

Electron microscopy

Fractions were fixed in 2% (v/v) formaldehyde/2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, for 18 h at 4 °C [29], followed by centrifugation in a Beckman Microfuge The pellets were post-fixed in 1% OsO_4 in 0.1 M-sodium cacodylate buffer, pH 7.4, for 1 h at 4 °C, stained *en bloc* with aq. 2% (w/v) uranyl acetate, then dehydrated and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate [30], and examined in a Philips CM12 electron microscope at 80 keV.

Reagents

Fetal-calf serum and RPMI 1640 were purchased from Gibco, Uxbridge, Middx., U.K. Reagents for electron microscopy were purchased from Polysciences, and electrophoresis reagents from Bio-Rad. All other reagents were purchased from Sigma Chemical Co., and were of the highest purity available.

RESULTS

Receptor-mediated endocytosis

Our first aim was to investigate the receptor-mediated endocytosis of IL1 by the mouse thymoma cell line, EL4 6.1. Our approach was to incubate the EL4 cells with 0.3 nM-^{125}I-IL1α for 18 h at 4 °C in order to achieve equilibrium binding to cell-surface IL1 receptors. After extensive washing in ice-cold medium, the cells were rapidly warmed to 37 °C and incubated for various periods of time. Endocytosis of surface-bound ^{125}I-IL1α was determined by vacuum filtration and washing of the cells in pH 3.5 buffer. Radioactivity which remained associated with the cell pellet after acid washing was considered to be intracellular, as > 95% of ^{125}I-IL1α bound to the cell-surface receptors of NaN_3-treated cells could be removed by acid washing (results not shown). By this technique, ^{125}I-IL1α could be determined inside the cell as soon as 10 min after warming to 37 °C, and internalization was seen to increase with time while the surface-bound IL1α showed a corresponding decrease (Fig. 1). Over the 6 h duration of the experiment, 80% of the originally surface-bound IL1α was internalized and 62% had remained within the cell. Recovery of the cell supernatant revealed that 10% of the ^{125}I-IL1α was released from the cell in a trichloroacetic acid-precipitable form and 8% as acid-soluble fragments.

On the basis of morphological data, it has been claimed that internalized IL1 is translocated to the nucleus [20,21]. In order to investigate these claims, we examined the endocytosis of ^{125}I-IL1α at 4, 16 and 37 °C, and its subsequent appearance in a nuclear-enriched cell fraction. EL4 cells were incubated for 18 h at 4 °C with ^{125}I-IL1α, followed by extensive washing in ice-cold medium. The cells were then further incubated at 4, 16 or 37 °C for various periods of time. Internalized ^{125}I-IL1α was determined by acid-washing the cells, and the nuclear-associated ^{125}I-IL1α was determined by washing the cells in

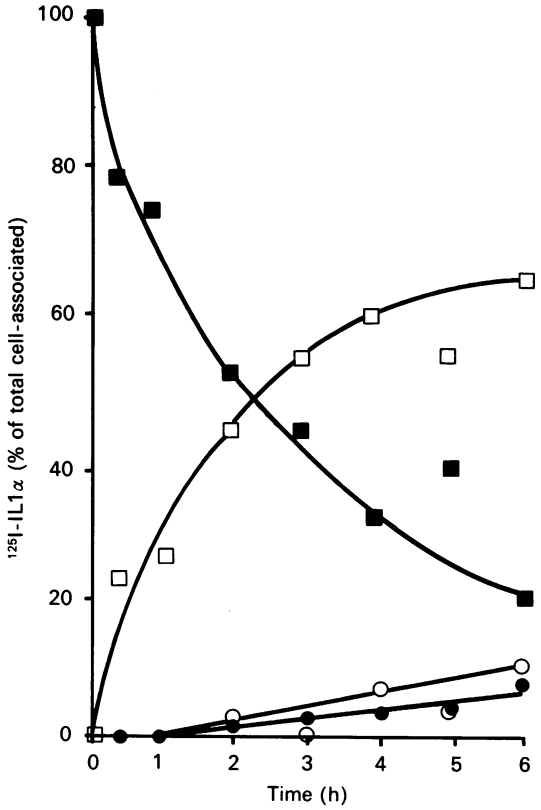

Fig. 1. Internalization and degradation of ^{125}I-IL1α by EL4 6.1 cells

Cell-surface receptors were saturated with ^{125}I-IL1α by incubating EL4 6.1 cells overnight with 0.3 nM-IL1α at 4 °C. Cells were then washed, resuspended in binding medium and rapidly transferred to 37 °C at zero time. At the indicated time points during the subsequent incubation at 37 °C, 150 μl samples were removed and the experiment was terminated by vacuum filtration of the cells. Total cell-associated (■) and internalized (□) ^{125}I-IL1α was determined by rapidly washing the cells retained on the filter mat, with either medium or pH 3.5 buffer respectively. The filtration plate was subsequently dried for 20 min in a 80 °C oven, the individual filter mats were punched out, and the bound radioactivity was counted. Radioactive material released from the cells into the medium during the incubation period was analysed by precipitation with trichloroacetic acid. Cell-surface receptors were saturated with ^{125}I-IL1α at 4 °C as described above, followed by transfer to 37 °C. At the indicated time points the cells were centrifuged and the supernatant was collected and precipitated with 20% trichloroacetic acid. Over the time course of the experiment, both acid-soluble (●) and -precipitable (○) ^{125}I was released into the supernatant. The results represent specific binding, and are expressed as percentages of the total cell-surface-associated ^{125}I-IL1α at zero time.

