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Identification and Initial Characterization of an Autocrine Pheromone Receptor in the Protozoan Ciliate *Euplotes raikovi*

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Abstract. The polypeptide pheromone Er-1, purified from the ciliate *Euplotes raikovi* of mating type I and genotype *mat-1/mat-1*, was iodinated with ^{125}I-Bolton-Hunter reagent to a sp act of 0.45–0.73 μCi/μg of protein. This preparation of ^{125}I-Er-1 bound specifically to high affinity binding sites on the same cells of mating type I. Binding of ^{125}I-Er-1 occurred with an apparent K_d of 4.63 $\pm$ 0.12 $\times$ 10^{-9} M in cells in early stationary phase. It was estimated that these cells carry a total number of $\sim$5 $\times$ 10^7 sites/cell, with a site density that falls in the range of 1,600–1,700/μm^2 of cell surface. Unlabeled Er-1, other homologous pheromones such as Er-2 and Er-10, antibodies specific for Er-1, and human IL-2 were shown to act as effective inhibitors of specific binding of ^{125}I-Er-1 to mating type I cells. The "autocrine" nature of the identified specific high affinity binding sites for Er-1 was further substantiated by cross-linking experiments. These experiments revealed that mating type-I cell membranes contain one protein entity of M_r = 28,000 that is capable of reacting specifically with the homodimeric native form of Er-1.

CHEMICAL cell signals are constitutively secreted by some species of ciliated protozoa and distinguish two or more cell classes (or "mating types") within a given species (reference 16 for review). Their presence in cell filtrates is usually revealed by the formation of (homotypic or intraclonal) mating pairs between cells of the same type suspended with a filtrate recovered from another cell type. These signals, or mating type factors, in the wake of their first chemical characterization in *Blepharisma japonicum* (reference 22, for review), were denominated "gamones" (21), a term derived from the contraction of "gametenhormone" (11), and defined "specific signal substances for interactions between cells complementary for fertilization that lead them to unite" (21). This definition expressed the view that ciliate mating type interactions, reputed similar in many respects to interactions between animal gametes in fertilization, involve phenomena of specific and mutual cell–cell recognition and activation in function of mating. This view, widely held since Sonneborn's discovery of mating types in *Paramecium aurelia* (27), was rationalized (12, 14, 23) by a model (known as "gamone-receptor hypothesis"), which assumes, quoting Kuhlmann and Heckmann (14), that "cells express receptors only for those gamones they do not synthesize themselves." Yet, univocal conceptual support and direct experimental evidence were never produced to corroborate this assumption.

More recently, mainly on the basis of (*a*) experimental data obtained on the mating type interactions and expression in *Euplotes raikovi* (16), (*b*) a reinterpretation of the studies of mating control in *B. japonicum* (16), and (*c*) intriguing (although rather neglected) observations that ciliate mating type factors may also exert mitogenetic effects (30, 31), whereby these factors are better considered as multifunctional, or pleiotropic, molecules capable of causing a range of effects rather than only inducing mating, it was proposed (16) that mating types evolved and basically function in ciliates as a purposeful and cell-specific mechanism of self recognition. The fundamental assumption intrinsic in the concept of self recognition is that, like in the "autocrine" secretion defined by Sporn and Todaro (28), the primary target of each mating type factor, renamed "mating pheromones" (16) and, hereafter, simply "pheromones," is a functional cell surface receptor produced by the same cell that secretes the pheromone.

The importance of autocrine and paracrine messengers in endocrine systems has been appreciated for some tissues of advanced vertebrates (3). Many of the tissue polypeptide growth factors function in this manner where systemic transport, a common element of the more classic endocrine substances, is replaced by other mechanisms, primarily diffusion (13). Autocrine interactions, in which the cells that synthesize and release the hormonal agent also bind and respond to it, are clearly widely distributed and may be particularly important in development. They also seem to characterize many transformed cells and therefore may be a major manifestation of oncogenesis.

In this report, we describe the identification of a high affinity autocrine receptor for the pheromone Er-1[1] (abbreviation of Euplomone *r*-1; reference 4) of *E. raikovi*, whose production in cells of mating type I is controlled by the allele *mat-1* at the mating type (*mat*) locus (20). In *E. raikovi*, the *mat* locus is highly polymorphic, as in many other species of *Euplotes* (for example see reference 9), and is represented by alleles codominantly expressed and inherited in 1:1 association with one pheromone (17). Protein amounts in the range of 1.4–3.5 mg (active at picomolar concentrations) can be obtained for each pheromone from 10 liters of cell filtrates (24). Among the five polypeptide pheromones of *E. raikovi* so far purified (4, 24, 26), Er-1 is the best characterized. The structure of the mature, secreted form has been determined by amino acid sequence analysis (25) and that of the precursor by molecular cloning and cDNA sequencing (20). The secreted form is a single chain polypeptide of 40 residues, with three disulfide bonds; its native structure is probably a dimer (or a larger aggregate) between identical units tightly associated in a noncovalent manner (25).

This finding provides direct evidence that the mechanisms of autocrine secretion and self recognition do not constitute an exclusive property of pluricellular complex organisms, but rather have their origins in unicellular eukaryotes.

Materials and Methods

Materials

[^{125}I]Iodine and [^{125}I]*p*-hydroxyphenylpropionic acid, *N*-hydroxysuccinimide ester (^{125}I-Bolton-Hunter reagent) were purchased from Amersham International, Amersham, UK; routine analytical grade reagents, sea salts, aprotinin from bovine lung (bPTI), octyl-β-glucopiranoside, PMSF, EDTA, and DMSO from Sigma Chemical Co., Poole, UK; Bio-Gel P-10, low molecular weight standards, Triton X-100, and PAGE reagents from Bio-Rad Laboratories, Richmond, CA; BSA from Serva Feinbiochemica GMbH & Co., Heidelberg, FRG; glutaraldehyde (25% in water) from Fluka Biochemica, Bucks, Switzerland; BA 85 nitrocellulose (0.45 μm) from Schleicher & Schuell, Inc., Keene, NH; polyethylene glycol (PEG 6,000) from Merck & Co., Rahway, NJ; bovine insulin, human choriogonadotropin (hCG), human epidermal growth factor (hEGF), human interleukin 1-β (hIL-1β), and human interleukin 2 (hIL-2) from Boehringer Mannheim Biochemicals, Indianapolis, IN; disuccinimidyl suberate (DSS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *n*-hydroxysulfosuccinimide (Sulfo-NHS), and 1,3,4,6-tetrachloro- 3-diphenylglycoril (iodogen) from Pierce Chemical Co., Rockford, IL; human gonadotropin releasing hormone (hGNRH) from Calbiochem-Behring Corp., San Diego, CA; chicken gonadotropin releasing hormone (cGNRH) was kindly provided by Dr. A. Polzonetti (Camerino, Italy).

