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Expression of a mutated ras gene in Dictyostelium discoideum alters the binding of cyclic AMP to its chemotactic receptor

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Summary

Dictyostelium discoideum cells contain a ras gene that codes for a polypeptide that is highly homologous to the human ras proteins. Extra copies of the wild-type gene or a gene carrying a missense mutation in codon 12 (ras-Gly12 and ras-Thr12, respectively) have been introduced into Dictyostelium cells by transformation.

We have investigated the properties of the chemotactic cell surface cyclic AMP receptor in crude membrane preparations of wild-type Dictyostelium cells and ras-Gly12 and ras-Thr12 transformants. In vitro, an ATP- and Ca2+-dependent reduction of the number of cyclic AMP receptors was observed in membranes from all three cell types. The number of available receptors was decreased maximally by about 50%. In the presence of ATP the half-maximal Ca2+ concentration required for this process was about 10^-5 M in wild-type and ras-Gly12 membranes, and less than 10^-7 M in ras-Thr12 membranes. Addition of GTP (but not GDP) or the phorbol ester PMA (phorbol-12-myristate-13-acetate) reduced the Ca2+ requirement of the process in wild-type and ras-Gly12 membranes to the physiological level of less than 10^-7 M. In membranes derived from ras-Thr12 cells addition of GTP or PMA had no effect.

The results indicate that Dictyostelium cells contain a cyclic AMP receptor-controlling pathway that can be activated in vitro and involves a GTP-binding protein and a Ca2+ plus ATP-dependent activity, possibly protein kinase C. It is concluded that the ras protein specifically interacts with this pathway; the pathway appears to be constitutively activated by the mutated ras gene product.

Key words: ras gene, chemotactic cyclic AMP receptor, D. discoideum, protein kinase C.

Introduction

Mammalian ras genes code for 21 000 M r proteins, which bind GTP and show GTPase activity (Manne et al. 1984, 1985; McGrath et al. 1984). Mutations at various positions in the ras coding region reduce the GTPase activity of the protein, can induce a transformed phenotype and have been associated with tumorigenicity (Taparowsky et al. 1982; Balmain & Pragnett, 1983; Fasan et al. 1984; Seeburg et al. 1984; Dcr et al. 1986). The cellular slime mould Dictyostelium discoideum contains a single ras gene that codes for a polypeptide highly homologous to mammalian ras proteins (Reymond et al. 1984). Extra copies of the wild-type ras gene (ras-Gly12) or of a gene with a missense mutation at codon 12 (ras-THR12) were introduced into Dictyostelium cells by transformation (Nellen et al. 1984). Expression of the mutated gene has been reported to result in aberrant morphogenesis and enhanced cyclic AMP-induced desensitization of guanylate cyclase (Reymond et al. 1986; Van Haastert et al. 1987). No effect of overexpression of the wild-type ras gene has been observed to date.

We have investigated the binding of cyclic AMP to the chemotactic cell-surface receptor in crude membrane preparations of wild-type D. discoideum cells and ras-Gly12 and ras-Thr12 transformants. Our results indicate that the ras protein is involved in a process that reduces the total number of available cyclic AMP receptors in vitro. This receptor loss appeared to be the result of an ATP- and Ca2+-dependent activity, possibly protein kinase C.
Materials and methods

Materials

[5'-3H]cyclic AMP (1-55 TBq mmol⁻¹) was purchased from Amersham International (UK), cyclic AMP and dithiothreitol from Serva (Heidelberg, FRG) and 5'-AMP, GDP, GTP and Gpp(NH)p (guanyl-imidodiphosphate) from Boehringer-Mannheim, FRG). PMA (phorbol-12-myristate-13-acetate), 4α-PDD (4α-phorbol-12,13-didecanoate) and geneticin were from Sigma.

Methods

Isolation of crude D. discoideum membranes. Transformants of D. discoideum strain Ax3 were made and grown as described by Reymond et al. (1986). Wild-type Ax3 cells and the transformed cell lines ras-Glyl2 and ras-Thrl2 were grown to a density of 5×10⁶ cells ml⁻¹, collected by centrifugation, washed once with 15 mM-potassium/sodium phosphate buffer (pH 6.5) and resuspended in the same buffer at a density of 10⁷ cells ml⁻¹. After starvation for 6 h at 22°C, the cells were washed once in homogenization medium (HM), containing 40 mM-Hepes–NaOH (pH 7.7), 1 mM-dithiothreitol, 0.5 mM-EDTA, 250 mM-sucrose and the following protease inhibitors: 5 mM-benzamidin, 100 μg ml⁻¹ aprotinin, 50 μg ml⁻¹ trypsin inhibitor, 20 μg ml⁻¹ antipain and 0.1 mM-phenylmethylsulphonyl fluoride (PMSF). Subsequently, the cells were resuspended in the same buffer (10⁸ cells ml⁻¹) and lysed by nitrogen cavitation (Janssens et al. 1986). The lysate was centrifuged for 10 min at 10,000 g at 4°C and the pellet was resuspended in HM. The crude membrane preparation was stored in liquid nitrogen.

