EXPERIMENTAL CELL RESEARCH **241,** 253–259 (1998)
ARTICLE NO. EX984056

Structure–Function Relationships of Pheromones of the Ciliate *Euplotes raikovi* with Mammalian Growth Factors: Cross-Reactivity between E*r*-1 and Interleukin-2 Systems

Adriana Vallesi,* Giovanna Giuli,* Paolo Ghiara,† Giuseppe Scapigliati,‡ and Pierangelo Luporini*,[1]

*Dipartimento di Biologia Molecolare, Cellulare e Animale, University of Camerino, 62032 Camerino (MC), Italy; †Dipartimento di Immunologia, IRIS, Chiron Biocine Spa, via Fiorentina 1, 53100 Siena, Italy; and ‡Dipartimento di Scienze Ambientali, Tuscia University, via de Lellis, 01100 Viterbo, Italy

Diffusible protein signals of the ciliate *Euplotes raikovi*, denoted as pheromones, have functionally been linked with prototypic growth factors of animal cells by the demonstration that they not only induce a temporary cell union in mating pairs, by acting in a paracrine-like fashion, but can also bind to cells in autocrine fashion and promote their vegetative (mitotic) proliferation. It is now shown that pheromone E*r*-1 is capable of binding to the α and β chains of the multimeric IL-2 receptor on mammalian cells and that IL-2 can, in turn, bind to the putative cell receptor of this pheromone. Similarities in the IL-2 and E*r*-1 structures support these findings and raise controversial implications with regard to their evolutionary significance.

INTRODUCTION

Diffusible protein cell markers, which distinguish otherwise morphologically similar cell (mating) types and are now usually referred to as (mating) pheromones [1–3], have been purified from the extracellular environment of protozoan ciliates such as *Blepharisma* [4], *Dileptus* [5], and *Euplotes* [6–8]. In *Euplotes raikovi*, comparative studies of their structures [9, 10] and analysis of Mendelian genetics of their control have shown that they form a large (indefinite) family of homologous proteins that most likely includes a number of subfamilies [1, 11]. Each family member, designated as E*r*-1, E*r*-2, and so forth (where E*r* stands for any *E. raikovi* pheromone and the number matches that of the cell type from which the pheromone is produced), appears to be controlled by one of a series of codominant alleles inherited at a single genetic locus in the cell germinal nucleus (micronucleus) [12] and as a rule contains 37–40 amino acid residues with six cysteines involved in the formation of three conserved intrachain disulfide bridges [13].

Different cellular responses are elicited by the E*r* molecules according to whether they bind to the same cells from which they are secreted in an autocrine fashion or to other cells in a paracrine-like (heterologous) fashion: vegetative cell proliferation is promoted in the former case and cell union in mating pair (conjugation) is promoted in the latter one [14]. The cell-surface receptors responsible for these E*r*-cell binding reactions have been identified with membrane-bound proteins whose extracellular COOH-terminal region is identical, at least in its amino acid sequence, to the secreted pheromone [15]. A unique genetic element, amplified to thousands of copies in the cell somatic nucleus (macronucleus) [16], specifies both the membrane-bound and the soluble E*r* forms in each cell type through a mechanism of alternate splicing of the primary transcripts [15].

In experiments undertaken to characterize functionally the type-I cell pheromone receptor (hereafter abbreviated as E*r*-1R, with reference to the primary autocrine activity of this receptor in binding the type-I cell pheromone E*r*-1), some mammalian protein hormones were assayed as potential competitors of the E*r*-1/E*r*-1R binding reactions [17]. Among them, human interleukin-2 (IL-2) and the epidermal growth factor (EGF) unexpectedly behaved as inhibitors, yet to distinct degrees of efficacy, of these reactions. In support of the remarkably stronger inhibitory activity exhibited by IL-2 with respect to EGF, some similarities were identified in the IL-2 and E*r*-1 amino acid sequences at the level of two short segments, one spanning five and the other seven amino acid residues, bridged together by a disulfide bond [17]. An additional structural similarity between E*r*-1 and IL-2 was then

[1] To whom correspondence and reprint requests should be addressed.

