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Primary structure of *Euplotes raikovi* pheromones: Comparison of five sequences of pheromones from cells with variable mating interactions

(homology/ciliated protozoa/cell recognition/signal transduction/hydrophilicity profiles)

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ABSTRACT **The amino acid sequences of five pheromones, E*r*-2, E*r*-3, E*r*-9, E*r*-11, and E*r*-20, secreted by cells of different mating types of the ciliated protozoa *Euplotes raikovi*, have been determined by automated Edman analyses of the whole proteins and germane fragments. In each case, the molecular mass was determined by plasma desorption or laser desorption mass spectrometry and was in excellent agreement with the calculated values. Where available, the determined sequences were also in accord with the corresponding segments of the precursor molecules predicted from relevant nucleic acid sequences. Of the five, two were found to be identical (E*r*-2 and E*r*-9) and one (E*r*-3) was identical to a pheromone previously sequenced (E*r*-1), even though mating pair formation was found to take place (although to a limited extent) when cells secreting those pheromones were combined in a mixture. Comparison of the five unique sequences suggested a closer relationship between E*r*-1 (E*r*-3) and E*r*-10 and between E*r*-11 and E*r*-20 (44% and 56% identity, respectively) than was generally observed among the other members. This pairing was also supported by hydrophobicity analyses. Interestingly, E*r*-20 cannot, as a rule, induce cell union in any of the other cell types, including cells secreting E*r*-11, despite the fact that E*r*-20 and E*r*-11 are the most similar of the five unique sequences. Thus sequence identity and secondary structure profiles are not a good indicator of biological relatedness as manifested in heterologous receptor interaction.**

Cell recognition and signal transduction by polypeptide ligands, such as growth factors and hormones, constitute a major feature of the cells of higher eukaryotes (1, 2). However, such processes are also found, albeit generally in simpler forms, in unicellular eukaryotic organisms, and they may represent the evolutionary forerunners to the sophisticated regulation mechanisms of the more recent species.

An excellent example of transmembrane signaling in a unicellular eukaryote is that regulated by the pheromones of ciliated protozoa (3). Species of the genus *Euplotes* are endowed with highly polymorphic systems of mating types (4) (as phenotypic classes of vegetative, or asexual, cells) that are distinguished from one another by small secreted polypeptides (pheromones) (5, 6), the synthesis of which is controlled by a series of multiple alleles at the single Mendelian locus *mat* (7, 8). The pheromone secreted by one cell type may bind to cells of compatible mating types and induce the formation of mating pairs. However, during vegetative life, these molecules act in an autocrine fashion (9), supposedly to control self-recognition and cell growth (3, 9). Both these binding processes appear to be mediated through cell-surface receptors that are thought to arise from alternate splicing of the transcripts from the same gene that produces the pheromone precursor (10).

After the development of a reproducible protocol for the isolation of homogeneous pheromones from the standardized media of *Euplotes raikovi* (11, 12), two pheromones, designated E*r*-1 and E*r*-10, were purified and sequenced (13, 14). The structural characterization of the corresponding cDNA clones has shown that the pheromones are synthesized as precursors that contain a putative signal peptide and a prosegment amino terminal to the mature sequence, an organization typical of many secreted proteins (15, 16).

Pheromones from other *E. raikovi* cell types, with various degrees of mating interactions, have now been isolated and their amino acid sequences have been determined. As discussed in this report, they are part, along with E*r*-1 and E*r*-10, of a homologous family that shows clear subgroupings and a conservation of residues presumably germane to a common conformation and preserved functions.

EXPERIMENTAL PROCEDURES

Pheromones and Cells. Sources of pheromones E*r*-2, E*r*-3, E*r*-9, and E*r*-11 were cells of known genotype at the *mat* locus (3) and belonged to clones $1bF_113$ (obtained from the wild strain 13 deposited at the Culture Collection of Algae and Protozoa, Ambleside, U.K., under the accession no. 1624/18), $1aF_127$, $1aF_11N$, and $1aF_13N$, respectively. These clones each represent a different mating type, based on the formation of mating pairs in mixtures prepared between any two of them. Numerous pairs formed in any clone combination except those involving clones producing E*r*-2 and E*r*-9, where the number of pairs were substantially reduced and, usually, did not exceed 5% of mixed cells. Pheromone E*r*-20 was obtained from cells of the wild strain GA-4, which show mating incompatibility, yet share a common gene pool, with cells of all the other clones described above (A. Valbonesi, C. Ortenzi, C.M., and P.L., unpublished observations).