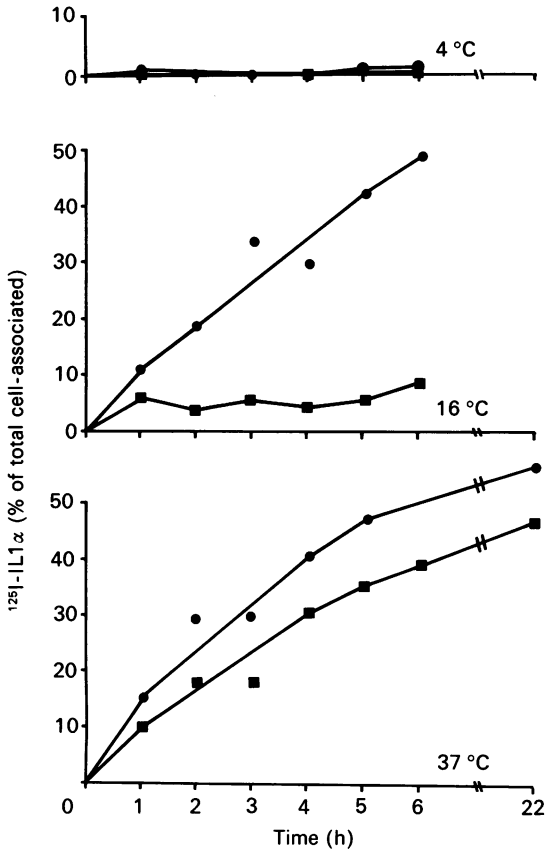

Fig. 2. Temperature-dependence of internalization and translocation of ^{125}I-IL1α to the nucleus of EL4 6.1 cells

Cell-surface receptors were saturated with ^{125}I-IL1α by incubating EL4 6.1 cells overnight with 0.3 nM-^{125}I-IL1α at 4 °C. Cells were washed, resuspended in binding medium, and re-incubated at 4, 16 or 37 °C. At the indicated time points during the subsequent incubation, 150 μl samples of the suspension were removed and the reaction was terminated by vacuum filtration. The amount of radioactivity present inside the cells (●) and that bound to the nucleus (■) were determined by washing four times with acid buffer (pH 3.5) and 2% Triton buffer respectively. After 20 min in a 80 °C oven, the filter mats were punched out and the bound radioactivity was counted. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled IL1α. The results represent specific binding, and are expressed as percentages of the total cell-surface-associated ^{125}I-IL1α at zero time.

2% Triton X-100 (Fig. 2). As previously demonstrated, washing whole cells in 2% Triton X-100 effectively solubilizes all membranous components of the cell, and leaves behind the nucleus and cytoskeleton [31,32]. Using this technique we demonstrated that at 4 °C there is no significant endocytosis or transport to the Triton-insoluble fraction. This failure to detect ^{125}I-IL1α associated with the Triton-insoluble fraction after binding to the cell surface at 4 °C suggests that IL1 receptors present on the plasma membrane are effectively solubilized by the Triton wash, and do not remain associated with the cytoskeleton/nuclear pellet. At both 16 °C and 37 °C, endocytosis of ^{125}I-IL1α can be demonstrated to occur with similar kinetics; however, transport to the Triton-insoluble fraction takes place only at 37 °C, and not at 16 °C. Blockade of transport to the Triton-insoluble fraction at 16 °C further demonstrates that the cytoskeleton/nuclear pellet is not contaminated with membrane-bound IL1. These observations revealed that at 37 °C 73% of the internalized ^{125}I-IL1α is associated with the Triton-insoluble fraction.

The blockade of ^{125}I-IL1α translocation to the Triton-insoluble fraction at 16 °C implicates the acidic endo-

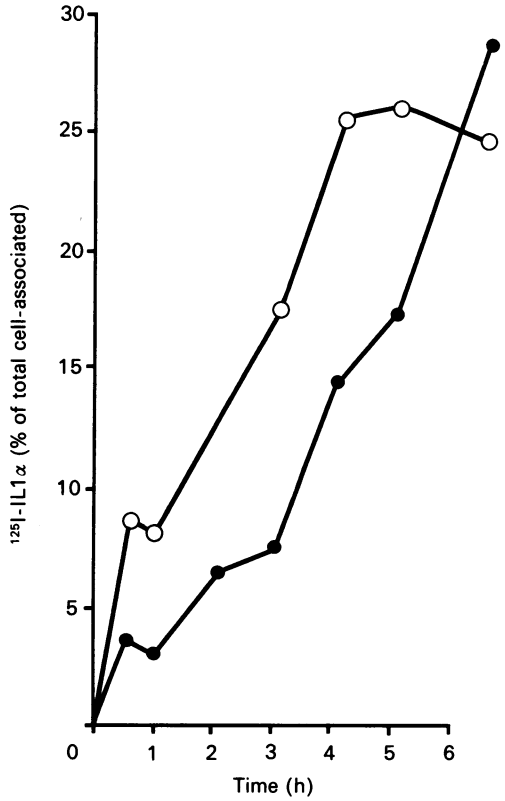

Fig. 3. Effect of chloroquine on nuclear binding of ^{125}I-IL1α by EL4 6.1 cells

