Autocrine, Mitogenic Pheromone Receptor Loop of the Ciliate Euplotes raikovi: Pheromone-Induced Receptor Internalization

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The ciliate Euplotes raikovi produces a family of diffusible signal proteins (pheromones) that function as prototypic growth factors. They may either promote cell growth, by binding to pheromone receptors synthesized by the same cells from which they are secreted (autocrine activity), or induce a temporary cell shift from the growth stage to a mating (sexual) one by binding to pheromone receptors of other, conspecific cells (paracrine activity). In cells constitutively secreting the pheromone Er-1, it was first observed that the expression of the Er-1 receptor “p15,” a type II membrane protein of 130 amino acids, is quantitatively correlated with the extracellular concentration of secreted pheromone. p15 expression on the cell surface rapidly and markedly increased after the removal of secreted Er-1 and gradually decreased in parallel with new Er-1 secretion. It was then shown that p15 is internalized through endocytic vesicles following Er-1 binding and that the internalization of p15/Er-1 complexes is specifically blocked by the paracrine p15 binding of Er-2, a pheromone structurally homologous to, and thus capable of fully antagonizing, Er-1. Based on previous findings that the p15 pheromone-binding site is structurally equivalent to Er-1 and that Er-1 molecules polymerize in crystals following a pattern of cooperative interaction, it was proposed that p15/Er-1 complexes are internalized as a consequence of their unique property (not shared by p15/Er-2 complexes) of undergoing clustering.

In association with the evolution of systems of multiple cell (mating) types controlling self/nonself recognition phenomena, species of Euplotes synthesize families of structurally homologous soluble proteins that confer on cells a chemical specificity, with each protein representing a diffusible chemical signal (pheromone) that distinguishes one cell type from all the others (17). Euplotes raikovi is the species with which these pheromones, designated Er-1, Er-2, and so forth, have been better characterized with regard to their structure and function. Pheromones are small molecules of 38 to 50 amino acids with a common architecture based on a bundle of three α-helices fastened together by three conserved disulfide bonds (16, 26, 35). They have at least two activity tasks, each acting as both prototypic autocrine growth factors and paracrine mating (sexual) signals (31). Upon binding their own pheromones, which cells constitutively secrete into the extracellular environment throughout the cell cycle, cells grow vegetatively and divide mitotically. When cells bind a nonself pheromone from another cell type, they temporarily arrest their growth and differentiate. When cells constitutively secrete into the extracellular environment for both autocrine and paracrine pheromone binding and its intracellular domain to trigger a mitogenic transduction pathway. The field addressing the issue of how cells can discriminate between autocrine and paracrine pheromone binding and accordingly mount a growth or mating response was advanced by the identification of the pheromone receptors as part of a study of pheromone gene structure and expression (20). These genes, which are transcriptionally active in thousands of copies in the somatic cell macronucleus (15), generate primary transcripts that undergo a process of alternative splicing that creates two closely related transcripts, one of which codes for the prepropheromone. The translation product of this transcript is proteolytically processed into the pheromone. The second transcript includes the prepropheromone sequence and a novel N-terminal sequence. When its translation product is processed, the entire C-terminal prepropheromone is retained as an extracellular peptide anchored to the cell surface through the “pre” transmembrane segment, and the N terminus is intracellular (13, 20). It is this membrane-bound pheromone isoform that cells utilize as a specific pheromone receptor (24).