Cyclic AMP binding assays. Crude membranes (final protein concentration 0.5 mg ml⁻¹) were incubated for 2 min at 22°C in 100 μl of 20 mM-potassium phosphate buffer (pH 7.0). When indicated, ATP, GTP, GDP, PMA or Ca²⁺ was present, in combinations and at concentrations given in the text. A Ca²⁺-EGTA buffer (1 mM) was used to control the free Ca²⁺ concentration (Bartfai, 1979). Cyclic AMP binding was initiated by adding 10 nM-[³H]cyclic AMP (1.78 TBq mmol⁻¹) in the presence of 20 μM-5'AMP and 10 μM-dithiothreitol, which inhibits phosphodiesterase activity (Green & Newell, 1975). After equilibration for 5 min at 0°C, the amount of bound [³H]cyclic AMP was determined by measuring the radioactivity in the membrane pellet after 2 min centrifugation at 10,000 g. Non-specific binding was determined by equilibration of [³H]cyclic AMP in the presence of 0.1 mM unlabelled cyclic AMP.

Results and Discussion

Incubation of crude D. discoideum membranes in the presence of ATP and increasing Ca²⁺ concentrations reduced equilibrium cyclic AMP binding to the chemotactic receptor (measured at 10 nM-cyclic AMP) by up to about 50% (Fig. 1A). In the absence of ATP no effect of Ca²⁺ was observed. Millimolar Ca²⁺ concentrations were required to reduce cyclic AMP binding in membranes derived from control cells and from ras-Glyl2 transformants. For membranes from ras-Thrl2 transformants submicromolar Ca²⁺ concentrations (i.e. in the physiological range) were already sufficient to induce the maximal decrease in cyclic AMP binding.

The Ca²⁺ requirement of the process in control membranes and ras-Gly12 membranes could be shifted to lower concentrations by the addition of 0.1 mM-GTP (or the non-hydrolysable analogue Gpp(NH)p, result not shown) (Fig. 1B). In ras-Thrl2 membranes the Ca²⁺ dependency of the process was not affected by GTP (Fig. 1B). GDP did not change the Ca²⁺ sensitivity in any of the membrane preparations (Fig. 1C).

These data indicate that a GTP-binding protein is involved in a Ca²⁺-dependent process that results in decreased cyclic AMP–receptor binding in vitro. Furthermore, the results are consistent with the hypothesis that the mutated, oncogene-like ras-Thrl2 gene codes for a protein that is irreversibly activated by GTP (Chiarugi et al. 1985; Fleischman et al. 1986).
Fig. 2. Effect of phorbol ester PMA and phorbol ester analogue 4α-PDD on cyclic AMP binding to the chemotactic receptor. Crude membranes from wild-type Ax3 cells were incubated for 2 min at 22°C with PMA (concentrations indicated in the figure) or 4α-PDD (40 μM), in the presence of 1 mM-ATP and 10⁻⁷ M-Ca²⁺. Subsequently, the membranes were equilibrated for 5 min at 0°C with 10 nM-[³H]cyclic AMP in the presence of 20 μM-5'-AMP and 10 mM-dithiothreitol. The membranes were then sedimented by centrifugation for 2 min at 10,000 g and the amount of specifically bound [³H]cyclic AMP in the membrane pellet was determined. Each value is the mean of the results of a determination in triplicate of an experiment reproduced twice.