All pheromones, except E*r*-20, were purified as described previously (11, 12). For E*r*-20, the last step of the purification procedure consisted of a reversed-phase chromatography using a Supercosil LC 318 column (Supelchem, Milan) connected to an HPLC system. In a continuous 20–50% acetonitrile gradient in 0.1% trifluoroacetic acid, at a constant flow rate of 0.7 ml/min, E*r*-20 was eluted at 37%. As a rule, 100–200 μg of pure E*r*-20 was recovered from 10 liters of supernatant.

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Abbreviations: E*r*-, *Euplotes raikovi* pheromone; Cm-, carboxymethyl-; PTH, phenylthiohydantoin.

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Materials. Cyanogen bromide, guanidine hydrochloride, iodoacetic acid (recrystallized before use), and carboxypeptidase Y were from Sigma; trifluoroacetic acid was from Pierce; iodo[1-^{14}C]acetic acid was from New England Nuclear; HPLC-grade solvents were from Fisher; and gas-phase sequencer chemicals were from Applied Biosystems. All other chemicals were of reagent grade.

Reduction and *S*-Carboxymethylation. *S*-Carboxymethylation was carried out as described previously (13); *S*-carboxymethylated products are indicated by Cm-.

Cleavage with Cyanogen Bromide and Separation of Peptides. Cm-E*r*-2 (0.5 mg) was treated with a 50-fold molar excess of CNBr over total estimated methionine residues in 1 ml of 70% (wt/wt) formic acid. After 24 hr under nitrogen at room temperature, the reaction mixture was diluted with 15 vol of distilled water and lyophilized (17). The resulting peptides were resolved by reversed-phase HPLC on a Vydac C_{18} column (4.6 × 250 mm), equilibrated in 5% acetonitrile with 0.1% trifluoroacetic acid, using an acetonitrile gradient. Cm-E*r*-9 was treated similarly.

Carboxyl-Terminal Analysis. Five nanomoles of Cm-E*r*-2 was dissolved in 0.02 M sodium phosphate, pH 6.0, and carboxypeptidase Y was added to a final mole ratio of 1:100 (18). Aliquots were removed at designated times, acidified with 1 M HCl, and centrifuged, and the recovered supernatant was dried prior to derivatization and amino acid analysis.

Amino Acid Analysis. Duplicate samples (0.5 nmol of pheromone or 10% of peptide fraction from reversed-phase chromatography) were hydrolyzed in 6 M HCl for 22 hr at 100°C under reduced pressure and dried. The phenylthiocarbamoyl amino acid derivatives were quantified by reversed-phase HPLC on a Hewlett–Packard 1090 instrument (19).

Amino Acid Sequence Determination. Sequence analysis was performed using either an Applied Biosystem 470A gas-phase sequencer and phenylthiohydantoin (PTH) derivatives were identified by a Hewlett–Packard 1084B instrument equipped with an Altex Ultrasphere ODS column, or a 477A pulse-liquid sequencer with an on-line 120A PTH-amino acid analyzer. Aliquots from each cycle were subjected to scintillation counting to determine the position of *S*-[^{14}C]carboxylmethylated cysteine residues.

Mass Spectrometry. The molecular masses of all five pheromones were determined by ^{252}Cf plasma desorption mass spectrometry, while those of E*r*-11 and E*r*-20 were also determined by matrix-assisted laser desorption mass spectrometry. The analyses were performed on time-of-flight mass spectrometers, constructed at the Rockefeller University (20, 21), as previously described (13, 22). The mass accuracy for both techniques was better than 2 parts in 10^4. Approximately 1 nmol of protein was used for the plasma desorption analyses and 1 pmol for the laser desorption analyses.

Hydropathy Analysis. Hydrophilicity profiles of the pheromone sequences were predicted according to parameters suggested by Hopp and Woods (23), using Apple DNA Inspector II software.