For type I cells secreting the pheromone Er-1, it was previously shown that the pheromone receptor, represented by a 15-kDa protein of 130 amino acids, originally designated “Er-1m” and hereafter called “p15,” can effectively utilize its extracellular domain for both autocrine and paracrine pheromone binding and its intracellular domain to trigger a mitogenic transduction pathway (24). In the 55-amino-acid sequence of this intracellular domain, there are potential sites for the phosphorylation of protein kinases as well as for receptor associations with GTP binding proteins. We have now analyzed the destiny of this 15-kDa receptor protein following its autocrine binding association with the secreted pheromone Er-1. Evidence is reported here that cell growth-promoting p15/Er-1 complexes undergo ligand-induced endocytosis and that this endocytosis is specifically blocked by exposing p15 to form paracrine mating-inducing complexes with Er-2, a pheromone that is structurally homologous to Er-1 and can bind to p15 in competition with Er-1 (16, 23). This finding thus implies that Er-1-induced p15 internalization is a key step in the transduction pathway underlying the autocrine pheromone receptor loop that promotes cell growth in E. raikovi.

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MATERIALS AND METHODS

Cells and pheromones. The cells used for this study belonged to a set of strains expanded from exconjugants of mating pairs induced to form in the wild-type strain 13 (deposited under accession no. 1624/18 at the Culture Collection for Algae and Protozoa, Ambleide, United Kingdom) and carrying a known homologous combination at the mating-type (mut) locus specific for the synthesis of the pheromone Er-1 only. They were grown in the green alga Danilella tertiolecta with a controlled rhythm of 8 h of weak light and 16 h of darkness at a temperature of 22°C. Purified pheromone preparations were obtained by a standard chromatographic purification procedure (18).

Antibodies. Polyclonal and immunoglobulin M (IgM) monoclonal antibodies to the (Er-1-like) p15 ectodomain were raised against purified Er-1 preparations (14, 20), and those to the p15 endodomain were raised against a synthetic peptide overlapping the sequence Cys27-Arg-Ser-Asn-Cys-Val-Gly-Pro-Leu-Asn-Ser-Ile-Asn-Arg80, which was identified as potentially immunogenic, in the p15 N-terminal region (24). All other antibodies were commercially available and included monoclonal anti-Rab5 and anti-biotin antibodies from BD Biosciences Pharmingen (San Diego, Calif.) and Molecular Probes (Leiden, The Netherlands), respectively, Alexa flour 488 goat anti-rabbit and 594 goat anti-mouse secondary antibodies from Molecular Probes (Leiden, The Netherlands), and peroxidase-conjugated anti-rabbit secondary antibodies from Amershams Bio-Sciences (Little Chalfont, England).

Cell fractions and Western blot analysis. Particulate and cytoplasmic cell fractions were prepared from counted suspensions of 106 cells that were: (i) cultured in seawater at room temperature with continuous stirring and then stopped by the addition of 2 mM Tris-HCl, pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Mo.), (ii) sonicated on ice for 1 to 2 s, and (iv) centrifuged in microcentrifuge tubes at 14,000 rpm for 5 min at 4°C. Separated fractions were analyzed under a phase-contrast microscope for their contents, suspended in Laemmli sodium dodecyl sulfate (SDS) sample buffer, and heat denatured by boiling for 5 min. Analysis by Western blotting was carried out on equivalent aliquots blotted onto polyvinylidene di-fluoride filters (Amershams Biosciences) after separation in 15% SDS-polyacrylamide gels. The filters were probed with the primary antibodies (diluted 1:1,000) overnight at 4°C and with peroxidase-conjugated secondary antibodies (diluted 1:5,000) for 1 h at 37°C. The immunoblots were enhanced with a chemiluminescence detection kit (Amershams Bio-Sciences), and preimmune sera were used under identical conditions to verify their specificities.

Pheromone labeling. Purified Er-1 and Er-2 preparations were conjugated with Texas Red-succinimidyl ester (Tr) or 6-[(biotinoyl)amino] hexanoic acid, sulfo-pheromones were separated from the unreacted reagent by extensive dialysis or freshly prepared 0.2 M glycine solution in bicarbonate buffer, pH 8.5. Labeled room temperature with continuous stirring and then stopped by the addition of 400-μg/ml aldehyde and 0.2 mg/ml leupeptin and were fixed with 4% formaldehyde in PBS overnight at 4°C and with peroxidase-conjugated secondary antibodies (diluted 1:1,000) at 37°C. The immunoblots were enhanced with a chemiluminescence detection kit (Amershams Bio-Sciences), and preimmune sera were used under identical conditions to verify their specificities.