0014-4827/98 $25.00

revealed by analysis of NMR spectroscopy and X-ray crystallography of the tertiary conformations of E*r*-1 and other E*r*'s [10, 18], all of which appeared to be exclusively based on a helical bundle fold, as occurs in the case of IL-2 and its homologous cytokines [19–22].

In the light of these observations, we studied more throughly to what extent specific cross-binding reactions can occur between the E*r*-1 and IL-2 systems. The results of this investigation, described here, confirm the IL-2 capacity to bind the E*r*-1-like extracellular domain of E*r*-1R and, more important, show that E*r*-1 can, in turn, bind to basic components of the IL-2 receptor (IL-2R) on the surface of IL-2 target cells. Although these results imply the existence of functional interrelationships between E*r*-1 and IL-2 of potential interest also in an applicative perspective, they did not provide any crucial information for linking these interrelationships causally to the structural similarities that were detected in these molecules.

MATERIALS AND METHODS

Cells and culture conditions. Type-I cell cultures of *E. raikovi*, with a known homozygous genotype at the Mendelian locus *mat* containing information for the synthesis of both E*r*-1 and E*r*-1R [15], were derived from an offspring clone of the wild-type parental strain No. 13 (that has been deposited at the Culture Collection of Algae and Protozoa, CCAP, Ambleside, UK, under the Accession No. 1624/18). They were grown on green algae *Dunaliella tertiolecta* under controlled conditions, as described in detail elsewhere [23]. Before use, cells were concentrated and resuspended in new volumes of artificial sea water to remove debris and at least part of secreted E*r*-1 from the extracellular environment.

Cultures of the cell line CTLL-2 were kindly supplied by Dr. G. Forni (University of Turin). They were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 50 μg/ml of gentamycin sulfate supplemented with 5% heat-inactivated fetal bovine serum in the presence of recombinant IL-2 according to standard procedures [24]. Cells were washed twice with RPMI 1640 medium and 10% FCS and mantained without IL-2, for 1 h, before use.

Reagents. Bolton-Hunter reagent ([^{125}I]*p*-hydroxyphenylpropionic acid, *N*-hydroxysuccinimide ester) was purchased from Amersham International (Amersham, UK); molecular weight standards, Triton X-100, and PAGE reagents were from Bio-Rad Laboratories (Richmond, CA); recombinant human IL-2 and monoclonal anti-mouse antibodies IL-2 receptor/CD 25 clone AMT-13 were from Boeringher Mannheim (Indianapolis, IN); RPMI 1640 medium was from Gibco (Paisley, Scotland); fetal bovin serum was from Hyclone (Logan, UT); [^{125}I]IL-2 (sp act, 41 μCi/μg) was from NEN Dupont (Wilmington, DE); IL-2 and recombinant human IL-2R α and β subunits were from R&D Systems (Minneapolis, MN); BSA was from Serva Feinbiochemica GMbH & Co. (Heidelberg, Germany); BA 85 nitrocellulose (0.45 μm) was from Schleicher & Schuell, Inc. (Keene, NH); routine analytical grade reagents, sea salts, L-glutamine, gentamycin sulfate, PMSF, EDTA, and DMSO were from Sigma Chemical Co. (St. Louis, MO); and disuccinimidyl suberate (DSS), 1-ethyl-3-(3-dimethylaminopropil)-carbodiimide hydrochloride (EDC), and Sulfo-NHS were from Pierce Chemical Co. (Rockford, IL).

Purification and radioiodination of Er-1. Homogeneous samples of E*r*-1 were obtained through standard procedures of pheromone purification [23] and were radiolabeled using the Bolton-Hunter reagent [17]. The specific activity of [^{125}I]E*r*-1 ranged from 1 to 2.5 μCi/μg.

Cell membrane preparation from E. raikovi. For membrane preparation, cells were first lysed by suspension with hypotonic buffer (Tris–HCl, 2.5 mM, pH 7.5) and subsequent sonication for 3 s in ice. After removal of unbroken cells and nuclei from lysates by centrifugation at 1000*g*, 10 min, the supernatants were centrifuged at 50,000*g*, 30 min, washed twice with stabilization buffer (0.1 M $NaHCO_3$, 0.3 M NaCl, and 1 mM $MgCl_2$, 1 mM PMSF, pH 8.3), and immediately used or stored in liquid nitrogen before use.