Cells and Pheromones

Cells of *E. raikovi* used were of clone laF$_1$13 obtained as sexual offspring (17) of the wild-type strain 13 (19), deposited (collection number, 1624/19) at the Culture Collection for Algae and Protozoa (CCAP), The Ambleside Laboratories, UK. They were supplied with green algae *Dunaliella tertiolecta* grown in artificial sea water and maintained under controlled conditions, at 24°C, as described elsewhere in detail (17).

Cells of *E. rariseta* belong to strain Ges$_3$ (6), of *Paramecium tetraurelia* to strain No. 51 (provided by Dr. T. Harumoto, Camerino), and of *Blepharisma japonicum* to strain "Rlog-I" (provided by Dr. A. Miyake, Camerino).

Pheromones Er-1, Er-2, and Er-10 were purified according to published procedures (4).

Preparation of Er-1 Antiserum

Antiserum was prepared by immunizing a New Zealand white rabbit with purified 1-mg samples of Er-1. For the immunization, Er-1 samples dissolved in physiological solution were mixed with equal volumes of Freund's incomplete adjuvant and injected subcutaneously into the rabbit. Booster injections (1-mg each) were given every 2 wk for 2 mo. The rabbit was bled after every immunization and the antibody titer was assayed by ELISA tests.

Preparation of Radiolabeled Er-1

Radiolabeling of Er-1 was carried out essentially according to the manufacturer's instructions. Briefly, 0.5 mCi of ^{125}I-Bolton-Hunter reagent (2,000 Ci/mMol) were incubated for 20 min, at 4°C, with 10 μg of Er-1 in 10 μl of 0.1 M Na_2BO_3, pH 8.8. The reaction was terminated by the addition of 100 μl of 0.2 M glycine, 0.1 M Na_2BO_3, pH 8.5. After 5 min at 25°C, 300 μl of 6 M guanidine-HCl were added to the incubation mixture. The radiolabeled Er-1 molecules were separated from unincorporated Bolton-Hunter reagent by chromatography on a Bio-Gel P-10 column (0.5 × 10 cm) equilibrated with PBS, pH 7.5. The preparation was then applied to a Superose-12 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M Tris, 0.15 M KCl, pH 7.5. The biologically active fractions, assayed according to standard methods (17) and corresponding to the maximal specific activity, were pooled, stored at −20°C, and used within 14 d.

Bioassay of Radiolabeled Er-1

The biological activity of ^{125}I-Er-1 was evaluated according to standard parameters (17), based on the percentage of tester cells induced to form mating pairs upon suspension with serially diluted pheromone samples. Tester cells were taken from clone 1bF$_1$13 of mating type II (17).

Preparation of Cells

Before use in the experiments, cells were washed and resuspended with fresh medium, i.e., artificial sea water for *Euplotes* and synthetic medium (21) for *Blepharisma* and *Paramecium*, in order to remove unbound (secreted) pheromones and catabolites from the extracellular environment. Cells were fixed with 0.25% glutaraldehyde in the presence of 0.2% BSA. After 1 h of fixation at 4°C, glutaraldehyde was removed by cell washing with three volumes of fresh medium containing 0.2% BSA.

Preparation of Cell Cortex Fractions

A 90-nm-thick cell cortex delimits the cell surface of *E. raikovi* (5); it consists of the plasma membrane plus two continuous alveolar membranes enclosing protein plates. Fractions of this cortex were prepared from cell cultures (5 liters), washed, resuspended at a density of ~12 × 10^3 cells/ml with fresh artificial sea water, and harvested by centrifugation at 600 *g* for 10 min, at 4°C. Harvested cells were suspended for 1 min with stabilization buffer (0.1 M $NaHCO_3$, 0.5 mM EDTA, 1 mM PMSF, pH 7.2) containing 0.5% Triton X-100, and then precipitated by centrifugation (1 h, 50,000 *g*), at 4°C. To remove Triton X-100 (that under these conditions only causes solubilization of cytoplasmic membranes, without disrupting cell cortex) and the pheromone activity from the supernatant, the pellet was resuspended and centrifuged at least three times with the same buffer without Triton X-100. The final preparation, suspended in 10 ml of stabilization buffer and diluted 1:500 before being used in binding experiments, could be stored for ~1 mo at −80°C without any appreciable loss in the pheromone binding activity.

Extraction of Soluble Binding Sites

Cell cortex preparations, obtained as described above, were suspended with octyl-β-glucopyranoside (final concentration, 1%) in the presence of 1 mM PMSF, and stirred overnight at 4°C with an end-over-end mixer. Insoluble material was removed by centrifugation (4 h, 50,000 *g*), at 4°C, and the supernatant (solubilized membranes) was recovered. The final protein content was estimated (2), using BSA as a standard, to be 15–20 mg/ml, with an average recovery of 3.5 μg protein for 10^3 cells. The preparation of solubilized membranes was diluted 1:10 in stabilization buffer before to be used in binding experiments.

1. *Abbreviations used in this paper*: bPTI, aprotinin from bovine lung; c, chicken; DSS, disuccinimidyl suberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carboniimide hydrochloride; Er-1, pheromone of *Euplotes raikovi* of *mat-1/mat-1* genotype and mating type I phenotype; GNRH, gonadotropin releasing hormone; h, human; hCG, human choriogonadotropin; PEG, polyethylene glycol; Sulfo-NHS, *n*-hydroxysulfosuccinimide.