Cell-surface receptors were saturated with ^{125}I-IL1α by incubating EL4 6.1 cells overnight with 0.3 nM-^{125}I-IL1α at 4 °C. Cells were then washed, resuspended in binding medium without (●) or with (○) 100 nM-chloroquine, and at zero time rapidly transferred to 37 °C. Samples of the suspension were removed at the indicated time points, and terminated by vacuum filtration. The total cell-associated and nuclear-associated ^{125}I-IL1α was determined by washing with medium and 2% Triton buffer respectively. Non-specific binding was measured in the presence of 100-fold molar excess of unlabelled IL1α. The results represent specific binding, and are expressed as percentages of total cell-surface-associated ^{125}I-IL1α at zero time.

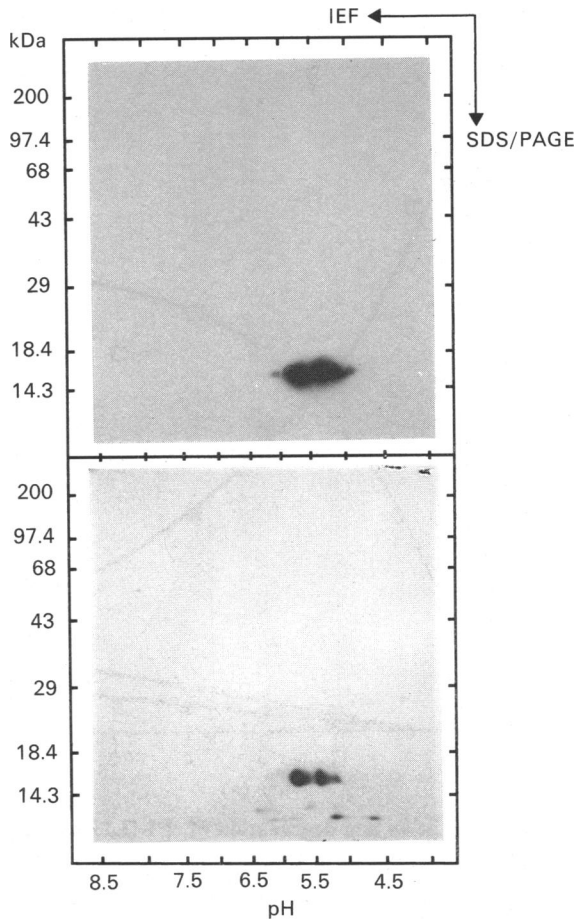

Fig. 4. Two-dimensional SDS/PAGE analysis of internalized ^{125}I-IL1α

EL4 cells were incubated with ^{125}I-IL1α for 4 h at 37 °C. The cells were washed and the nuclei were isolated by washing the cell pellet with buffer containing 2% Triton X-100. The Triton-insoluble pellet was resuspended in 9 M-urea and analysed by two-dimensional SDS/PAGE in accordance with O'Farrell [26] (lower panel). For comparative purposes a sample of ^{125}I-IL1α was analysed under identical conditions (upper panel). Abbreviation: IEF, isoelectric focusing.

somal compartment in the internalization pathway. To investigate this possibility further, we examined the influence of the lysosomotropic agent chloroquine on the transport of ^{125}I-IL1α from the cell surface to the Triton-insoluble fraction. Fig. 3 shows that in the presence of 100 nM-chloroquine, there is an increased rate of transport of ^{125}I-IL1α to the Triton-insoluble fraction. The possible routing of IL1 through the acidic compartments of the cell suggested that the observed accumulation of the ^{125}I-IL1α in the Triton-insoluble fraction might represent proteolytic fragments of the labelled protein. To examine this possibility, a Triton-insoluble fraction was made from cells previously incubated with ^{125}I-IL1α for 5 h at 37 °C. This preparation was analysed by two-dimensional PAGE and compared with the original ^{125}I-IL1α preparation (Fig. 4). Analysis by this technique reveals two spots both with an apparent molecular mass of 17 kDa, which represent the amidated (pI 5.45) and deaminated (pI 5.2) forms of the protein. These are produced in almost equal amounts in the *Escherichia coli* expression system used to produce the recombinant IL1α [33]. Analysis of the ^{125}I-IL1α which accumulates in the Triton-insoluble fraction shows it to be mostly intact IL1α, although some proteolytic fragments can be detected.