SDS–PAGE and autoradiography. For autoradiography, 10% or 12.5% SDS–PAGE gels were run essentially according to Laemmli [25], stained with Coomassie blue R-250, dried, and exposed to Kodak X-Omat Ar films, at −70°C.

Cross-linking. Cross-linker DSS, previously dissolved in DMSO, was used at the final concentration of 100 μg/ml and EDC at the final concentration of 25 mM together with the enhancer Sulfo-NHS at the final concentration of 5 mM. Mixtures incubated with the cross-linker were then maintained under gentle shaking, at room temperature for 30 min. Before analysis on SDS–PAGE, mixtures between [^{125}I]IL-2 and material from *E. raikovi* were washed twice with 6 vol of 10 mM Tris–HCl, pH 7.4, 1 mM EDTA; the other ones, between [^{125}I]E*r*-1 and material from CTLLs, twice with 3 vol of PBS and 0.1% BSA.

RESULTS

IL-2 Binding to Er-1R

We first sought to provide more direct evidence, than had been earlier reported from competition binding assays [17], that IL-2 can specifically bind to E*r*-1R on the type-I cell surface. Affinity cross-linking experiments were carried out by incubating intact *E. raikovi* cells, or their membrane preparations with [^{125}I]IL-2 and the homofunctional cross-linker DSS, and monitoring the formation of radiolabeled protein complexes by SDS–PAGE and autoradiography. As shown in Fig. 1, only one principal species of complexes was revealed, and its formation appeared to represent a time-dependent and specific process. The intensity of the relative autoradiographic band, in fact, clearly increased the longer were the intervals of cell incubation with the radioligand, and no band was practically visible after an IL-2 or E*r*-1 excess was added to the incubation mixtures. Moreover, the molecular mass of 30 kDa shown by these radiolabeled complexes was fully consistent with the conclusion that they were derived from an association of [^{125}I]IL-2 with E*r*-1R in a 1:1 ratio, since the masses of these molecules have been calculated to be about 15.5 kDa [19, 20] and 14.5 kDa [15], respectively.

This conclusion was then confirmed by results of two other experiments designed on the basis of the notion that the extracellular domain of E*r*-1R, deputed to bind IL-2, and soluble E*r*-1 are structurally identical to each other [15] and may thus mimic each other in relevant functional activities. (i) Cross-linked protein complexes of about 21 kDa were obtained by mixing [^{125}I]IL-2 with E*r*-1 (molecular mass, about 4.5 kDa), as well as [^{125}I]E*r*-1 with IL-2 (as can be seen in Fig. 2, lane 3 of

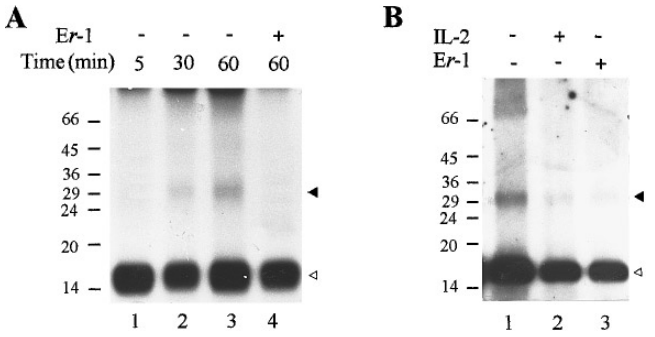

FIG. 1. Electrophoretic and autoradiographic analysis of [^{125}I]IL-2 cross-linking to intact cells and membrane preparations from *E. raikovi*. (A) Incubation of 1-ml cell samples (cell concentration, 2×10^4/ml), at 4°C, with 1 nM [^{125}I]IL-2 for 5, 30, and 60 min in absence (−) (lanes 1–3) or presence (+) (lane 4) of 500-fold molar excess of E*r*-1. (B) Incubation of 200-μl samples of membrane preparations (protein concentration, 200 μg/ml), at 4°C for 60 min, with 5 nM [^{125}I]IL-2, in absence (lane 1) or presence of 100-fold molar excess of IL-2 (lane 2) or 500-fold molar excess of E*r*-1 (lane 3). In each autoradiogram, filled and open arrowheads indicate a 30-kDa band and free radioligand, respectively, and the positions of the molecular mass (in kilodaltons) of standard proteins are shown on the left side.

the autoradiogram marked D and relative to experiments described in the next section). (ii) On the other hand, radiolabeled protein complexes were not revealed by analysis of cells which were incubated with [^{125}I]IL-2 after having been suspended with antibodies raised against E*r*-1 [15] (data not shown).