Binding Experiments

In experiments involving cells and cell cortex preparations, samples were incubated with the radioligand in the presence of 0.2% BSA and applied to wells of a spotting manifold (Bio-Dot; Bio-Rad Laboratories) assembled with a BA 85 nitrocellulose membrane. Unbound radioactivity was washed away under vacuum by addition of 3 vol of either sea water (for cells) or stabilization buffer (for cortex preparations), both containing 0.2% BSA. The nitrocellulose membrane was then cut into small pieces, each one corresponding to one well, and the radioactivity detected on a gamma counter (RackGamma II; LKB Instruments, Inc., Gaithersburg, MD).

In experiments involving solubilized membranes, the reactions with the radioligand were carried out in the presence of 0.2% BSA and 0.5% octyl-β-glucopyranoside and terminated by the addition of 2 vol of calf serum and 4 vol of 20% PEG 6,000. Samples were centrifuged (10 min, 12,000 *g*) at 4°C, the supernatants decanted, and pellets resuspended in 8 vol of 12.5% PEG 6,000 and centrifuged again. The radioactivity of each pellet was eventually counted.

Specific binding was determined by measuring the difference between total bound radioactivity and nonspecific binding determined in parallel series of replicated samples incubated with the radioligand in the presence of a 100-fold molar excess of Er-1.

Cross-linking Experiments

Solubilized membranes (25-μl samples diluted in equal volume of stabilization buffer) were mixed with ^{125}I-Er-1 (100 ng), at 0°C, in the presence or absence of 25-fold or greater molar excess of Er-1. After 25 min, the reaction mixtures were incubated, at 24°C for 40 min, with aliquots of DSS (freshly prepared dissolving 0.25 mg/ml in DMSO), or with aliquots of freshly prepared EDC (100 mM in 50 mM PBS, pH 6.5) and enhancer Sulfo-NHS (50 mM in 50 mM PBS, pH 6.5).

PAGE and Autoradiography

PAGE was carried out under nondenaturing conditions essentially according to Dewald et al. (7), and under denaturing and reducing conditions according to Laemmli (15). Gels were either stained with Coomassie blue R-250, or dried and exposed to Kodak X-Omat Ar films at −80°C.

Results

Preparation of ^{125}I-Er-1

To identify and characterize Er-1-receptors on mating type-I cells, we prepared radiolabeled Er-1. Since Er-1 consists of a single polypeptide chain with four tyrosine residues (20, 25), we first tried a tyrosine-directed method of iodination, i.e., iodogen (18). This method proved unsuccessful, however. High specific radioactivity was introduced, but Er-1 bioactivity was completely lost. The ^{125}I-Bolton-Hunter reagent was therefore chosen to radioiodinate Er-1, as it offered the advantage of introducing, under mild reaction conditions, the bulky iodine solely at the amino terminus because of the absence of other amino groups (20, 25).

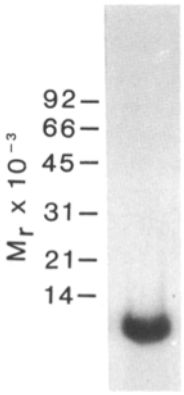

Figure 1. SDS and autoradiographic analysis of ^{125}I-Er-1. ^{125}I-Er-1 (specific radioactivity, 0.73 μCi/μg) was boiled for 6 min in sample buffer containing 2% SDS and 5% 2-mercaptoethanol, and applied (1.5×10^4 cpm) to a linear gradient (5–20%) gel. The migration positions of marker proteins are indicated.

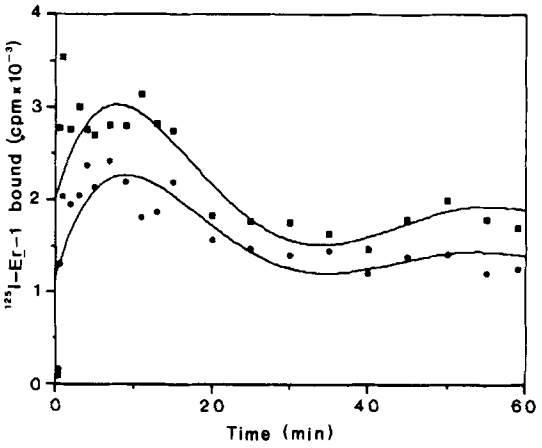

Figure 2. Time course of binding of ^{125}I-Er-1 to living cells of mating type I. A sample of cells in early stationary phase was suspended (cell density, 10^4/ml) with 300 ng/ml of ^{125}I-Er-1 (1.54×10^3 cpm/ng), at 24 (■) or 0°C (●), and aliquots (250 μl) withdrawn at progressive times. The data, of one experiment taken as representative, have been interpolated and represent specific binding.

At the end of the reaction the labeled Er-1 molecules were completely separated from unincorporated Bolton-Hunter reagent by chromatography on a Bio-Gel P-10 column, and then applied to a Superose-12 column operated on a HPLC system to obtain biologically active fractions with the maximal specific activity. With this iodination method we were regularly able to obtain ^{125}I-labeled preparations of Er-1 with specific activities in the range of 0.45–0.73 μCi/μg, that showed no appreciable loss of biological activity for at least 2 wk when stored at −20°C. As shown in Fig. 1, on analysis by SDS-PAGE and subsequent autoradiography of the gel, these preparations typically contained a single molecular species which migrated as a well defined band of M_r = 5,000. In some experiments, a minor component of M_r = 9,500–10,000, that is in the area expected for Er-1 homodimers, was also evident (data not shown).

Time Course of ^{125}I-Er-1 Binding

We prelimarily assayed binding of ^{125}I-Er-1 to living cells of mating type I as a function of time, maintaining the radioligand concentration constant at the physiological level of 300 ng/ml. Cells used were in early stationary phase, i.e., ones that, after 1 d of starvation (during which cell division continues for one or two generations), start accumulating in the G_1 stage of the cell cycle (8).