Confocal immunofluorescence microscopy. The expression of p15 was considered in this context in terms of the relative p15 concentrations in the particulate and cytoplasmic cell fractions, with the former fraction carrying cell ghosts and membranes and the latter carrying cytosol plus endosomal vesicles. Evidence for this vesicular assignment of the endosomal compartment was provided by the utilization of monoclonal antibodies to an established endosomal marker in mammalian cells, i.e., the Rab5 protein (36, 37). In a Western blot analysis of vesicles prepared from cytoplasmic fractions by centrifugation at 200,000 × g, it was first observed that these antibodies generate a band of the size (25 kDa) expected for a candidate Rab5 protein (data not shown) and then, by confocal microscopy, that they and p15 antibodies corecognize the same discrete structural units in the cell cytoplasm (data shown below).

RESULTS

p15 expression depends on the extracellular concentration of secreted pheromone. The expression of p15 was considered in this context in terms of the relative p15 concentrations in the particulate and cytoplasmic cell fractions, with the former fraction carrying cell ghosts and membranes and the latter carrying cytosol plus endosomal vesicles. Evidence for this vesicular assignment of the endosomal compartment was provided by the utilization of monoclonal antibodies to an established endosomal marker in mammalian cells, i.e., the Rab5 protein (36, 37). In a Western blot analysis of vesicles prepared from cytoplasmic fractions by centrifugation at 200,000 × g, it was first observed that these antibodies generate a band of the size (25 kDa) expected for a candidate Rab5 protein (data not shown) and then, by confocal microscopy, that they and p15 antibodies corecognize the same discrete structural units in the cell cytoplasm (data shown below).
washed free of their secreted pheromone and resuspended for 3 to 4 h (i.e., the interval previously observed to be appropriate for optimal p15 expression in the particulate fraction) in new volumes of fresh seawater with the addition of 300 ng/ml of a purified Er-1 preparation or in new volumes of fresh seawater with no pheromone addition. As shown in Fig. 2A, cells incubated without Er-1 carried p15 primarily concentrated in the particulate fraction, while cells incubated with Er-1 concentrated p15 in the cytoplasmic fraction compared to the particulate fraction.

The effective formation of complexes between p15 and Er-1 was verified by a cross-linking experiment carried out on cell lysates (instead of whole cells) to optimize the availability of p15 molecules for Er-1 binding and cross-linking. These lysates were prepared from cells 3 h after the shift to fresh seawater and were incubated with a preparation of Er-1 conjugated with biotin at its N terminus, and the incubation mixtures were exposed to the heterobifunctional cross-linker EDC (links molecules between their free NH$_2$ and COOH groups). A Western blot analysis of these mixtures was carried out with monoclonal anti-biotin antibodies to distinguish between p15 molecules cross-linked into complexes with Er-1 and molecules not bound to Er-1. As shown in Fig. 2B, p15 appeared to be cross-linked with Er-1 in a quantitatively minor type of complex of about 20 kDa, indicative of a 1:1 stoichiometric association between the two molecules, and in two quantitatively major types, of about 35 and 66 kDa, indicative of p15/Er-1 oligomeric associations.