Er-1 Binding to IL-2R

To explore the capacity of *E. raikovi* pheromones to bind to IL-2R, experiments were carried out on the continuous murine T lymphocyte cell line CTLL-2 that is totally dependent on IL-2 binding for its survival and proliferation [26].

Binding of IL-2 to intact CTLLs was found to be not significantly inhibited by E*r*-1, nor could E*r*-1 replace IL-2 in sustaining CTLL proliferation or bind to living CTLLs with an affinity measurable with usual procedures such as the silicone-oil/paraffin-oil separation method [26] and centrifugation (unpublished observations). Nevertheless, initial evidence that E*r*-1 can effectively bind to CTLL membrane components, either individually and/or in synergistic association with IL-2, was derived from the following results. (i) Detergent CTLL extracts, which were separated on a gel electrophoresis system run under native conditions, transferred to nitrocellulose filters, and incubated with [^{125}I]IL-2 or [^{125}I]E*r*-1, produced a single band that was apparently equivalent on the two autoradiograms (data not shown). (ii) Rates of [^{3}H]thymidine incorporation were measured to be usually higher in CTLLs that had been previously suspended in a medium containing IL-2 plus E*r*-1 or other Er's such as E*r*-2 and E*r*-10 used at 1–10 μM concentrations, than in one containing IL-2 alone (data not shown).

To identify CTLL membrane components responsible for E*r*-1 binding, intact CTLLs were incubated with [^{125}I]E*r*-1 in the presence of the irreversible, bifunctional cross-linker EDC (used in place of DSS because [^{125}I]E*r*-1 bears only reactive carboxyl groups and no free amino groups) and then analyzed by SDS–PAGE and autoradiography. As shown in Figs. 2A and 2B, two major bands denoting the formation of radiolabeled complexes of about 60 and 74 kDa were revealed by this analysis, together with a minor and less compact one usually migrating in a range from 32 to 40 kDa. All three bands sharply decreased their intensity, or disappeared altogether, after addition of an IL-2 excess to the incubation mixtures or after exposition of CTLLs to antibodies blocking IL-2R [27]. Nevertheless, only the two most prominent bands deserved primary interest; the third one was taken as presumptive evidence of a partial breakdown of the principal protein complexes. In effect, their relative molecular masses of 60 and 74 kDa were closely consistent with an association of one 4.5-kDa molecule of [^{125}I]E*r*-1 with the two functional subunits of the IL-2R structure that are represented by proteins of 55 and 70 kDa and usually denoted as IL-2Rα chain and IL-2Rβ chain, respectively. These two IL-2R chains are coexpressed in CTLLs [26], and IL-2Rα is capable of binding IL-2 independently of both IL-2Rβ and the third IL-2R subunit (the IL-2Rγ chain) that are not capable; nevertheless, IL-2/IL-2Rα binding occurs with a relatively low affinity of the order of 10^{-8} M and without eliciting signal transduction [28–30].

Since IL-2Rα and IL-2Rβ are commercially available in the form of recombinant, soluble proteins of 36 and 30 kDa, respectively, it was possible to assess the [^{125}I]E*r*-1 capacity to bind these molecules in solution. As shown in Figs. 2C and 2D, this analysis fully validated this [^{125}I]E*r*-1 capacity, since it evidenced the specific and reversible formation of radiolabeled protein complexes of the expected molecular masses of 41 and 35 kDa. From the results of this analysis, it was in addition possible to appreciate a stronger [^{125}I]E*r*-1 binding to the 36-kDa IL-2Rα protein than to the 30-kDa IL-2Rβ protein. The band at 41 kDa was in fact consistently seen to be always more intense and prominent than the other one at 35 kDa as well as to be accompanied with at least another slower-migrating band denoting a tendency of the [^{125}I]E*r*-1 complexes with the 36-kDa protein to undergo associations of a higher order.