As shown in Fig. 2, binding proceeded very rapidly and with a similar trend at either 24 or 0°C, although it was initially appreciably lower (26–28%) in cells incubated at 0°C. Cell-bound radioactivity reached a peak within 5–10 min of incubation; then, it decreased until a nearly constant level of about half of the initial maximal amount remained associated with the cells after about 30 min.

Subsequently, an experiment of time-course binding of ^{125}I-Er-1 to cell cortex preparations was conducted (data not shown). The radioligand binding was again very rapid; how-

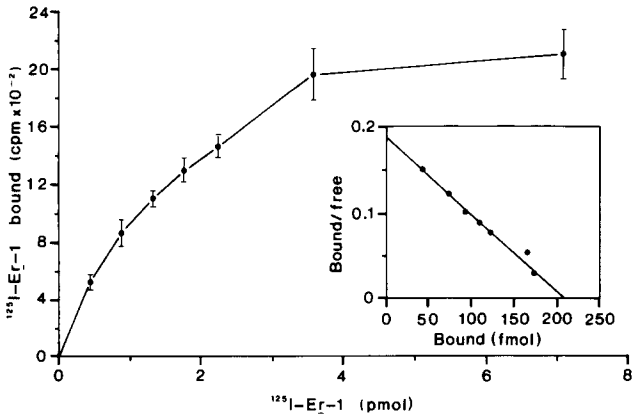

Figure 3. Concentration dependence and Scatchard analysis of ^{125}I-Er-1 binding to cells of mating type I in early stationary phase. Cells (2.5×10^3) were incubated for 1 h with increasing concentrations of ^{125}I-Er-1 in final volumes of 250 μl, at 24°C. Each value is the mean (±SD) of six to eight determinations obtained from three experiments and represents specific binding. Nonspecific binding was in the range of 39–58% of total binding.

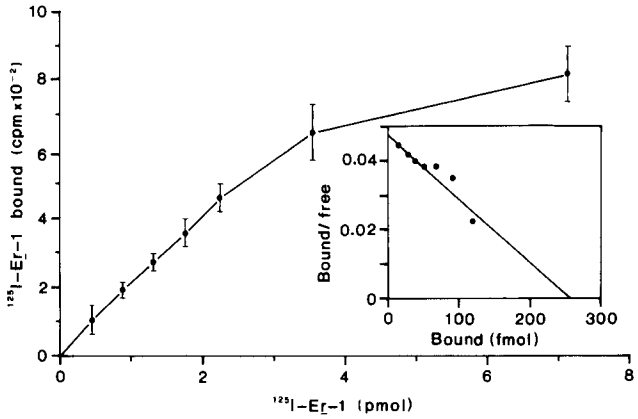

Figure 5. Concentration dependence and Scatchard analysis of ^{125}I-Er-1 binding to cell cortex fractions of mating type I cells. Cell cortex samples (230 μl) were incubated for 1 h with increasing concentrations of ^{125}I-Er-1 in final volumes of 250 μl, at 24°C. Each value is the mean (±SD) of four determinations obtained from two experiments and represents specific binding. Nonspecific binding was in the range of 44–60% of total binding.

ever, once a peak was reached, values of bound radioactivity remained virtually unchanged. This difference in the time-course patterns of ^{125}I-Er-1 binding, between living cells and cell cortex preparations, was taken as presumptive evidence that an active, complex regulation of pheromone binding takes place in living cells. Therefore cells were fixed before being used intact in the next experiments in order to avoid interference either by cellular metabolic processes or by passive radioligand uptake through the cell cytostome. This fixation was carried out with 0.2% glutaraldehyde, which also has been successfully used in human cell lines to study interactions of high affinity receptors with polypeptide hormones, such as EGF (1).

^{125}I-Er-1 Concentration-dependent Equilibrium Binding

Direct binding experiments were first carried out by incubating increasing concentrations of ^{125}I-Er-1 with cells of logarithmically and asynchronously growing cultures, deprived of food immediately before being used, and cells in early stationary phase. As shown in Figs. 3 and 4, the plots of specific counts bound as a function of radioligand concentration (assuming binding of the dimeric form of Er-1) indicate that the binding is a dose-responsive and saturable process in both cases examined. Scatchard analyses of the data indicated a single class of high affinity binding sites for Er-1 and an apparent equilibrium dissociation constant (K_d) equivalent between cells in early stationary phase (K_d = 4.63 ± 0.12 [SD] × 10^{-9} M) and in growth phase (K_d = 4.99 ± 0.19 [SD] × 10^{-9} M). In the former, however, the average number of binding sites per cell was nearly twice that in the latter (5.0×10^7 vs. 2.9×10^7). On the basis that the estimated total surface area of *E. raikovi*, calculated as the surface of

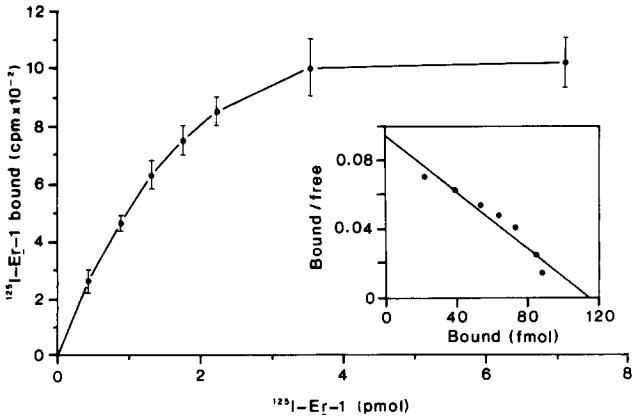

Figure 4. Concentration dependence and Scatchard analysis of ^{125}I-Er-1 binding to cells of mating type I in growth phase. Experimental conditions and values reported as in legend to Fig. 3. Nonspecific binding was in the range of 45–56% of total binding.