In order to confirm that internalization of ^{125}I-IL1α was a receptor-mediated event, we used two approaches. Firstly we pretreated EL4 cells with trypsin, under conditions which remove > 90% of the cell-surface receptors (results not shown). These 'shaved' cells were incubated with ^{125}I-IL1α at 37 °C, in the presence of cycloheximide to prevent the re-expression of newly synthesized receptors, and transport of ligand to the Triton-insoluble fraction was determined (Table 1). The data demonstrate that in the absence of cell-surface receptors translocation of ^{125}I-IL1α to the Triton-insoluble fraction is blocked. In order to support this finding, we examined internalization of ^{125}I-IL1α in an

EL4 6.1 subclone, EL4.3, which is known to lack surface IL1 receptors [34]. These cells are unable to bind or internalize ^{125}I-IL1α, so confirming the role of the IL1 receptor in endocytosis and nuclear translocation (results not shown).

Equilibrium binding studies

Studies were performed to investigate binding of ^{125}I-IL1α to azide-treated EL4 cells and nuclei purified from these cells by a modification of published fractionation techniques (see the Materials and methods section). Contamination of the nuclear fraction with plasma membranes would clearly invalidate this approach. In order to identify any such contamination, we labelled the cell-surface receptors with ^{125}I-IL1α at 4 °C, and subsequently isolated the nuclei. By measuring the specific radioactivity associated with the nuclear fractions, we could thus quantify contamination of the nuclei with plasma-membrane IL1 receptors. By this method we were able to show that 2.8% of plasma-membrane-bound ^{125}I-IL1α was recovered in the P2 fraction of isolated nuclei (Table 2). Consequently this P2 fraction, which was used in the subsequent binding studies, contains significant contamination from the plasma membrane. However, to eliminate the possibility that in our equilibrium binding studies ^{125}I-IL1α associated with the nuclear fraction was due to this membranous contamination, on termination of each experiment the P2 nuclear pellet was washed four times in buffer containing 2% Triton X-100, to produce the P3 fraction. As shown in Table 2, this action removed virtually all detectable contamination by plasma membranes. The quality of the isolated nuclear preparation used in these binding studies is of critical importance. To validate our approach further, we determined the yield of DNA and protein in each fraction. As shown in Table 2, the P2 fraction contained 35% of the total DNA present in the homogenate. Comparison of the protein/DNA ratio in the various fractions revealed that the P2 nuclear fraction was enriched 8.3-fold relative to the homogenate. We were unable to determine accurately the protein/DNA ratio in the Triton-washed P3 fraction, owing to interference by the detergent in the assay procedures, and the tendency of the P3 fraction to agglutinate. The P2 and P3 fractions were also examined for purity by electron microscopy. As shown in Fig. 5, the P2 fraction revealed a low level of contamination with membranous material; however, no significant contamination with identifiable organelles could be detected in any section. After Triton washing, the P3 fraction was free of any detectable membranous contamination. The nuclear envelope, which is clearly present in the P2 fraction, is removed by Triton washing, and there is also an increased nuclear

Table 1. Effects of trypsin treatment on ^{125}I-IL1α internalization by EL4 6.1 cells

Cells were incubated in the absence and presence of 1 mg of trypsin/ml for 20 min at 37 °C. The cells were washed, and incubated at 37 °C in the presence of 0.5 mM-cycloheximide and 0.3 nM-^{125}I-IL1α. At the times indicated, 150 μl samples were removed, and binding was terminated by vacuum filtration. Nuclear-associated ^{125}I-IL1α binding was measured by washing the cell pellet four times with 2% Triton X-100 buffer. After 20 min in a 80 °C oven, the individual filter mats were punched out, and the radioactivity was measured in a γ-radiation counter. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled IL1α. The results represent specifically bound radioactivity, expressed as a percentage of total cell-associated ^{125}I-IL1α at zero time.

Time (h)	Nuclear-associated label (%)	
	+Trypsin	No trypsin
1	0.0	2.8
2	0.0	2.4
3	0.0	8.6
4	2.7	17.7
5	1.2	20.2

Table 2. Purification of nuclei from EL4 cells

Cell-surface IL1 receptors were saturated by incubating cells overnight at 4 °C with 0.3 nM-^{125}I-IL1α. Cells were washed and resuspended in 1.5 mM-$MgCl_2$. After 5 min on ice the suspension was homogenized with 20 strokes of a tight Dounce homogenizer. The homogenate was centrifuged (600 $g_{av.}$ for 10 min), and the supernatant (S1) discarded. The pellet (P1) was resuspended in 0.25 M-sucrose buffer, overlaid on a 2 M-sucrose cushion and centrifuged at 80000 $g_{av.}$ for 1 h with no brake. The sucrose overlay, the interface and the cushion were discarded. The pellet (P2) was resuspended in RPMI medium and washed twice, followed by two washes with Triton buffer (P3). Samples of each fraction were counted for radioactivity. Non-specific binding was determined in the presence of a 100-fold molar excess of unlabelled IL1. The specific radioactivity in each fraction is given as a percentage of that in the original homogenate, and was used to quantify the distribution of plasma-membrane IL1 receptors. The total protein and DNA in each fraction were determined as described in the Materials and methods section. Values represent means ± S.D. from three separate experiments.