RESULTS

Amino Acid Sequence of E*r*-2 and E*r*-9. The amino acid sequence of E*r*-2 (and E*r*-9) was determined by automated Edman degradation and carboxypeptidase Y digestion of reduced and carboxymethylated E*r*-2 and from similar analyses of the CNBr peptides. As shown in Fig. 1, and predicted from amino acid analyses, there are five CNBr fragments; each was isolated and entirely sequenced (Table 1). The order of four of these was provided by the sequence determination of the whole molecule; the position of the fifth fragment is implied by difference. Direct evidence was provided by the partial sequence of peptide CB-4-5, which arose from incomplete cleavage of the Met-Thr bond at positions 26-27. Peptides corresponding to residues 27–34 and 35–40, which presumably were formed by acid cleavage of the Asp-Pro bond at 34-35, were also isolated (data not shown). The carboxyl-terminal Leu-Pro sequence was confirmed by timed release of carboxypeptidase Y products. This sequence coincides with that predicted from the cDNA sequence coding for the precursor (16).

The amino acid sequence of E*r*-9 was found to be identical to that of E*r*-2 by using a similar approach, which was confirmed by the molecular weight determination obtained with the mass spectrometer. To understand why cells secreting sequentially identical pheromones behave like cells of different mating types and form mating pairs (although to a limited extent) requires further investigation (see also *Discussion*).

Amino Acid Sequence of E*r*-11 and E*r*-20. Nearly complete sequence information for the pheromones E*r*-11 and E*r*-20 was obtained from direct sequence analysis of the intact mature pheromones (Table 2). In each case, 36 cycles were completed, providing for the positive identification of 30 amino acids [the 6 half-cystine residues were not modified and were therefore only inferred from the related sequences of E*r*-1 and E*r*-10 (13, 14)]. Compositional (data not shown) and mass spectral analyses (Table 3) suggested that E*r*-20 contained an additional residue of serine and that E*r*-11 had additional residues of serine, proline, and phenylalanine.

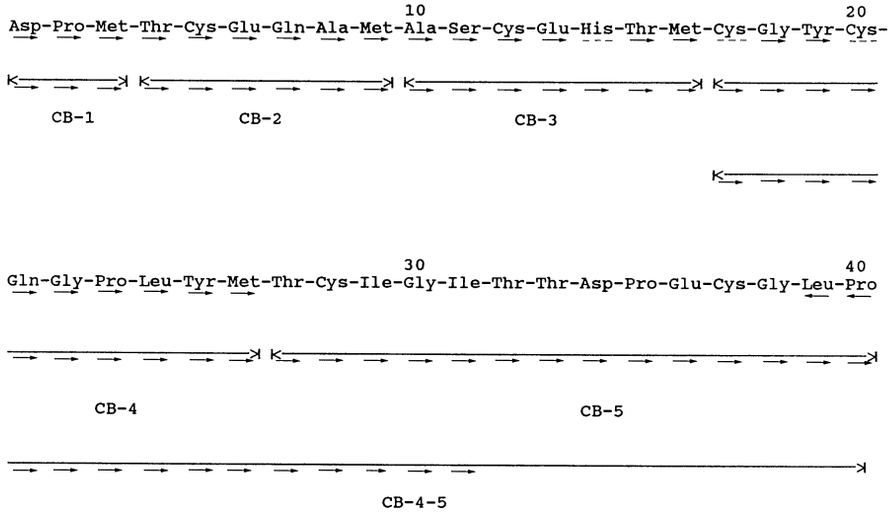

FIG. 1. Summary of the complete amino acid sequence determination of the pheromone E*r*-2. The peptides obtained from cyanogen bromide cleavage are designated as CB and have been numbered according to the theoretical order in which they appear in the sequence. Peptides indicated by two numbers connected with a hyphen result from partial cleavage. Residues directly identified by automated Edman degradation and carboxypeptidase Y digestion are marked by → and ←, respectively.