**p15 internalization is specifically induced by Er-1 binding and mimicked by antibody binding.** To verify that p15 internalization is correlated with autocrine Er-1 binding, we analyzed the effects generated on this internalization by the competitive, paracrine binding of Er-2 to p15. Er-2 is a pheromone that is structurally homologous to Er-1 (16) and to which Er-1-secreting cells respond by switching from the growth stage to the sexual stage of mating pair formation. This analysis was first based on an extracellular protease assay performed with cell cultures that were previously induced to express maximal p15 concentrations 3 to 4 h after the shift to new volumes of fresh seawater (hereafter denoted as “3-h-shifted cells”) and then resuspended under one of three sets of conditions, i.e., with Er-1 at 22°C, with Er-2 at 22°C, or with Er-1 at 4°C (a temperature incompatible with vesicle endocytosis). Cell samples were removed at progressive intervals, incubated with proteinase K or mock treated without proteinase K, and probed for a Western blot with antibodies to the p15 endodomain to deduce from the extent of enzymatic digestion whether the cytoplasmic localization of p15 was reduced by its protease digestion on the cell surface or if p15 was protected from the protease inside endocytic vesicles. As shown in Fig. 3, immu-
norecognized bands were invariably intense only for cells suspended with E<sub>r</sub>-1 at 22°C, consistent with p15 internalization induced by E<sub>r</sub>-1 binding and consequent protected p15 cytoplasmic localization. In contrast, cells suspended with E<sub>r</sub>-2 at 22°C revealed bands with markedly reduced intensities, similar to cells that were conditioned by the temperature to arrest their endocytic traffic.

These differences in cellular localization between p15/E<sub>r</sub>-1 and p15/E<sub>r</sub>-2 complexes were next visualized by laser confocal microscopy of 3-h-shifted cells that were exposed to Texas Red-labeled preparations of E<sub>r</sub>-1 and E<sub>r</sub>-2 (hereafter denoted E<sub>r</sub>-1-Tr and E<sub>r</sub>-2-Tr, respectively) prior to be incubated in vitro, first with antibodies directed to the p15 endodomain and then with Alexa fluor 488-labeled (green) secondary antibodies. After 10 min of interaction with E<sub>r</sub>-1-Tr or E<sub>r</sub>-2-Tr, the cells revealed a similar punctate colocalization (yellow) of green and red fluorescence all over their surfaces, indicative of the effective formation of p15/E<sub>r</sub>-1-Tr or p15/E<sub>r</sub>-2-Tr complexes. Over the next 20 to 40 min, however, the cells sharply diverged regarding the distribution of their fluorescent signals according to whether they were interacting with E<sub>r</sub>-1-Tr or E<sub>r</sub>-2-Tr. As shown in Fig. 4A, cells forming p15/E<sub>r</sub>-1-Tr complexes became densely decorated with overlaying red and green spots (mostly 1 to 2 μm in diameter), some of which appeared to crowd into a specific cell district (coincident with the cell left front-lateral side) characterized by the sites of insertion of the adoral ciliary membranelles and some of which appeared localized inside the cell cytoplasm. This intracellular localization thus indicated that the p15/E<sub>r</sub>-1-Tr complexes were internalized, and two lines of evidence supported the concept that this internalization occurred through endosomal vesicles. First, the ciliary membranelle district (where the overlaid red and green spots crowded) is the cell’s elective site of endocytic activity (2). This activity can in fact be carried out through tiny invaginations of the only “naked” plasma membrane, known as parasomal sacs, which are locally quite numerous because of the interruptions generated through the thick alveolar layer of the ciliate cortex by the insertions of the ciliary membranelles. Second, cells suspended for 20 min in vivo with E<sub>r</sub>-1 and then subjected in vitro to double immunorecognition with antibodies to p15 and to the early endosome.
mal marker Rab5 (36, 37) revealed spots of colocalization between the p15 and the Rab5 signals, as shown in Fig. 4B. In contrast to the case for cells forming p15/Er-1-Tr complexes, the cytoplasm of cells forming p15/Er-2-Tr complexes remained free of any relevant staining, as shown in Fig. 4C, and no clustering of fluorescent spots occurred in correspondence with the base of the adoral membranelles, thus indicating that these complexes are not internalized. Rather, they appeared to persist all over the cell surface and seemed to eventually concentrate preferentially on cilia of membranelles and on cirri, which are involved in establishing the initial, loose cell-cell mating adhesions.