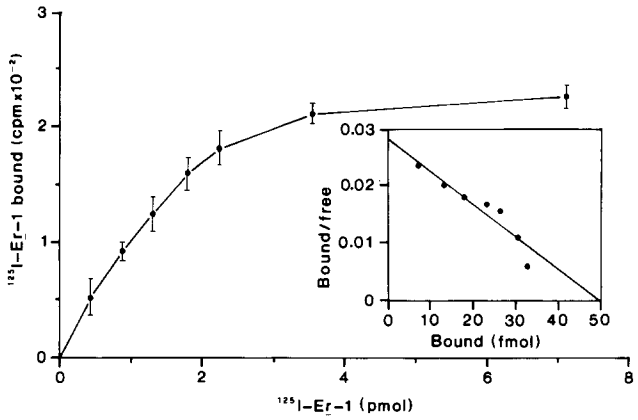

Figure 6. Concentration dependence and Scatchard analysis of ^{125}I-Er-1 binding to solubilized membrane preparations of mating type I cells. Membrane samples (5 μl containing ~9 μg of protein) were incubated for 1 h with increasing concentrations of ^{125}I-Er-1 in final volumes of 250 μl, at 24°C. Each value is the mean (±SD) of four determinations obtained from two experiments and represents specific binding. Nonspecific binding was in the range of 50–67% of total binding.

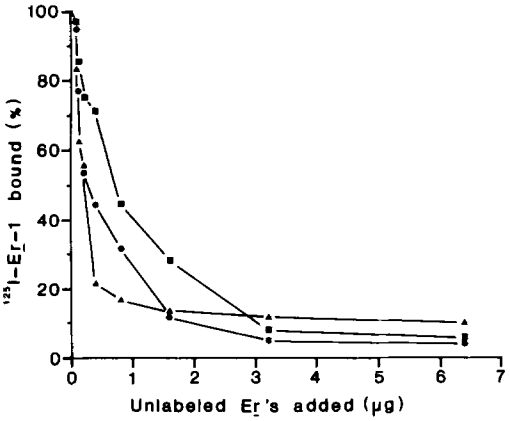

Figure 7. Displacement of ^{125}I-Er-1 by increasing concentrations of Er-1 and two other homologous pheromones. Cells (2.5×10^3) in early stationary phase were incubated with samples consisting of saturating amounts of 5.5×10^{-12} M of ^{125}I-Er-1 plus serially increasing amounts of Er-1 (▲), Er-2 (●), and Er-10 (■) for 1 h, in final volumes of 250 μl at 24°C. The reported values represent specific binding and are of one experiment taken as representative. Nonspecific binding was in the range of 11–42% of total binding.

a half ellipsoid of $40 \times 15 \times 15$ μm plus the surface of an estimated number (2,300) of cirral and membranellar cilia (each one matching a cylinder of dimensions 0.25×15 μm), is ~30,000 μm², the maximal Er-1 binding site density on mating type-I cells is in the range of 1,600–1,700 molecules/μm².

The effect of increasing concentrations of ^{125}I-Er-1 was then determined for binding to cell cortex and solubilized membrane preparations of cells in early stationary phase. The data (illustrated in Figs. 5 and 6) confirm that the binding reaction is a saturable process and involves a single class of sites. In addition, no net change of slope (K_d = 6.76 ± 0.29 [SD] × 10^{-9} M) was observed in a Scatchard plot of specific counts bound to solubilized membranes, whereas a conspicuous change occurred in the plot of binding to cell cortex preparations (K_d = 2.59 ± 0.97 [SD] × 10^{-8} M). This observation, therefore, provides evidence that pheromone binding sites were effectively extracted by the detergent and maintained in a functional state.

Binding Site Competition

Samples of early stationary cells were incubated with sets of mixtures consisting of a saturating amount of ^{125}I-Er-1 plus serially increasing quantities of Er-1, or of the two other allelic pheromones Er-2 and Er-10. These pheromones were purified from *E. raikovi* of genotype *mat-2/mat-2* (17, 24) and *mat-10/mat-10* (26), respectively, and show only ~40% identity with Er-1 (26; Raffioni, S., R. A. Bradshaw, and P. Luporini, manuscript submitted for publication). However, they are similar with respect to biological activity, native molecular weight, pI, and most likely three-dimensional structure as suggested by the conservation of half-cystine residues. The results, shown in Fig. 7, indicate that the amount of ^{125}I-Er-1 bound to the cells was reduced, in a dose-dependent manner, to the amounts of Er-1 and, as well, of the two other pheromones contained in the mixtures. Therefore, Er-1 binds reversibly to the cells and both Er-2 and Er-10 prove to be as effective as Er-1 in the competition assay.

^{125}I-Er-1 binding specificity to mating type-I cells was further examined by including rabbit antiserum to Er-1 in the binding medium and using various unlabeled proteins as competitors.

The antiserum used at a dilution of 1:100 eliminated 82% of ^{125}I-Er-1 binding, while the binding eliminated by the preimmune serum (used in parallel and at the same dilution) reached 40%.

As summarized in Table I, among a panel of human polypeptide growth factors tested at increasing molar concentrations, IL-2 was found to function as a very effective competitor: 50-fold molar excess inhibited 90% of ^{125}I-Er-1 binding. Also EGF and IL-1β, although at a markedly less extent than IL-2, showed an inhibitory effect on the radioligand binding. On the other hand, the inhibition by insulin was insignificant, and bPTI, hCG, hGNRH, and cGNRH had no effect on ^{125}I-Er-1 binding.

Binding of ^{125}I-Er-1 to Other Ciliates

The capacity of three different species of ciliates, *Euplotes rariseta*, *Paramecium aurelia*, and *Blepharisma japonicum*, to specifically bind ^{125}I-Er-1 was examined using the same experimental procedure as that of the equilibrium binding experiments carried out on *E. raikovi* (and illustrated in Figs. 4 and 5). None of the species tested specifically bound any ^{125}I-Er-1.