Table 1. Amino acid sequence data for the pheromone E*r*-2 and the peptides derived from CNBr cleavage

	Cm-E*r*-2*			Cm-E*r*-2*			CB-1		CB-2		CB-3		CB-4		CB-5		CB-4-5	
Cycle	Residue	pmol	Cycle (cont.)	Residue	pmol	Cycle	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol	Residue	pmol
1	Asp	105	16	Met	23	1	Asp	93	Thr	65	Ala	929	Cys	—	Thr	154	Cys	—
2	Pro	134	17	Cys	—	2	Pro	25	Cys	—	Ser	376	Gly	218	Cys	—	Gly	233
3	Met	96	18	Gly	95	3	(Met)†	—	Glu	55	Cys	—	Tyr	308	Ile	222	Tyr	324
4	Thr	25	19	Tyr	17	4			Gln	93	Glu	432	Cys	—	Gly	122	Cys	—
5	Cys	—	20	Cys	—	5			Ala	46	His	100	Gln	181	Ile	187	Gln	355
6	Glu	22	21	Gln	14	6			(Met)†	—	Thr	156	Gly	139	Thr	89	Gly	158
7	Gln	74	22	Gly	12	7					(Met)†	—	Pro	90	Thr	96	Pro	216
8	Ala	82	23	Pro	8	8							Leu	152	Asp	49	Leu	207
9	Met	82	24	Leu	8	9							Tyr	120	Pro	63	Tyr	211
10	Ala	112	25	Tyr	10	10							(Met)†	—	Glu	75	Met	20
11	Ser	81	26	Met	10	11									Cys	—	Thr	45
12	Cys	—				12									Gly	37	Cys	—
13	Glu	93				13									Leu	28	Ile	62
14	(His)	—				14									Pro	18	Gly	65
15	(Thr)	—				15											Ile	52

Values represent the yield, in pmol, for each sequencer cycle of peptides. Serine was quantitated as the sum of PTH-Ser and its reduced form. Residues in parentheses indicate qualitative identification of PTH derivatives. Cysteine was identified on the basis of radioactivity. Amounts analyzed: CM-E*r*-2, 250 pmol; CB-1, 300 pmol; CB-2, 200 pmol; CB-3, 2000 pmol; CB-4, 500 pmol; CB-5, 500 pmol; and CB-4-5, 500 pmol.
*E*r*-2 was sequenced following reduction and [^{14}C]carboxymethylation.
†Methionine was detected as homoserine.

These assignments have been confirmed by sequence analysis of the corresponding cDNAs.

Amino Acid Sequence of E*r*-3. The complete amino acid sequence of pheromone E*r*-3 was determined from direct sequence analysis of the intact protein and from the peptides derived from CNBr and performic acid oxidation of the protein (data not shown). As confirmed by mass spectrometry (Fig. 2; Table 3), this pheromone was indistinguishable in sequence from E*r*-1 (13), even though (as in the case of cells secreting E*r*-2 and E*r*-9) a low percentage of cells formed mating pairs when mixed together.

Mass Spectral Analyses. The molecular masses of the pheromone subunits were independently determined by mass spectrometry. A representative mass spectrum (of E*r*-3) is shown in Fig. 2. In each case, measured values were in agreement with the calculated molecular masses determined from the proposed sequences to within the accuracy of the analyses (2 parts in 10^4). In addition, the analyses yielded three other observations: (*i*) The preparations of E*r*-2 used showed small amounts of material with twice the molecular mass of the major peak, suggesting that the dimeric form [which is apparently the major species in solution (9, 13)] is very stable. Although the solution dimers are unlikely to be covalently linked, small amounts of material (as observed here) might have arisen from artifactual cross-linking. (*ii*) The preparation of E*r*-20 contains a species with a mass of 4010.4, ≈15 atomic mass units greater than the calculated value. This material most likely arises from the oxidation of the single methionine residue to the corresponding sulfoxide derivative, a modification that can occur readily in proteins in oxidizing conditions (24). However, it may also indicate the presence of a quantitatively minor species of pheromone (see below). (*iii*) No evidence for any subform of E*r*-3, relative to E*r*-1 (13), was observed.

DISCUSSION

These analyses, taken with those reported previously (13, 14), bring to seven (five of which are unique) the number of known *E. raikovi* pheromone sequences. A comparative alignment of these molecules is shown in Fig. 3. Clearly, all five proteins form a homologous family, extending the conclusion reached on more limited information (14) to a broader basis. However, subtle relationships are discernible in this larger comparison that were not evident before.