Fraction	Sp. radioactivity of plasma-membrane IL1α		Protein (mg)	DNA		Protein/DNA
	(c.p.m./fraction)	(% of homogenate)		(mg)	(yield, % of homogenate)	
Homogenate	143224	100	7.92±1.35	2.02±0.54	100	3.92
S1	120453	84.1	3.80±0.60	1.03±0.36	45.7±11.6	3.68
P1	27568	19.3	2.40±0.34	1.03±0.32	47.3±18.3	2.33
P2	4063	2.8	0.36±0.11	0.76±0.14	35.2±9.7	0.47
P3	8	0.006				

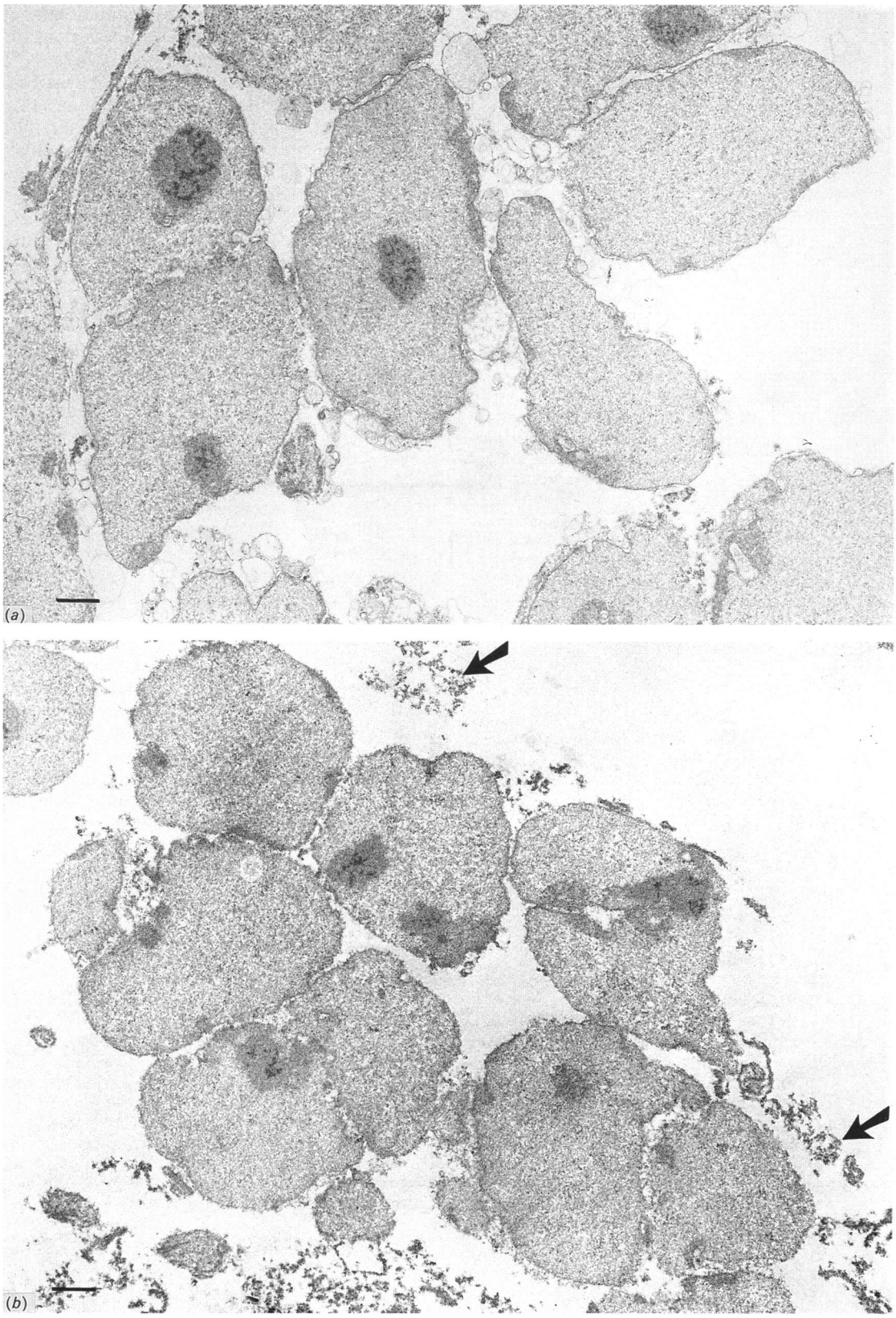

Fig. 5. Electron microscopy of isolated nuclei

(*a*) Electron micrograph of the P2 nuclear fraction. The isolated nuclei have well-preserved morphology, and retain their nuclear envelope. The preparation is essentially free of cellular organelles, and only small quantities of membranous debris and cytoplasmic material are evident. Bar = 1 μm. (*b*) Electron micrograph of the Triton-washed P3 nuclear fraction. No membranous material is evident in this fraction, although some breakage of the nuclei is seen (arrow). Triton washing has removed the nuclear envelope. Bar = 1 μm.