Since we have previously reported evidence that antibodies binding to p15 promote a cell growth response (31), as does the autocrine Er-1 binding shown above to induce p15 internalization, we used confocal microscopy to analyze whether p15/antibody complexes were internalized similarly to p15/Er-1 complex. For this analysis, 3-h-shifted cells were incubated in vivo with a preparation of monoclonal Er-1 antibodies of the IgM class directed to the (Er-1-like) p15 ectodomain; samples taken over time were then processed in vitro with antibodies directed to the p15 endodomain. Two types of secondary antibodies, one of which fluoresces green and the other of which fluoresces red, were eventually utilized to recognize the p15/antibody complexes. As shown in Fig. 5, these complexes followed a vesicular pattern of internalization that appeared overall to be equivalent to that of the p15/Er-1-Tr complexes. The only appreciable difference was that a shorter time was required to observe their internalization. After 10 min of incubation with the Er-1 antibodies, cells were usually observed to already carry several fluorescent cytoplasmic spots, and after 40 min, their surfaces were practically free of any relevant signal, with all fluorescent spots having migrated inside the cytoplasm.

FIG. 5. Confocal microscopic analysis of p15 internalization induced by antibodies to the p15 ectodomain. After the indicated times of in vivo incubation with antibodies to the p15 ectodomain (red), cells were permeabilized (10 min) and exposed to antibodies to the p15 endodomain (green). Arrows indicate examples of overlay between the p15 signal and the signal of the antibodies to the p15 ectodomain. Each confocal image shown corresponds to the middle image from an 8- to 10-section series collected every 1 μm.

FIG. 6. Schematic representation of differences between p15 autocrine binding to Er-1 (A) and paracrine binding to Er-2 (B). The Er-1 and Er-2 configurations are based on nuclear magnetic resonance determinations (21, 25). The three helices are numbered h1, h2, and h3 starting from the molecule’s amino terminus, the disulfide-bonded cysteines are represented as gold spheres, and the overall distribution of the electrostatic potentials on the three faces are color-coded white, red, and blue to indicate neutral, negative, and positive potentials, respectively. The three-helix configuration of the p15 ectodomain was extrapolated from the equivalence of the primary structure of this domain with Er-1 (20). Clustering of the p15/Er-1 complexes was based on a pattern of cooperative association determined for the Er-1 molecules in the crystal structure (33). This pattern requires that every molecule utilizes all three of its helices to associate with two other molecules: one association (dimer 1) involves helix-1/helix-1 (yellow/yellow) and helix-2/helix-2 (red/red) interactions, and the second association (dimer 2) involves helix-3/helix-3 (green/green) interactions. Variations in the charge and structure of Er-2 helices 2 and 3 (hatched red and green cylinders) with respect to their counterparts in the p15 ectodomain would interfere with the formation of the type 2 dimer and restrain the association between p15 and Er-2 to only the type 1 dimer involving binding forces essentially provided by the conserved helix 1 (yellow cylinder). The molecular amino and carboxyl termini are indicated by N and C, respectively.
DISCUSSION

Signal-induced receptor internalization through endocytic vesicles has usually been viewed as a mechanism that cells utilize to attenuate signaling and degrade their surface receptors (19). However, it is increasingly appreciated that an additional major function of this mechanism is represented by the activation of transduction pathways that regulate the cell response to the binding of regulatory chemical signals (11, 12, 32). Convergent evidence supports the concept that receptors, whether tyrosine kinase or G protein coupled, are delivered to “signaling endosomes” upon their ligand-induced phosphorylation to maintain more long-lasting effective interactions with downstream signaling molecules of the mitogen-activated protein kinase pathways (3, 5, 9, 28, 34). At the same time, the activation of basic components of mitogen-activated protein kinase pathways has been shown to be suppressed in response to inhibition of the internalization of a variety of structurally diverse receptors, such as the adrenergic, insulin, and insulin-like growth factor I receptors (1, 6, 7).