In the alignment shown, there are only 7 residues common to the five unique sequences, the amino-terminal aspartic acid and the 6 half-cystine residues, and their conservation has been deliberately contrived by the insertion of a number of gaps. This is justified on the basis that the pairing of half-cystines is conserved in E*r*-1 and E*r*-2 (and presumably in all of the pheromones) (A. Stewart, S.R., T. Chaudhary, B.T.C., P.L., and R.A.B., unpublished observations) and thereby they provide a convenient benchmark for alignment based on a retained structural feature. Similarly the amino-terminal aspartic acid, as a common feature of all *E. raikovi* pheromones studied to date, may reflect a structural requirement for the processing enzyme that releases the mature form from the precursor. This conclusion is supported by the observations that the adjacent residue (providing the carboxyl group to the scissile bond) is variable (ref. 16; A. La Terza and C.M., unpublished observations).

There are 11 additional sites in which three or more of the pheromones share a common residue (marked by asterisks in Fig. 3); interestingly, 7 of these are clustered in the amino-terminal region, including all the residues between Cys I and Cys II. The absence of gaps in this region (discounting those inserted to align the amino-terminal Asp) further suggests that this portion of the pheromone molecule is well conserved and probably reflects a common feature, such as the interface between the subunits of the dimer. In contrast, the carboxyl-terminal domain (commencing after Cys III) is clearly less well conserved. Of the 22 positions between Cys II and Cys VI, 8 require one or more gaps for optimal alignment, and only 3 of the positions share a common residue in three or more sequences (discounting Cys residues). Nonetheless, there are patterns of relatedness that reveal subgroupings of the pheromones (see below). This may reflect a function for this region that is more closely related to unique properties, such as receptor interactions. It should be noted that there is additional heterogeneity in the carboxyl-terminal segment that results from the variable length of the polypeptides (and the alignment used). Four of the five sequences differ in length, which can be conveniently marked from Cys VI. Thus, E*r*-10 has only one residue in this "extension," while E*r*-11 and E*r*-20 have five, with E*r*-1 (E*r*-3) and E*r*-2 (E*r*-9) being intermediate with four and three, respectively. This segment does contain two of the positions with three or more conserved residues, suggesting it may have some functional importance as well.

Table 2. Sequence analysis of the pheromones Er-11 and Er-20

	Er-11		Er-20	
Cycle	Residue	pmol	Residue	pmol
1	Asp	1141	Asp	439
2	Glu	1193	Ile	1095
3	(Cys)	ND	(Cys)	ND
4	Ala	1732	Asp	307
5	Asn	1366	Asp	386
6	Ala	1683	Ala	781
7	Ala	1583	Val	906
8	Ala	1519	Ala	879
9	Gln	1085	Gln	451
10	(Cys)	ND	(Cys)	ND
11	Ser	712	Ser	302
12	Ile	1075	Met	590
13	Thr	679	Thr	397
14	Leu	855	Leu	629
15	(Cys)	ND	(Cys)	ND
16	Asn	613	Gln	344
17	Leu	810	Leu	532
18	Tyr	657	(Cys)	ND
19	(Cys)	ND	Tyr	286
20	Gly	473	Asn	230
21	Pro	393	Thr	202
22	Leu	491	Glu	101
23	Ile	521	Ile	313
24	Glu	263	(Cys)	ND
25	Ile	404	Glu	44
26	(Cys)	ND	Leu	106
27	Glu	144	Ser	75
28	Leu	235	Val	79
29	Thr	153	Ile	95
30	Val	177	Gly	57
31	Met	172	Ser	38
32	Gln	111	(Cys)	ND
33	Asn	97	Gln	16
34	(Cys)	ND	Pro	26
35	Glu	40	Pro	30
36	Pro	31	Phe	15

Amounts analyzed: Er-11, 3000 pmol; Er-20, 2000 pmol. ND, not determined; identity implied from other Er sequences. Ser was quantitated as the sum of PTH-Ser and its reduced form.

The percentage of identities ranges from 23% to 56%, with the majority in the 25–30% group. Considering that about half the identical residues are contributed by the half-cystines and the amino terminus, this level of relatedness is only minimal for homologous proteins. Two pairs stand out as exceptions to this pattern—i.e., Er-1/Er-10 and Er-11/Er-20. These show a distribution of conserved residues that is more uniform than the overall pattern, particularly in the Er-11/Er-20 pair. Remarkably, cells secreting Er-20 do not form mating pairs with any of the cells producing the other pheromones, including Er-11. At the same time, all of the other cell types do display good mating interactions, indicating receptor cross-reactions.