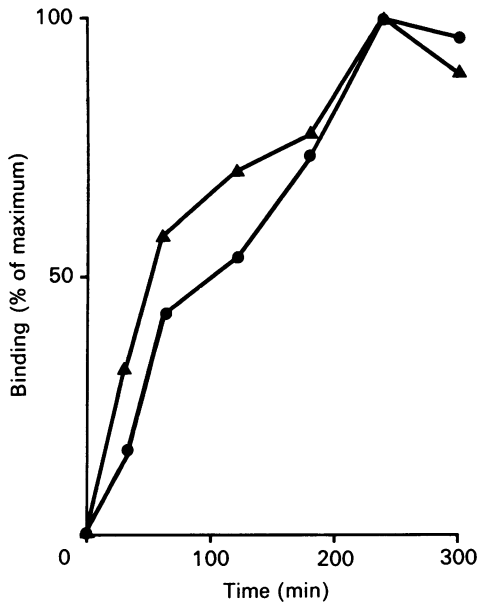

Fig. 6. Kinetics of ^{125}I-IL1α binding to azide-treated cells (▲) and isolated nuclei (●) from EL4 6.1 cells

Nuclei were isolated as described in the Materials and methods section. Isolated nuclei (●) or azide-treated cells (▲) were incubated at 37 °C in the presence of 0.3 nM-^{125}I-IL1α. At the indicated time points the binding was terminated by vacuum filtration, and the cells or nuclei were rapidly washed four times with buffer. The filter plate was dried for 20 min in a 80 °C oven, the individual filter mats were punched out, and the bound radioactivity was counted. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled IL1α. The results represent specific binding, and are expressed as percentages of the maximum radioactivity bound.

disruption in P3. The possibility exists that ^{125}I-IL1α may bind to cytoskeletal elements which might be contaminating the P3 nuclear fraction. However, it has been shown that the Triton-insoluble cytoskeleton can be disrupted by treatment at pH 11 [35]. Thus, to examine this potential artifact, after binding of ^{125}I-IL1α to the P2 fraction the nuclei were washed three times in Triton X-100 buffer and three times in pH 11 buffer. ^{125}I-IL1α specifically bound to the Triton-washed nuclei could not be washed off at pH 11, suggesting that binding was not to cytoskeleton contained within the pellet (results not shown).

Having established our nuclear preparation, we examined the binding kinetics of ^{125}I-IL1α to both the intact EL4 cells and purified Triton-washed nuclei. Our results show that in both cases specific binding of the ligand can be measured and equilibrium is reached after 4 h incubation at 37 °C (Fig. 6). Equilibrium binding studies were subsequently performed on both intact EL4 cells and purified nuclei (Fig. 7). Analysis of the binding data by Scatchard transformations revealed a single high-affinity site on intact cells, with a dissociation constant (K_d) of 45±3 pM, and 1750±60 receptor sites per cell. Scatchard analysis of binding to the purified nuclei was also consistent with a single high-affinity site, with a K_d of 17±2 pM, and 79±12 binding sites per nucleus. Unlabelled IL1β and IL1α were both equally effective in inhibiting specific binding of ^{125}I-IL1α to whole EL4 cells and islated nuclei (results no shown).

DISCUSSION

Receptor-mediated endocytosis is known to be an important component of the overall response of cells to many biologically important molecules (for reviews, see [36,37]). The fate of these ligands once internalized varies

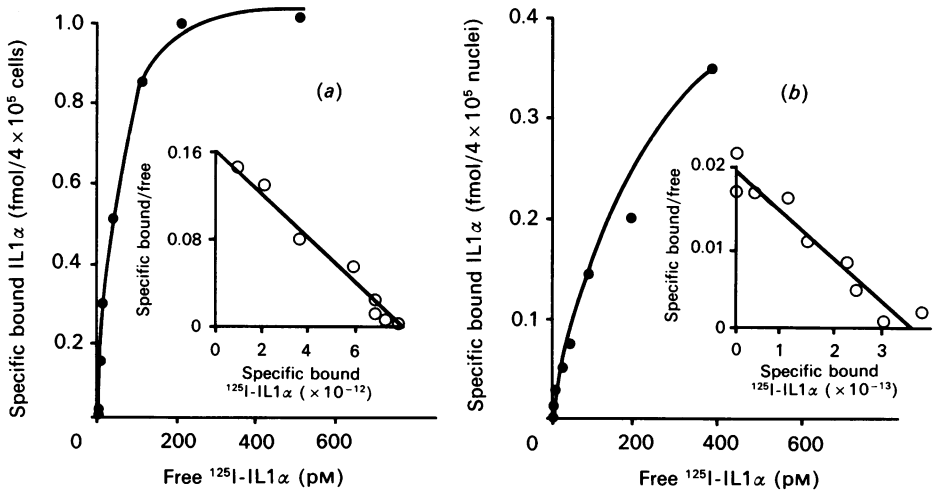

Fig. 7. Equilibrium binding of ^{125}I-IL1α to the cell surface and nuclei isolated from EL4 6.1 cells