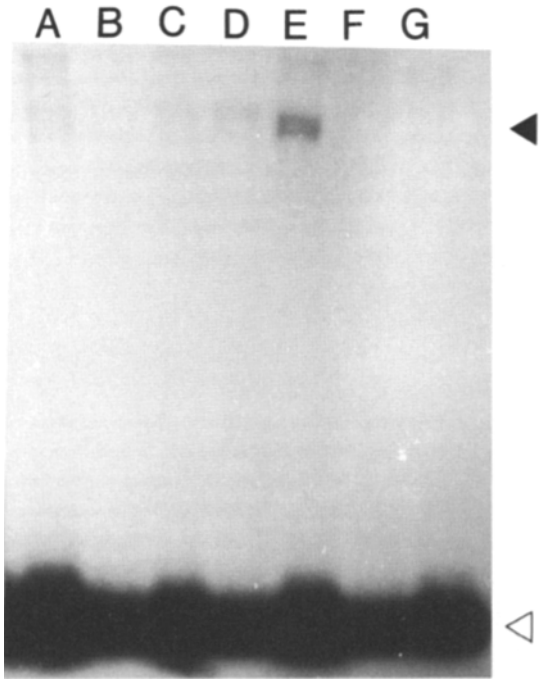

Figure 8. Triton X-100 PAGE and autoradiographic analysis of solubilized membrane preparations of mating type I cells cross-linked to ^{125}I-Er-1. Reaction mixtures between membrane samples and ^{125}I-Er-1 were incubated, in the presence or absence of an excess of Er-1, with DSS (5 mM) and analyzed on a linear gradient (5–20%) gel containing 0.1% of Triton X-100. Lane *A*, without DSS, as a control. Lanes *B–D*, with 100-, 50-, 25-fold excess of Er-1, respectively. Lane *E*, without Er-1. Lanes *F* and *G*, ^{125}I-Er-1, without membrane fractions, incubated with or without DSS, respectively, as controls. The solid arrowhead indicates the complex of interest and the open one indicates ^{125}I-Er-1.

Table I. Inhibition of Specific ^{125}I-Er-1 Binding to Mating Type I Cells by Various Purified Proteins

Excess molar concentration	bPTI	hCG	hGNRH	cGNRH	Insulin	hEGF	hIL-1β	hIL-2
					% of inhibition			
Equimolar	<5	<5	<5	<5	<5	<5	<5	<5
10-fold	<5	<5	<5	<5	<5	10	9	44
25-fold	<5	<5	<5	<5	11	24	15	61
50-fold	<5	<5	<5	<5	18	47	34	90
100-fold	<5	ND	<5	<5	26	ND	ND	90

^{125}I-Er-1 (5.5×10^{-12} M) was incubated at 24°C with mating type I cells (2.5×10^3 in 250 μl) in the presence or absence of increasing concentrations of each protein. After 1 h of incubation, specifically bound ^{125}I-Er-1 was quantified. Values represent the mean of four determinations obtained from two experiments.

Cross-linking of ^{125}I-Er-1 to Membrane Proteins

To identify the Er-1 binding sites on mating type I cells, affinity cross-linking experiments, using either the bifunctional reagent DSS, or the heterofunctional EDC in association with the enhancer Sulfo-NHC, were carried out. Solubilized membrane fractions were incubated with ^{125}I-Er-1, in the presence or absence of an excess of Er-1 to delineate the specificity of binding, and the resulting complexes were exposed, under mild cross-linking conditions, to relatively low concentrations of DSS (5 mM) or EDC (20 mM) for 40 min. As shown in Figs. 8 and 9, the autoradiographic analysis of the labeled proteins after electrophoresis on different polyacrylamide gel systems revealed only one principal species, which was cross-linked and migrated as a sharp band of M_r = 38,000 under denaturing and reducing conditions (Fig. 9). Interestingly, this band was not evident when the DSS–cross-linked complexes were run under denaturing conditions (data not shown). This is probably due to single amino groups in Er-1 subunits, the site of reactivity for DSS. Variations in reaction conditions, such as doubling the incubation time and/or the cross-linker concentrations, did not result in the appearance of additional bands, nor of substantial amounts of high molecular mass material at the top of the separating gel.

Discussion

By using the pheromone Er-1 purified from *E. raikovi* cells of mating type I and radiolabeled to high specific activity with ^{125}I, we provided the first direct evidence of the specific interaction of a ciliate pheromone with the cell surface. This interaction was shown to entail pheromone binding, rapid and of high affinity, to specific sites exposed on the same cells that produce the pheromone. These binding sites were saturable and showed K_d values of the order of 10^{-9} M, and not of 10^{-12} M as earlier calculated and preliminarily reported in abstracts (Ortenzi, C., C. Miceli, P. Luporini. *J. Protozool.* 1989. 36:3A–23A). The binding of the radiolabeled pheromone was inhibited by the same native pheromone, as well as by other homologous and structurally related pheromones. If, as we believe, the identified binding sites represent the Er-1 receptor, it may be suggested that: (*a*) as with other polypeptide hormones, the first step in the action of ciliate pheromones involves interaction with a specific cell surface receptor; and (*b*) as this receptor is expressed by the same cells that produce the pheromone to which it specifically binds, it represents a functional unit of an autocrine secretion system. The latter conclusion strongly substantiates that ciliate mating types constitute a mechanism for a self recognition process and, at the same time, challenges the "gamone-receptor hypothesis" based on the assumption that the mating type expression involves suppression of the receptor for the pheromone (gamone, sex substance) that the cell produces (12, 14). Furthermore, this conclusion well accounts for the results of Mendelian genetic analyses of mating type inheritance so far carried out in many different species of *Euplotes* (e.g., reference 9). All these results in fact suggest that both the molecules relevant to the mating type expression of the cell (i.e., the pheromone and its receptor), are both controlled by genes lying in the same locus *mat*. The molecular basis of this control is currently under investigation.