The validity of this indication was confirmed by using the 30-kDa IL-2Rβ and 36-kDa IL-2Rα proteins,

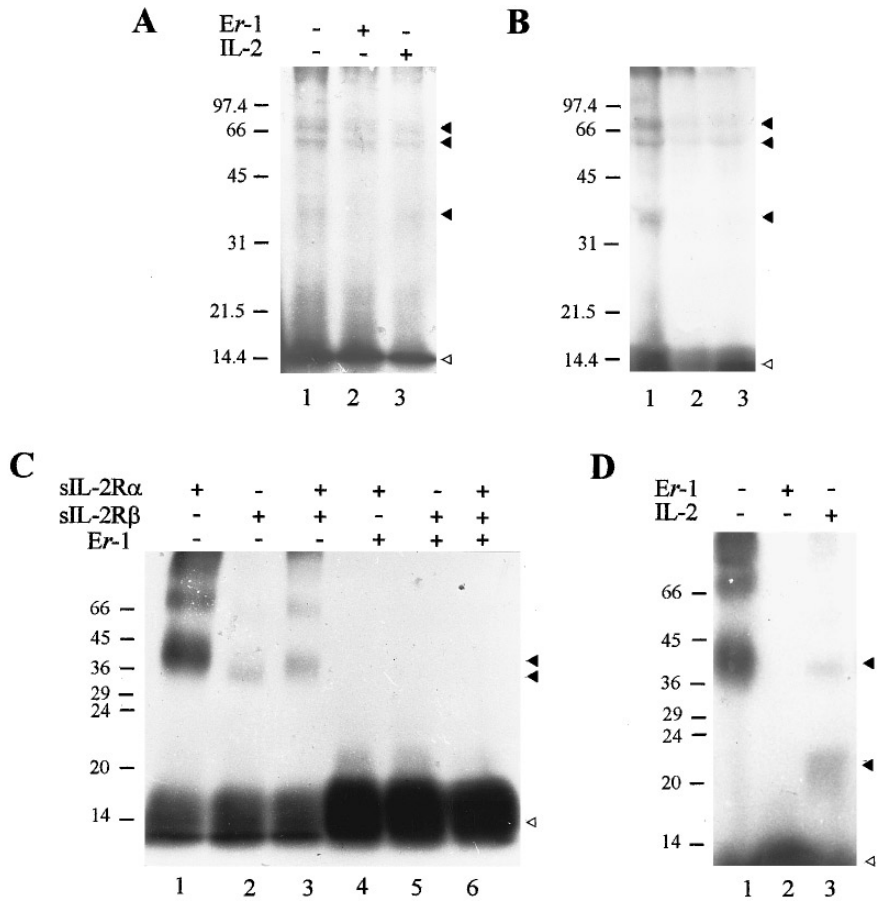

FIG. 2. Electrophoretic and autoradiographic analysis of [^{125}I]E*r*-1 cross-linking to CTLLs and to IL-2Rα and IL-2Rβ chains. (A) Incubation of 200-μl cell samples (cell concentration, 5×10^6/ml), in ice for 1 h, with 75 nM [^{125}I]E*r*-1 in PBS, pH 7.4, containing 0.1% BSA, in absence (lane 1) or presence of either 50-fold molar excess of E*r*-1 (lane 2) or IL-2 at equimolar concentration (lane 3). (B) Incubation of 200-μl cell samples (cell concentration, 5×10^6/ml) with 75 nM [^{125}I]E*r*-1 as in A, without (lane 1) or with 8 and 16 μg/ml monoclonal IL-2R antibodies (lanes 2 and 3, respectively). (C) Incubation of 50-μl samples of IL-2Rα and IL-2Rβ chains, for 1 h in ice, either individually (protein concentration, 55 and 66 nM, respectively) (lanes 1 and 2) or mixed together (protein concentration, 27 and 33 nM, respectively) (lane 3), with 750 nM [^{125}I]E*r*-1 in PBS buffer, pH 7.4, in the absence or presence of a 200-fold molar excess of E*r*-1 (lanes 4–6). (D) Incubation of IL-2Rα chain as in C, in the absence (lane 1) or presence of either 200-fold E*r*-1 molar excess (lane 2) or 2.5-fold IL-2 molar excess (lane 3). Filled arrowheads indicate radiolabeled protein complexes of about 74, 60, and 40 kDa in A and B, of about 41 and 35 kDa in C, and of about 41 and 21 kDa in D. In each autoradiogram, a open arrowhead indicates free radioligand, and the positions of the molecular mass (in kilodaltons) of standard proteins are reported on the left side.