Table 3. Mass spectrometric analysis of the pheromones Er-2, Er-3, Er-9, Er-11, and Er-20

	Molecular mass, atomic mass units			
Pheromone	Major peak	Minor peak	Calculated	Method*
Er-2	4296.5	8592.0	4297.0	PD
Er-3	4410.5	—	4410.9	PD
Er-9	4296.2	—	4297.0	PD
Er-11	4202.8	—	4202.8	PD, LD
Er-20	3996.2; 4010.4	—	3995.6	PD, LD

The mass accuracy for both techniques was better than 2 parts in 10^4. Approximately 1 nmol of protein was used for plasma desorption analyses and 1 pmol for the laser desorption analyses.
*PD, plasma desorption mass spectrometry; LD, laser desorption mass spectrometry.

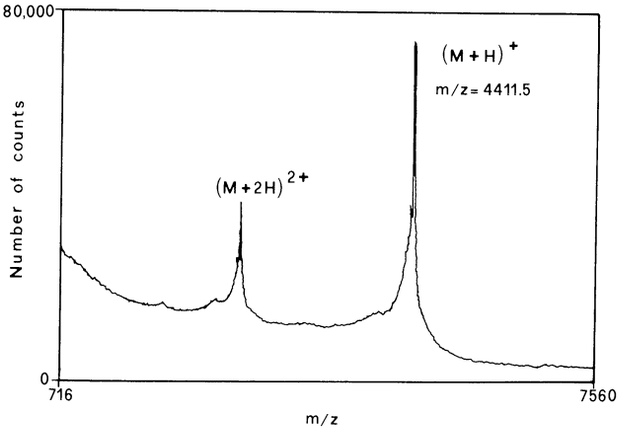

FIG. 2. ^{252}Cf plasma desorption mass spectrum of Er-3 between mass-to-charge ratio (m/z) 716 and 7560. Two dominant peaks are observed, corresponding to single protonated Er-3, designated $(M + H)^+$, and doubly protonated Er-3, designated $(M + 2H)^{2+}$. Note that the m/z axis as plotted is not linear.

Er-2 does not clearly belong with either of the other two pairs, although it does show a little greater similarity to Er-10 and Er-11 than to Er-1 or Er-20, suggesting that it occupies an intermediate position. This conclusion is emphasized by the hydropathy plots shown in Fig. 4. The pairing of Er-1/Er-10 and Er-11/Er-20 is even more obvious, with the two corresponding sets of profiles nearly superimposable. The Er-1/Er-10 pair have a dominant hydrophilic peak between residues 20 and 30 followed by a substantial hydrophobic trough. Er-11/Er-20, on the other hand, show a major hydrophobic region between residues 10 and 20 and a much less pronounced hydrophobic region between residues 20 and 30. Importantly, Er-2 is unique in having a major hydrophobic trough in this latter region. Although the main chain conformations are certainly likely to be similar, given the restriction

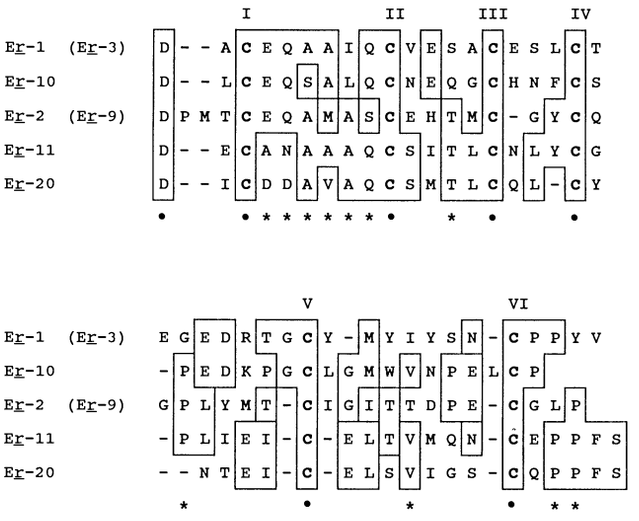

FIG. 3. Comparison of the amino acid sequences of the pheromones Er-1/Er-3, Er-10, Er-2/Er-9, Er-11, and Er-20. Alignment has been maximized through the insertion of gaps. Identical residues are enclosed in boxes. The half-cystine residues are indicated by Roman numerals. Dots and asterisks mark amino acids shared by all five sequences and by at least three sequences, respectively. The sequences of Er-1 and Er-10 are taken from refs. 12 and 13, respectively.