Preliminary structural characterization of the Er-1/receptor complex suggests a molecular mass of the complex of ~38 kD. If it is assumed that the biologically active form of Er-1 is a dimer of ~10 kD and that it participates in a 1:1 ratio in the formation of these complexes, then the membrane protein would be ~28 kD. The number of Er-1 receptors per cell was calculated from Scatchard plots to reach a maximum of $\sim 5 \times 10^7$ in early stationary phase cells, uniformly accumulated in the G_1 stage of the cell cycle, whereas it was about half in cells in asynchronous multiplication. Although this seems to be a high number of receptors for a single cell, the density of these receptors, estimated to be 1,600–1,700/μm^2 of cell surface, is quite comparable to that calculated for receptors of polypeptide hormones in numerous types of mammalian cells, where the plasma membrane is much less extended than in an *E. raikovi* cell. In agreement with these calculations, we have observed (unpublished data), using Er-1 conjugated to fluorescent dyes, that pheromone binding sites are distributed over the entire cell surface, and seem to be particularly dense in the cytostomal area and in the surrounding compound ciliary organelles.

In addition to Er-1, other pheromones, such as Er-2 and Er-10, displaced ^{125}I-Er-1 binding to mating type I cells, consistently with predicted similarities of three-dimensional structure based on sequence analyses (20, 25; Raffioni, S., R. A. Bradshaw, and P. Luporini, manuscript submitted for publication) and the commonality of immunological epitopes (10) among these homologous polypeptides. The relative inhibitory capacities of Er-2 and Er-10 were quantitatively correlated to, and apparently effective as native Er-1, as argued from a comparison (illustrated in Fig. 7) of the patterns of ^{125}I-Er-1 binding inhibition. This observation provides direct evidence that the same cell and, presumably, the same recep-

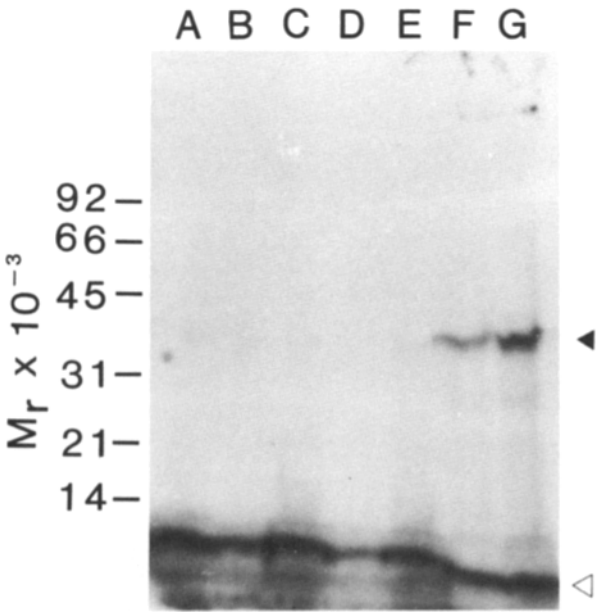

Figure 9. SDS-PAGE and autoradiographic analysis of solubilized membrane preparations of mating type I cells cross-linked to ^{125}I-E*r*-1. Reaction mixtures between membrane samples and ^{125}I-E*r*-1 were incubated, with or without an excess of E*r*-1, with different concentrations of EDC and 5 mM of enhancer Sulfo-NHS. Before being loaded on a linear gradient (5–20%) gel, the mixtures were suspended in sample buffer containing 2% SDS and 5% 2-mercaptoethanol, and boiled for 6 min. Lanes *A–C*, 25-, 50-, and 100-fold excess of E*r*-1, respectively, and 25 mM EDC plus Sulfo-NHS. Lanes *E–G*, 10, 20, and 25 mM EDC, respectively, plus Sulfo-NHS. Lane *D*, ^{125}I-E*r*-1, without membrane fractions, incubated with 25 mM EDC plus Sulfo-NHS, as control. The solid arrowhead indicates the cross-linked complex of interest and the open one indicates the monomeric form of ^{125}I-E*r*-1 (which migrates faster in lanes *F* and *G*, probably as a consequence of an increased concentration of urea generated by the EDC reaction.) The migration positions of marker proteins are indicated.

tor can bind with comparable affinities qualitatively different (i.e., "self" and foreign or "nonself") pheromones. The study of how this differential receptor binding (i.e., "autocrine" and "paracrine") may actually be capable of promoting two separate cellular responses, one addressed to maintain the cell vegetative growth and the other to interrupt this growth and prepare the cell to mate, is crucial for a better understanding of ciliate mating type mechanism. Regardless of the functional responses elicited, the presence of autocrine receptors on such ancient, single-celled organisms indicates that "self-induction" in hormone-like systems is a mechanism that developed very early in evolution.

Among the molecules tested for this capability to inhibit ^{125}I-E*r*-1 binding, hIL-2 unexpectedly was a very effective competitor. A comparison of the amino acid sequences of E*r*-1 (20, 25) and hIL-2 (29) reveals some similarity in two segments, each one surrounding a half-cystine residue. One segment consists of five amino acid residues: Ile^{8}-Gln-Cys-Val-Glu in E*r*-1 and Leu^{56}-Gln-Cys-Leu-Glu in hIL-2; the other contains seven residues: Thr^{26}-Gly-Cys-Tyr-Met-Tyr-Ile in E*r*-1 and Thr^{102}-Phe-Met-Cys-Glu-Tyr-Ala in hIL-2. Only two of seven residues are actually conserved in this latter comparison but there is a concentration of sulfur-containing and aromatic amino acids that may increase the significance. In addition, it has been found (Eardley, D., and C. Miceli, unpublished results), that E*r*-1 monoclonal antibodies raised against E*r*-1, and produced by clone mabE*r*-1/g (10), block the proliferation of mouse T cell lines that are totally IL-2 dependent.

These results suggest a structural relatedness between *E. raikovi* pheromones and mammalian lymphokines. Whether this relatedness results from a genuine evolutionary divergence cannot be ascertained without further information, such as the three-dimensional structure of both molecules.