previously adsorbed to nitrocellulose filters, in quantitative [^{125}I]E*r*-1 binding assays. These filter-immobilized molecules were first assayed with increasing [^{125}I]E*r*-1 concentrations in the range from 10 to 320 nM, and the measures of the relative [^{125}I]E*r*-1 amounts specifically bound to IL-2Rα were in every case determined, as shown in Fig. 3, to be nearly twofold higher than to IL-2Rβ. Similar assays were then repeated to calculate the relative affinities of these [^{125}I]E*r*-1 binding reactions in saturation experiments (not shown). An affinity of the order of 10^{-6} M was calculated for the [^{125}I]E*r*-1 binding to IL-2Rα, while no saturation was reached and a value of affinity could not be calculated in the case of the IL-2Rβ chain. Nevertheless, a comparative analysis of the [^{125}I]E*r*-1 binding inhibition by IL-2 provided additional evidence that the [^{125}I]E*r*-1 binding to the IL-2Rα chain occurs with an affinity of at least one order of magnitude higher than to the IL-2Rβ chain. As reported in Table 1, while nearly all [^{125}I]E*r*-1 bound to IL-2Rα was replaced by IL-2 used at equimolar concentrations, fivefold molar concentrations of IL-2 replaced only 15% of [^{125}I]E*r*-1 bound to IL-2Rβ.

DISCUSSION

The results we have described provide evidence that the ciliate pheromone E*r*-1 can bind to the α and β

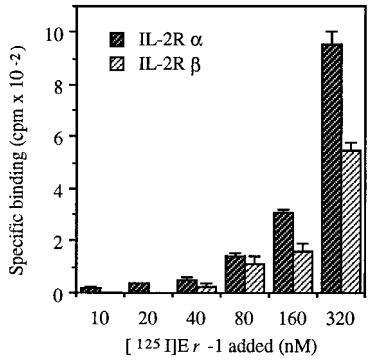

FIG. 3. Specific binding of [^{125}I]E*r*-1 to immobilized IL-2Rα and IL-2Rβ chains. Samples (25 ng) of soluble IL-2Rα and IL-2Rβ chains, diluted in PBS, pH 7.4, containing 1% BSA, were adsorbed to nitrocellulose filters (5 mm in diameter), which were then saturated with 3% BSA and incubated with increasing amounts of [^{125}I]E*r*-1, at room temperature for 30 min, under constant gentle agitation. At the end of incubation, filters were washed three times with PBS, pH 7.4, containing 1% BSA and 0.1% Triton X-100, and gamma counted. Nonspecific binding was determined in the presence of 200-fold molar excess of E*r*-1. Values are means of three independent measurements and error bars are SD.

chains of IL-2R, yet with different affinities, and confirm that IL-2 is able, in turn, to bind to the cell receptors of this pheromone. Before discussing whether and to what extent the occurrence of these cross-reactions reflects the existence of common structural traits in these two extracellular protein mediators, it is useful to reiterate some aspects of the biology of ciliate pheromones (only marginally mentioned in the Introduction) that are indicative, first of all, of the validity of a functional parallel between E*r*-1 and IL-2.