A new contribution to this emerging context of growth factor-receptor biology is provided here by the finding that the p15 pheromone receptor of E. raikovi is induced to undergo internalization via endocytic vesicles by autocrine binding of the cell’s own (self) pheromone, Er-1. This p15 internalization appears to be specifically required to activate the transduction pathway that controls the cell growth response, and the presence in the p15 cytoplasmic domain of two potential phosphorylation sites for protein kinases A and C, identified by the R/KXXS sequence (24), makes it likely that it involves an Er-1-induced p15 phosphorylation. Support for the cause-effect relationship that links Er-1-induced p15 internalization to the cell growth response was provided by the observation that cells no longer internalize p15 bound to another (nonself) pheromone such as Er-2, which was used in this study as a competitive analogue of Er-1. Rather, they respond to the Er-2-induced inhibition of p15 internalization by temporarily arresting their growth stage and shifting to the sexual stage of mating.

Since there were both internalization of the autocrine p15/Er-1 complexes and a failure to internalize paracrine p15/Er-2 complexes from the cell surface, there must be molecular mechanisms underlying p15’s discrimination between Er-1 and Er-2 binding. One model of these molecular interactions is proposed in Fig. 6, in which the internalization of the p15/Er-1 complexes depends on a phenomenon of oligomerization and clustering that is distinctive of these complexes. This hypothesis was suggested earlier by the finding that, like autocrine pheromone binding, antibodies binding to p15 promote cell growth (31). Considering the similarity of the p15 ectodomain and Er-1 (20, 24), it is a short extrapolation to propose the hetero-oligomerization of p15 and Er-1 from the crystal structural data of Er-1, which show homo-oligomerization (33). In addition, the Er-1 molecules pack in the crystal according to a pattern of cooperative protein-protein oligomerization, which is imposed by the capacity to interact with one another by means of all their surfaces provided by their three-helix bundle conformation. In practice, each molecule utilizes helices 1 and 2 to associate (and form one type of symmetrical dimer) with helices 1 and 2 of a second molecule and helix 3 to associate (and form a second type of asymmetrical dimer) with helix 3 of a third molecule. On the other hand, this cooperative model of molecular association appears not to be applicable, at least in principle, to the interactions of p15 with Er-2 due to significant variations in charge distribution that mainly involve and distinguish helices 2 and 3 of the Er-2 structure from their counterparts in the structure of the p15 ectodomain. These variations are clearly in conflict with the basic requirement of structural uniformity on which the Er-1 cooperative model of oligomerization is based. Rather, they imply that p15 associates with Er-2 according to a noncooperative pattern, presumably by binding forces principally provided by helix 1, which appears to be strictly conserved between the two molecules as well as between Er-1 and all other members of the E. raikovi pheromone family (16, 26, 35).

Whether p15 internalization occurs through the clathrin-mediated endocytosis machinery has not yet been directly investigated. Nevertheless, indirect evidence that it is precisely this machinery that is involved has been derived from observing (data not shown) that the internalization of the p15/Er-1 complexes is completely abolished in cells incubated with monodansylcadaverine, an inhibitor of the enzyme transglutaminase, which is essential for coated-pit formation (27), or exposed to hyperosmotic sucrose, which similarly prevents clathrin-dependent endocytosis (10). In addition, in the tail of the p15 endodomain there is a structural motif, identified by the sequence QYSL, that closely recalls the tyrosine-based internalization signals distinguished by the general sequence XYYXD, where “X” is any amino acid and “D” stands for a bulky hydrophobic side chain. These signals were originally identified in relation to transferrin receptor internalization (8) and were later shown to be used in general for the sorting of transmembrane proteins and for interactions of receptors, such as the epidermal growth factor receptor, with specific subunits of the clathrin adaptor complexes responsible for the formation of clathrin-coated endocytic vesicles (4, 22, 29, 30).

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RECEPTOR INTERNALIZATION IN EUPLOTES 1227

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