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References

1. Boonstra, J., P. van Maurik, L. H. K. Defize, S. W. de Laat, J. L. M. Leunissen, and A. J. Verkley. 1985. Visualization of epidermal growth factor receptor in cryosections of cultured A431 cells by immuno-gold labeling. *Eur. J. Cell Biol.* 36:209–216.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
3. Bradshaw, R. A., and M. B. Sporn. 1983. Polypeptide growth factors and the regulation of cell growth and differentiation. *Fed. Proc.* 42:2590–2591.
4. Concetti, A., S. Raffioni, C. Miceli, D. Barra, and P. Luporini. 1986. Purification to apparent homogeneity of the mating pheromone of *mat-1* homozygous *Euplotes raikovi*. *J. Biol. Chem.* 61:10582–10587.
5. Dallai, R., P. Salvatici, and P. Luporini. 1985. Plasma membrane specialization at the discharge site of the excretory pore-less vacuole of the ciliate *Euplotes raikovi*. *Tissue & Cell.* 17:309–320.
6. Dallai, R., C. Miceli, and P. Luporini. 1986. *Euplotes rariseta* Curds et. al. (Ciliophora, Hypotrichida) from the Somalian coast: description and preliminary observations on cyst induction and ultrastructure. *Monit. Zool. Ital.* 22:263–280.
7. Dewald, B., J. T. Dulaney, and O. Touster. 1974. Solubilization and polyacrylamide gel electrophoresis of membrane enzymes with detergents. *Methods Enzymol.* 32:82–91.
8. Dini, F., and P. Luporini. 1979. Preconjugant cell interaction and cell cycle in the ciliate *Euplotes crassus*: switching from the vegetative to the sexual stage. *Dev. Biol.* 69:506–516.
9. Dini, F., and P. Luporini. 1985. Mating-type polymorphic variation in *Euplotes minuta* (Ciliophora, Hypotrichida). *J. Protozool.* 32:111–117.
10. Fiori, P. L., C. Miceli, S. Raffioni, and A. Vallesi. 1990. Specific and common epitopes in mating pheromones of *Euplotes raikovi* revealed by monoclonal antibodies. *J. Protozool.* 37:187–190.
11. Hartmann, M., and O. Schartau. 1939. Untersuchungen uber die Befruchtungsstoffe der Seeigel. *I. Biol. Zentralbl.* 59:571–587.
12. Heckmann, K., and H. W. Kuhlmann. 1986. Mating type and mating inducing substances in *Euplotes octocarinatus*. *J. Exp. Zool.* 237:87–96.
13. James, R., and R. A. Bradshaw. 1984. Polypeptide growth factors. *Annu. Rev. Biochem.* 53:259–292.
14. Kuhlmann, H. W., and K. Heckmann. 1989. Adolescence in *Euplotes octocarinatus*. *J. Exp. Zool.* 251:316–328.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.
16. Luporini, P., and C. Miceli. 1986. Mating pheromones. *In* Molecular biology of ciliated protozoa. J. G. Gall, editor. Academic Press, Inc., New York. 263–299.
17. Luporini, P., S. Raffioni, A. Concetti, and C. Miceli. 1986. The ciliate *Euplotes raikovi* heterozygous at the *mat* locus coreleases two individual species of mating pheromones: genetic and biochemical evidence. *Proc. Natl. Acad. Sci. USA.* 83:2889–2893.
18. Markwell, M., and F. Fox. 1978. Surface-specific iodination of membrane proteins of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3alpha-diphenyl glycouril. *Biochemistry.* 17:4807–4817.
19. Miceli, C., A. Concetti, and P. Luporini. 1983. Isolation of the mating in-

ducing factor of the ciliate *Euplotes*. *Exp. Cell Res.* 149:593–598.
20. Miceli, C., A. La Terza, and M. Melli. 1989. Isolation and structural characterization of cDNA clones encoding the mating pheromone Er-1 secreted by the ciliate *Euplotes raikovi*. *Proc. Natl. Acad. Sci. USA.* 86:3016–3020.
21. Miyake, A. 1974. Cell interaction in conjugation of ciliates. *Curr. Top. Microbiol. Immunol.* 64:49–77.
22. Miyake, A. 1981. Cell interaction by gamones in *Blepharisma*. *In* Sexual Interactions in Eukaryotic Microbes. D. H. O'Day, and P. A. Horgen, editors. Academic Press, Inc., New York. 95–129.
23. Miyake, A. 1981. Physiology and biochemistry of conjugation in ciliates. *In* Biochemistry and Physiology of Protozoa. Vol. 4. M. Levandowsky, and S. H. Hutner, editors. Academic Press, Inc., New York. 211–230.
24. Raffioni, S., C. Miceli, A Concetti, D. Barra, and P. Luporini. 1987. Purification and characterization of new mating pheromones of the ciliate *Euplotes raikovi*. *Exp. Cell Res.* 172:417–424.
25. Raffioni, S., P. Luporini, B. T. Chait, S. S. Disper, and R. A. Bradshaw. 1988. Primary structure of the mating pheromone Er-1 of the ciliate *Euplotes raikovi*. *J. Biol. Chem.* 263:18152–18159.
26. Raffioni, S., P. Luporini, and R. A. Bradshaw. 1989. Purification, characterization and amino-acid sequence of the mating pheromone Er-10 of the ciliate *Euplotes raikovi*. *Biochemistry.* 28:5250–5256.
27. Sonneborn, T. M. 1937. Sex, sex inheritance and sex determination in *Paramecium aurelia*. *Proc. Natl. Acad. Sci. USA.* 23:378–385.
28. Sporn, M. B., and G. J. Todaro. 1980. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* 303:878–880.
29. Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin-2. *Nature (Lond.).* 302:305–310.
30. Tavrovskaja, M. W. 1974. Induction of cell division with culture fluid from clones of different mating types of *Dileptus anser* (Ciliata, Holotricha. Gymnostomatida). *Functional Morphology, Genetics, and Biochemistry of the Cell*, Academy of Science of USSR, Leningrad. 16:83–85. (In Russian.)
31. Yudin, A. L., S. Y. Afon'kin, and E. V. Parfenova. 1990. Mating pheromones in the ciliate *Dileptus anser*. *Tsitologia.* 32:107–116. (In Russian with English summary.)