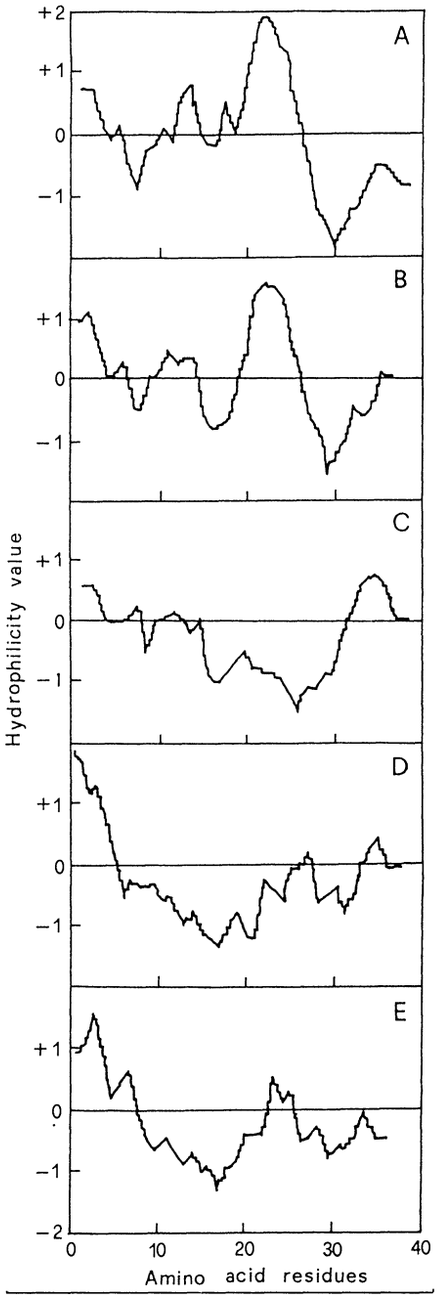

FIG. 4. Hydrophilicity profiles of the pheromones Er-1 (*A*), Er-10 (*B*), Er-2 (*C*), Er-11 (*D*), and Er-20 (*E*) calculated by the method of Hopp and Woods (23), using six residues as window size.

of the disulfide bonds, the hydrophobicity plots suggest local regions of difference may exist that could also affect quaternary interactions. The determination of the three-dimensional structure by both two-dimensional NMR and x-ray diffraction analyses (25) should provide significant insight into this issue.

The nature of pheromone–receptor interactions with *E. raikovi* cells is still uncertain. Preliminary characterization (9) of the receptor in cells producing Er-1 suggests that it is a membrane protein capable of interaction with Er-1 itself, to produce autocrine-like responses, as well as Er-2, Er-10, and Er-11, interactions that ultimately lead to cell mating union (3). However, since Er-1 binding to its own receptor does not induce mating, clearly there must be differences in the way these pheromones bind to their own receptor and those of cells secreting other pheromones. Since overall similarity is not correlated with these activities, it suggests that receptor binding may be controlled by only a small number of residues. An analogous situation is found with epidermal growth factor (EGF) and transforming growth factor α, two proteins that share only $\approx$25% identity but are capable of binding to the same (EGF) receptor (1).

The importance of as little as a single substitution to regional variation and hence to possible alterations in receptor interactions is emphasized by the observation (A. La Terza and C.M., unpublished results) that a second clone of the cDNA for Er-1, obtained from the same library as previously reported (15), contains a Pro → Leu substitution at position 38. Although the [Pro]Er-1 form is dominant, the presence of some [Leu]Er-1 may explain the weak but measurable ability of cells secreting Er-1 to form mating pairs when mixed with cells producing Er-3, which is sequentially identical to [Pro]Er-1. It is not known whether the Er-3 cells also possess a message for [Leu]Er-3. The location of this residue in the carboxyl terminus also points to this region as functionally important in receptor interactions.

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