The study of ciliate pheromones has for long been centered only on the bipolar rationale and vocabulary of sex, as their alternative denominations, "mating substances" and "gamones," which are still in use, clearly denote [31]. This pheromone link to regulatory functions of phenomena of mutual cell sexual recognition and activation to cross-fertilization reflects the persistent utilization that has been made, in every procedure of their identification and purification, of the most immediately perceivable activity that these molecules can exert on the formation of cell mating pairs. However, it is now known that these molecules: (i) are constitutively secreted throughout the whole clonal life cycle, also by cells which are in developmental or physiological stages not permissive of mating [14, 32]; (ii) promote the DNA synthesis and (mitotic) reproduction of the cells from which they are synthesized and to which they bind in an autocrine fashion [14]; and (iii) can compete with one another in binding to the same receptors [17, 33], relying on their equivalent conformations that assemble together common and unique structural motifs [10]. Thus, these properties not only indicate how pertinent is the qualification of ciliate pheromones as prototypic cell growth factors, capable of eliciting varied and context-dependent cell responses, but also suggest that their action on mating should be regarded as a secondary acquisition for favoring an intercellular gene exchange at the cost of a temporary arrest of the cell mitogenic activity [14].

The indroduction of these new concepts in our understanding of the pheromone biology adds significance to the similarities detected in the E*r*-1 and IL-2 structures. However, the implications that these similarities raise in terms of chance, convergence, or common ancestry, in addition to being intrinsically quite controversial, can hardly be decoded in absence of any aid from references to molecular structures of potential intermediates of these two phylogenetically distant molecular systems. Nevertheless, the study of mechanisms of cell recognition and immunity in invertebrates has shown that precursors of numerous cytokines, such as IL-1 and IL-6 and tumor necrosis factor, are already synthesized by very successful early groups of both the protostome and deuterostome lines [34–36, and references therein].

After similarities in E*r*-1 and IL-2 structures had been identified, other E*r*'s were structurally characterized [9, 11] and can now be considered together with E*r*-1 in the comparison with IL-2. As shown in Fig. 4, only the shorter of the two segments of five and seven residues that are here the matter of discussion appears to represent a recurrent motif of the whole E*r* family and hence to be worthy of attention. However, the location of this shorter segment in the E*r* structure

TABLE 1

Inhibition of Specific [^{125}I]E*r*-1 Binding to IL-2Rα and IL-2Rβ Chains by IL-2 and E*r*-1

Excess molar concentrations (folds)		Inhibition (%)	
IL-2	E*r*-1	IL-2Rα	IL-2Rβ
0, 5		40	<5
1		60	<5
2, 5		78	<5
5		85	15
	50	82	40
	100	98	63
	200	100	90

Note. Samples (25 ng) of IL-2Rα and IL-2Rβ chains were immobilized onto nitrocellulose filters and incubated, for 30 min at room temperature, with [^{125}I]E*r*-1 (500 nM) in the presence or absence of increasing concentrations of E*r*-1 and IL-2. After incubation, specifically bound [^{125}I]E*r*-1 was quantified. Values represent means of four determinations obtained from two experiments. Nonspecific binding was 30–40% of total binding.