Like GTP, the phorbol ester PMA lowered the Ca²⁺ requirement of membranes from wild-type cells and ras-Gly12 transformants (Fig. 1D). In the presence of 10⁻⁷ M-Ca²⁺ and 1 mM-ATP, PMA reduced cyclic AMP binding in these membranes by 30% and 21%, respectively, to a level close to that of ras-Thr12 membranes (Fig. 2C). A high Ca²⁺ concentration (10⁻⁴ M) was required to induce the same effect in membranes from control cells and ras-Gly12 transformants (Fig. 3A, B). These data show that the decrease in cyclic AMP binding that was induced by ATP and Ca²⁺ (as seen in Fig. 1) was mainly due to a decrease in available cyclic AMP receptor sites, rather than to a lowered affinity of the receptors. In intact D. discoideum cells, a similar effect on cyclic AMP receptors is seen after stimulation of cells with high (micromolar) concentrations of cyclic AMP (Klein & Juliani, 1977; Van Haastert, 1987a). This phenomenon is known as receptor-downregulation. It has been associated with desensitization of the receptor-stimulated adenylate cyclase activity (Van Haastert, 1987a), but the molecular mechanism underlying the downregulation is not known.

Besides the Ca²⁺ plus ATP-dependent reduction of the number of cyclic AMP binding sites, other conditions in vitro have been described that affect cyclic AMP-receptor properties of D. discoideum. Addition of GTP (or GDP) to isolated membranes results in a decrease in receptor affinity: the cyclic AMP receptor is converted from a slowly dissociating, high-affinity state to a fast-dissociating, low-affinity one (Janssens et al. 1986; Van Haastert et al. 1986). The total number of receptors is not affected. GTP and GDP are thought to bind to a guanine nucleotide-binding protein (G-protein), that interacts with the cyclic AMP receptor. As can be seen in Fig. 1C (data points at zero Ca²⁺), GTP and GDP both reduced equilibrium cyclic AMP binding by about 20% in all three membrane types. It must be noted that this value can vary considerably from preparation to preparation of membranes (Janssens et al. 1986; Van Haastert et al. 1986; M. E. E. Ludérous, unpublished). At high Ca²⁺ concentrations cyclic AMP–receptor binding in crude D. discoideum membranes at concentrations up to 40 μM (Fig. 2). The requirement for ATP and Ca²⁺, the stimulatory effect of phorbol ester and the phorbol ester specificity strongly suggest the involvement of protein kinase C in the regulation of cyclic AMP–receptor function (Kishimoto et al. 1980; Nishizuka, 1984; Ashendel, 1985; Bell, 1986). If this interpretation is correct, activation of D. discoideum protein kinase C requires a significantly higher PMA concentration than has been reported for protein kinase C from mammalian cells (Nishizuka, 1984).

In order to determine the cause of the decrease in cyclic AMP binding after incubation with ATP and Ca²⁺, equilibrium receptor binding studies were carried out (Fig. 3). In the presence of ATP, but in the absence of Ca²⁺, the three types of membrane preparations had similar cyclic AMP binding properties. Incubation with ATP and 10⁻⁷ M-Ca²⁺ dramatically reduced the total number of available cyclic AMP binding sites of ras-Thr12 membranes (Fig. 3C). A high Ca²⁺ concentration (10⁻⁴ M) was required to induce the same effect in membranes from control cells and ras-Gly12 transformants (Fig. 3A, B). These data show that the decrease in cyclic AMP binding that was induced by ATP and Ca²⁺ (as seen in Fig. 1) was mainly due to a decrease in available cyclic AMP receptor sites, rather than to a lowered affinity of the receptors. In intact D. discoideum cells, a similar effect on cyclic AMP receptors is seen after stimulation of cells with high (micromolar) concentrations of cyclic AMP (Klein & Juliani, 1977; Van Haastert, 1987a). This phenomenon is known as receptor-downregulation. It has been associated with desensitization of the receptor-stimulated adenylate cyclase activity (Van Haastert, 1987a), but the molecular mechanism underlying the downregulation is not known.

Mutated ras gene alters cyclic AMP receptor binding
Fig. 3. Equilibrium binding of cyclic AMP to the chemotactic receptor after preincubation with ATP and different free Ca\(^{2+}\) concentrations (Scatchard plots). Crude membranes of wild-type Ax3 (A) and the transformed cell lines ras-Gly12 (B) and ras-Thrl2 (C) were incubated for 2 min at 22°C in 20 mM-potassium phosphate buffer (pH 7.0), containing 1 mM-ATP plus 1 mM-EDTA (●), 0·1 μM-free Ca\(^{2+}\) (×), or 0·1 mM-free Ca\(^{2+}\) (O). Subsequently, the membranes were equilibrated for 5 min at 0°C with \[^{3}H\]cyclic AMP at concentrations between 0·1 nM and 300 nM in the presence of 20 μM-5′-AMP and 10 mM-dithiothreitol. The membranes were sedimented and the amount of receptor-bound \[^{3}H\]cyclic AMP was determined. Each point is the mean of the results of a determination in triplicate of an experiment reproduced two to three times.