Azide-treated cells (*a*) and isolated nuclei (*b*) were incubated with various concentrations of ^{125}I-IL1α for 4 h at 37 °C. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled IL1α. Binding was terminated by vacuum filtration followed by rapid washing. The filter mat was dried for 20 min in an 80 °C oven, the individual filter mats were punched out, and the bound radioactivity was counted. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled IL1α. Scatchard analysis of the binding data (insets) reveals 1750±60 receptors per cell with a K_d of 45±3 pM, and 79±12 receptors per nucleus with a K_d of 17±2 pM.

considerably, from lysosomal degradation to intracellular storage to transcytosis. Receptors also have diverse fates and may be degraded, re-cycled to the plasma membrane for re-use, or released from the cell with their ligand. Receptor-mediated endocytosis may also be involved in the signal-transduction mechanism for several hormones and growth factors. For example, the receptors for epidermal growth factor [38] and platelet-derived growth factor [39] are known to be rapidly internalized via coated pits upon binding their ligands. These hormones elicit acute responses which occur in seconds after binding to their receptor, such as the activation of the receptor's tyrosine kinase activity. However, they also elicit much slower responses, which occur many hours later, such as the initiation of DNA synthesis.

IL1 is known to bind to a specific cell-surface receptor and to be internalized [20–22]. It has also been reported to elicit a variety of acute changes in target cells, such as increasing cyclic AMP [12,13,40], the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the generation of an inositol trisphosphate second messenger [8], the cleavage of phosphatidylethanolamine [41] or phosphatidylcholine with the resultant generation of diacylglycerol [9,42], and the rapid phosphorylation on serine residues of an acidic cytosolic protein [43]. IL1 also induces a rapid and transient stimulation of the nuclear proto-oncogenes *c-fos* and *c-myc* [14,15,18]. However, despite many studies there appears to be no clear consensus as to how these early events following IL1 binding to its receptor can induce the wide range of cellular responses to IL1 which may manifest themselves hours or even days later (for reviews, see [1,44]). IL1 is known to regulate the transcription of many genes such as those coding for IL2 and the IL2 receptor [45], stromeolysin [17] and major histocompatibility complex class III in transfected mouse L cells [46]. A possible explanation for the influence exerted by IL1 on the transcription of certain genes is that IL1 is internalized and transported to the nucleus, where it may act directly, or via a receptor, on transcriptional control elements in the genome. The localization of endocytosed ^{125}I-IL1 by autoradiography is suggestive of IL1 translocation from the plasma membrane to the nucleus. Consequently, the aim of our study was to examine the endocytosis and intracellular fate of IL1 in the mouse thymoma cell line EL4 6.1.

We first examined the kinetics of ^{125}I-IL1α endocytosis at 37 °C. By binding the ligand to the cells at 4 °C followed by rapid warming to 37 °C, we were able to measure a pulse of receptor-mediated internalization. Our data revealed that ^{125}I-IL1α is endocytosed with a $t_{\frac{1}{2}}$ of approx. 2 h, which is in close agreement with previous determinations of 60–120 min [20] and 90 min [21]. However, this rate of endocytosis is considerably slower than that measured for IL2 [47], transferrin [48–50], epidermal growth factor [51,52], insulin [24,53,54], low-density lipoprotein [55] or asialoglycoprotein [56]. Over the 6 h period examined, 80% of the surface-bound ^{125}I-IL1α was internalized and only 8% was subsequently released from the cell as trichloroacetic acid-soluble fragments. This indicated that the endocytosed IL1α was accumulating intracellularly. We then went on to test whether the ^{125}I-IL1α was being transported to the nucleus, as has been suggested by autoradiographical studies. To do this, we allowed the EL4 cells to endocytose ^{125}I-IL1α, then at various time points the cells were lysed and washed in buffer containing 2% Triton X-100. The radioactivity remaining associated with the Triton-insoluble fraction was taken to be a measure of nuclear-bound ^{125}I-IL1α. Such a protocol would clearly eliminate the possibility of membrane-bound ^{125}I-IL1α contaminating the nuclei, since all such material would be solubilized by the detergent. However, it is possible that ^{125}I-IL1α was binding to the Triton-insoluble cytoskeleton, since nerve growth-factor receptors have been found associated with the cytoskeleton of PC12 cells [39,40]. The most important evidence in favour of nuclear translocation, rather than cytoskeleton binding, comes from comparison of receptor-mediated endocytosis at 4, 16 and 37 °C. At 4 °C, receptor-bound ^{125}I-IL1α was not internalized, and no ^{125}I-IL1α was associated with the Triton-insoluble fraction. This demonstrates that IL1 receptors are efficiently solubilized by the Triton wash, and do not remain associated with the insoluble residue. At 16 °C and at 37 °C the ligand was endocytosed at comparable rates; however, transport to the Triton-insoluble fraction only occurred at 37 °C. It is well established that at 16 °C endocytosis of surface-bound ligands occurs, but the internalized ligands become blocked in the endosomal compartment and fail to proceed to their subsequent cellular destination. The finding that ^{125}I-IL1α does not accumulate in the Triton-insoluble fraction at 4 °C or at 16 °C clearly demonstrates that our protocol for measuring nuclear transport is valid and not subject to contamination from either plasma-membrane-bound ^{125}I-IL1α or ^{125}I-IL1α present in endosomes. Our findings also reveal that over 70% of the internalized ^{125}I-IL1α is transported to the nucleus, which is considerably more than that estimated by autoradiography. Analysis of the nuclear-bound ^{125}I-IL1α by two-dimensional SDS/PAGE showed two spots, representing amidated and deamidated forms of IL1, with apparent molecular mass of 17 kDa. This demonstrates that most of the nuclear-bound material was indeed intact, although some small breakdown products could be detected.