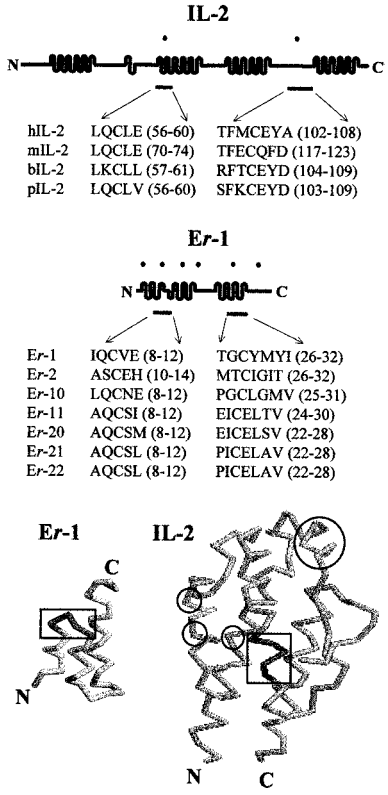

FIG. 4. Schematic representations of IL-2 and E*r*-1 structures. In the linear diagrams of the two molecules are shown helical regions (wavy line), approximate positions of Cys residues (dots), and the two segments (of five and seven amino acid residues) where amino acid sequence similarities were identified (bars). For each segment is reported (in the single-letter code) the amino acid sequence of IL-2 from various mammals (h, m, b, and p stand for human, murine, bovine, and porcine, respectively) and of other E*r*'s in addition to E*r*-1. In the 3-D diagrams are indicated the positions in IL-2 and E*r*-1 of the shorter sequence segment that is site of more significant similarities (squares) and the IL-2 binding sites to the IL-2Rα chain (large circle) and to the IL-2Rβ chain (small circles). (Data on IL-2 from Refs. 19, 20, 39; data on E*r*'s from Refs. 3, 9, 18, 37; the 3-D structures from Brookhaven Protein Data Bank, RasWin Molecular Graphics representation.)

does not appear to be equivalent with that assumed by its counterpart in IL-2. The former is substantially responsible for the formation of an interhelix loop [18, 37]; the latter lies inside one helix (helix B) that, moreover, does not contain any of the four sites of the IL-2 structure that have been shown, by site-directed mutagenesis experiments, to be more directly responsible for the IL-2 binding to the IL-2Rα and IL-2Rβ chains [38, 39]. The IL-2 IL-2Rα binding site is provided by a major cluster of four residues (Lys-35, Arg-38, Phe-42, and Lys-43) of a supplementary small helix (denoted as helix A′) arranged in a very extended loop connecting helices A and B of the IL-2 structure, while the IL-2 IL-2Rβ site is provided by three residues (Asp-20, Asp-84, and Asn-88) internal to helices A and C. In conclusion, at the level of amino acid sequences, the similarities of E*r*-1 to IL-2 seem to represent an instructive case of conservativism between evolutionarily unrelated molecules.

Some doubts on this conclusion arise with considerations on the gross three-dimensional configurations and the mode of receptor binding and activation of E*r*-1 and IL-2 and of the protein families that they represent. Every member of both families, in fact, adopts a molecular architecture with a core exclusively based on a bundle of antiparallel helices [10, 21, 22]. Much larger dimensions and four helices with an up–up–down–down topology obviously separate the architecture of the IL-2 family from that of E*r*-1, in which the helices are three and take on an up–down–up orientation. However, this separation is at least partly reduced, if also the presumed structure of the E*r* receptors, represented by 14.5-kDa membrane-bound isoforms of the same diffusible E*r*'s [2, 15], is taken into account. This structure should, in fact, contain a fourth transmembrane helix in addition to the three ones of the extracellular E*r*-like domain. Furthermore, it has been preliminarily observed that also the E*r* structure itself can accomodate a fourth helix, although exceptionally. This appears to be the case of a new *E. raikovi* pheromone (E*r*-23), the sequence of which has been recently determined to be of 52 amino acid residues with 10 cysteines, instead of the usual 37–40 with six cysteines (C. Miceli and G. Di Giuseppe, personal communication).

With regard to the behavior as receptor ligands shown by the members of the IL-2 and E*r*-1 families, it appears likely that the analogy is largely dependent on a common ability of these proteins to preserve their structural core of helices, irrespectively of the variations that may have profoundly separated their amino acid sequences. One reason of this preservation by the IL-2 family members appears to be their utilization of at least two distinct binding domains on their helical surfaces to promote an ordered or stepwise oligomerization of their receptor components [40–42]. In this context, it is worth noting how the sequential oligomerization of the trimeric IL-2R is usually induced by IL-2 through a first step of docking to the only subunit of the three that is ineffective in eliciting transduction [28, 43], namely, the IL-2Rα chain that we have shown to be most directly responsible for the E*r*-1 binding to IL-2R. An analogous behavior is apparently used by E*r*'s in their receptor binding reactions. A model, derived from analysis of E*r*-1 packing in the crystal structure, suggests that E*r*-1 receptor oligomerization is required for signalling and is obtained through the formation of at least two distinct types of dimers that derive from cooperative interactions of all the three

helices of neighboring molecules [18]. To clarify the nature and the evolutionary significance of the IL-2/Er-1 cross-reactions that we have described, more purposeful structural and biophysical analyses are necessary, and such studies might be instrumental also for the rational design of small cytokine mimics.

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Received June 23, 1997
Revised version received February 9, 1998