Fig. 4. Equilibrium binding of cyclic AMP to the chemotactic receptor in the presence or absence of Mg\(^{2+}\)-ATP (Scatchard plots). Crude membranes of wild-type Ax3 (A) and the transformed cell lines ras-Gly12 (B) and ras-Thrl2 (C) were preincubated in 20 mM-potassium phosphate (pH 7.0), containing 1 mM-ATP and 1 mM-EGTA for 2 min at 22°C in the absence (●) or presence (+) of 1 mM-MgCl\(_2\). \[^{3}H\]cyclic AMP binding curves were determined as described in the legend to Fig. 3. Each point is the mean of results of a determination in triplicate of an experiment reproduced twice.

the effect of GTP and GDP could not be observed. Van Haastert (1987b) has shown that the guanine nucleotide effect can be mimicked by incubating membranes with Mg\(^{2+}\)-ATP, thereby activating a putative endogenous protein kinase. We have compared the binding properties of the cyclic AMP receptors of membranes from wild-type cells and the two ras transformants after incubation with Mg\(^{2+}\)-ATP. Scatchard analysis in Fig. 4 reveals, under these conditions, a similar decrease in receptor affinity and an unaffected receptor number in all three membrane types.

Thus, overexpression of either the wild-type or the mutated ras gene did not affect this Mg\(^{2+}\)- dependent cyclic AMP-receptor regulating mechanism.

The data in Figs 1 and 3 indicate that activating a G-protein or putative protein kinase C had the same effect on cyclic AMP receptors as the presence of the mutated ras gene product. In several other receptor systems, receptor properties are controlled by protein kinase C (Shoyab et al. 1979; Brown et al. 1984; Klausner et al. 1984; Serra et al. 1986; Dawson et al. 1986). The activation pathway of this kinase has been studied extensively in mammalian cells (Nishizuka, 1984; Bell, 1986). In response to ligand binding, various receptors activate a phospholipase C (Berridge & Irvine, 1984), which catalyses the hydrolysis of phosphatidyl inositol 4,5-diphosphate to 1,2-diacylglycerol and inositol
1,4,5-trisphosphate. The latter is thought to release Ca\(^{2+}\) from intracellular stores, the former to activate protein kinase C, provided that Ca\(^{2+}\) is present (Kishimoto et al. 1980; Bell, 1986). Several recent reports suggest that a ras protein regulates the activity of phospholipase C, possibly by acting as a G-protein-like entity (Blackmore et al. 1985; Chiarugi et al. 1985; Cockcroft & Gomperts, 1985; Fleischman et al. 1986).

In Dictyostelium cells protein kinase C and phospholipase C have not been identified. However, it was recently shown that inositol 1,4,5-trisphosphate and Ca\(^{2+}\) are intracellular messengers for the activation of guanylate cyclase via the cyclic AMP receptor (Europe-Finner & Newell, 1986a,b; Small et al. 1986). Moreover, Europe-Finner et al. (1988) recently showed that ras-Thrl2 transformants contain an elevated level of inositol trisphosphate compared to cells carrying only the wild-type ras gene. These data suggest that the phosphatidyl inositol signal-transduction pathway is active in this lower eukaryote, and is controlled by a ras protein in a direct or indirect way.

Summarizing, our results indicate the involvement of the D. discoideum ras protein in a cyclic AMP receptor-controlling pathway in vivo, which depends on a protein kinase C-like activity. This pathway appears to be constitutively activated in membranes derived from cells that express the mutated ras-Thrl2 gene, resulting in the reduction of the number of cyclic AMP receptors. The D. discoideum ras transformants that were used in this study as well as in previous work (Reymond et al. 1986; Van Haastert et al. 1987) have been shown to contain two to four times more ras protein than untransformed cells (Reymond et al. 1986). Cyclic AMP-receptor binding to intact cells of these ras transformants was reported to be indistinguishable from binding to wild-type cells (Reymond et al. 1986). Interestingly, we have recently found that further increased expression levels of the mutated ras protein (in ras-Thrl2 transformants) resulted in a dramatic reduction of the number of available cell surface cyclic AMP receptors in vivo. A similar increase in the cellular level of wild-type ras protein (in ras-Gly12 transformants), on the other hand, did not affect the number of available receptors (M. E. E. Luderus, unpublished). These findings are in agreement with our present observations in vitro. Whether the ras protein in D. discoideum directly interacts with phospholipase C is under investigation.

References


phosphatidyl inositol-4,5-bisphosphate and catabolites. 


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