The blockade of IL1α transport to the nucleus at 16 °C shows that this ligand passes through an acidic endosomal or pre-lysosomal compartment after receptor-mediated endocytosis. Further circumstantial evidence supports this observation. We demonstrated that the rate of transport of ^{125}I-IL1α to the nucleus at 37 °C can be augmented when the cells are treated with the lysosomotropic agent chloroquine. This is similar to previous observations that epidermal growth factor accumulates in the nucleus of chloroquine-treated rat pituitary cells [38].

Nuclear transport of IL1α depends upon the cell-surface receptor, as revealed by several lines of evidence. Firstly, we removed surface IL1 receptors from the EL4 cells by brief digestion with trypsin. Under these conditions, no transport to the nucleus could be detected. Secondly, the EL4 subclone, EL4.3, which is deficient in surface IL1 receptors, is also incapable of transporting IL1 to the nucleus. This evidence eliminates the possibility of IL1 being routed to the nucleus by non-specific fluid-phase endocytosis.

Equilibrium binding studies were performed with ^{125}I-IL1α on intact EL4 cells and nuclei purified from these cells. The use of isolated nuclei for binding studies presents several technical difficulties. Firstly, isolated nuclei have a tendency to agglutinate, and consequently

all assays and procedures must be carried out on freshly prepared samples. Secondly, one must take into account possible contamination from IL1 receptors present in the plasma membrane or other cellular organelles. By binding the ligand to surface receptors at 4 °C, and isolating the nuclei, we were able to show that Triton-washed nuclei have less than 0.006% plasma-membrane contamination. This eliminates the possibility of membrane contamination being responsible for the observed nuclear binding. Moreover, morphological analysis of the Triton-washed P3 fraction clearly revealed it to be free from membranous contamination. Having validated our use of isolated nuclei for receptor–ligand-binding studies, our data demonstrated a single class of high-affinity binding site both on the plasma membrane and in the nucleus, with a K_d of 45 ± 3 pM and 17 ± 2 pM respectively. Assuming that one IL1α molecule binds to one binding site results in a calculation of 1750 ± 60 receptors per cell on the plasma membrane and 79 ± 12 receptors on the nucleus. Whether the nuclear receptor is identical with the plasma-membrane receptor requires investigation.

The nuclear translocation and actions of steroid and thyroid hormones are well documented. There is also growing evidence that some polypeptide hormones and nerve growth factors, such as epidermal growth factor [38], growth factor [31,32], platelet-derived growth factor [57] and insulin [24,58], may also be translocated to the nucleus, and that this may be a mechanism which mediates some of their long-term effects. Our data demonstrate that intact IL1α is efficiently transported by receptor-mediated endocytosis from the plasma membrane to the nucleus via an endosomal compartment. It is unknown whether the IL1 is translocated to the nucleus free or complexed to its surface receptor. Studies on protein import into the nucleus have revealed that the information required for targeting is encoded in a short polybasic amino acid sequence [59]. The best example of this is the SV40 large T-antigen sequence -PKKKRKV-. The importance of this sequence is confirmed by the finding of similar sequences on other viral and non-viral proteins which are targeted to the nucleus, such as steroid-hormone receptors [60] and nucleoplasmin [61]. Recent evidence suggests that the polybasic targeting sequence is recognized by a polyacidic sequence contained within a receptor localized to the nuclear pore [62]. The IL1 receptor contains a polybasic -VKKSRR- sequence in its cytoplasmic tail, which is similar to the nuclear targeting motif [6]. Both murine and human IL1α contain an -LKKRRL- sequence which is similar to the putative nuclear targeting motif, as does IL1β, i.e. -PKKKMEKR- [63]. The importance of such a sequence for IL1 or IL1-receptor routing to the nucleus remains to be proven. Site-directed mutants of IL1 lacking these polybasic sequences may, however, resolve this problem. Evidence from EL4 subclones which are defective in IL1 endocytosis suggests that ligand internalization is essential for the biological response of target cells to IL1. Such subclones are unable to secrete IL2 or express IL2 receptor in response to IL1 and PMA stimulation [64]. Our finding that IL1α binds to isolated nuclei with high affinity would suggest that the surface IL1 receptor may not be necessary for nuclear targeting of IL1α. This suggests that IL1α may be released from its receptor in the endosomal compartment through which the receptor–ligand complex is routed. The presence of nuclear binding sites for IL1 raises the possibility that the mechanism of action of IL1 may not be restricted to events at the plasma membrane, and additional studies are needed to identify the nuclear IL1 receptor and to evaluate the importance of the translocation of IL1 in eliciting biological responses within the cell.

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