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NOVEL GTP-BINDING PROTEINS IN PLASMA MEMBRANES OF THE FUNGUS METARHIZIUM ANISOPLIAE

Raymond J. St. Leger, Donald W. Roberts
and Richard C. Staples

Boyce Thompson Institute for Plant Research
Tower Road, Cornell University
Ithaca, NY 14853

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SUMMARY: We report the existence of several families of GTP-binding proteins in plasma membranes of Metarhizium anisopliae. Two proteins (18.4 and 24 kDa) resemble mammalian Gn-proteins in their being toxin insensitive, binding [α-^{32}P]GTP on nitrocellulose blots of sodium dodecyl sulfate (SDS)-polyacrylamide gels, and also in their immunological properties. Four other proteins (31-38.2 kDa) were similar except that they did not bind [α-^{32}P]GTP after treatment with sodium dodecyl sulfate. An 18.2 kDa cholera toxin substrate and three toxin insensitive bands (18.6, 18.8, and 24 kDa) are novel proteins antigenically related both to mammalian G-proteins and ras gene products. An additional 23 kDa pertussis toxin substrate (the major G-protein in a crude mycelial extract) reacted strongly with antisera to G-proteins but not with anti-ras serum. Other substrates ADP ribosylated by cholera toxin or botulinum D toxin were immunologically unreactive. Analysis of the structural and functional characteristics of these multiple GTP-binding proteins will promote a better understanding of signal transduction in fungi.

The generation of intracellular signals from chemically and physically coded information received by membrane receptors involves transduction by GTP-binding proteins (G-proteins) which regulate the ability of the receptor to interact with membrane bound proteins (enzymes, ion channels) of the cell (1). Metarhizium anisopliae is a commercially important entomopathogenic fungus which responds to touch and chemical stimuli by producing infection structures, including a hold fast (appressorium) and a penetration peg (2).

Ca^{2+}- and cyclic AMP- (cAMP) dependent phosphorylation events are involved in germination of conidia, growth, and differentiation (3, 4); and a membrane bound adenylate cyclase activity has been reported that is apparently involved in transmembrane signalling reactions (3, 4). The discovery that M. anisopliae also possesses a membrane bound protein tyrosine kinase (3) indicates that M. anisopliae is an ideal organism to analyze the biochemical and molecular processes involved in signal

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transduction and integration. To date, however, there has been no demonstration or analysis of the GTP-binding proteins of M. anisopliae; and information on GTP-binding proteins in other fungi is sparce. In yeast, the ras genes have been cloned and their products characterized (5). Six GTP-binding pertussis toxin substrates have been identified in crude mycelial extracts from the filamentous fungus, Neurospora crassa (6). In the light of this we have conducted a study which demonstrates the presence of multiple families of GTP-binding proteins in Metarhizium plasma membranes which bind [α-^{32}P]GTP or are recognized by antisera to mammalian ras-proteins or G-proteins. Three previously undescribed proteins are antigenically related both to mammalian G-proteins and ras gene products.

MATERIALS AND METHODS

Materials. The isolate of Metarhizium anisopliae (ME1) and its maintenance were described previously (2). Immuno-alkaline phosphatase reagents were from Promega. [^{32}P]NAD (15 Ci/mmol) was from NEN Research Products and [^{32}P]GTP (3,000 Ci/mmol) was from Amersham. Toxins and other chemicals were from Sigma.

Blotting with [α-^{32}P]GTP. Samples of purified plasma membranes or mycelial extracts prepared from M. anisopliae by the procedures described previously (3) were suspended in Laemmli (1970) buffer and the proteins separated by electrophoresis through an SDS-polyacrylamide gel (12.5% w/v acrylamide). The polypeptides were transferred to nitrocellulose by transverse electroblotting (7) and binding of [α-^{32}P]GTP was carried out essentially as in (8) except that the nitrocellulose was first incubated for 10 min in buffer containing 0.3% BSA. The blots were then incubated for 1 h in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 μM $MgCl_2$, 0.1% Nonidet P-40 and 1 μCi/ml [α-^{32}P]GTP. In some experiments unlabelled GTP or ATP was included in the incubation. The blots were washed and autoradiography carried out using Kodak XAR film.

Immunoblotting. Sample proteins were transferred to nitrocellulose and immunoblotted essentially as described previously (7). The blots were developed with alkaline phosphatase coupled antibody to mouse or rabbit IgG according to the ProtoBlot Western Blot AP system technical manual (Promega). Antibodies utilized were: R5,6, (kindly provided by Dr. G.M. Bokock, Scripps Clinic, La Jolla, CA 92037, USA), a rabbit polyclonal antibody prepared against bovine brain Gi and which reacts with the α and β subunits of Gi, Gn, and Go (11) and anti-ras p21 (pan) monoclonal antibody (Dupont, NEN) raised against a bacterial recombinant ras p21 protein.

Toxin ADP-ribosylation of membrane proteins. The reaction mixture (200 μl) contained 50 mM Tris-HCl (pH 7.5), 10 mM thymidine, 5 mM DTT, 1 mM $NADP^+$, 1 mM ATP, 3 mM phosphenolpyruvate, 10 μg/ml pyruvate kinase, 1 mM PMSF [^{32}P]NAD^+ (5 μCi/assay) and 50 μg plasma membranes. The reaction was started by the addition of 3 μg toxin followed by incubation at 27°C for 45 min; it was stopped by addition of 100 μl of 30% (w/v) trichloroacetic acid. Precipitated protein was analyzed by SDS-PAGE electrophoresis and autoradiography. Toxins utilized were botulinum neurotoxin D, cholera toxin, and pertussis toxin. The cholera and pertussis toxins were pre-activated with 50 mM DTT for 30 min at 30°C before use.

RESULTS AND DISCUSSION

We have compared the electrophoretic mobilities of ras and G-proteins in preparations from M. anisopliae, using as probes both [^{32}P]GTP and specific antibodies (Fig. 1). Two major [^{32}P]GTP binding proteins (18.4 and 24 kDa) were routinely detected in both crude extracts from differentiating cells and plasma membranes isolated from Sabouraud dextrose broth grown cultures (Fig. 1, lanes 9-11). In addition, the same proteins were also found to a much lesser extent in conidia and mitochondrial preparations (data not shown). The binding of [α-^{32}P]GTP was specific in that it was prevented by simultaneous addition of 10 μM GTP but was unaffected by 50 μM ATP or 20 μM GMP. Many G-proteins including Gs and Gi are unable to bind GTP after denaturation with SDS (8), and it is suggested that the technique is specific for low Mr Gn-proteins previously described in plant hypocotyl (9) and various mammalian membranes (10). The two [^{32}P]GTP binding proteins in Metarhizium resembled Gn-proteins in being toxin-insensitive and being recognizable by antiserum R5,6. The 18.4 kDa protein was also immunologically distinct from ras proteins and thus resembles a 27 kDa platelet Gn protein (10). The 24 kDa α[^{32}P]GTP binding band co-migrated with a ras immunoreactive protein. Two other closely spaced ras-immunoreactive bands (18.6 and 22.3 kDa) were also detected in membrane preparations (Fig. 1, lane 8). The

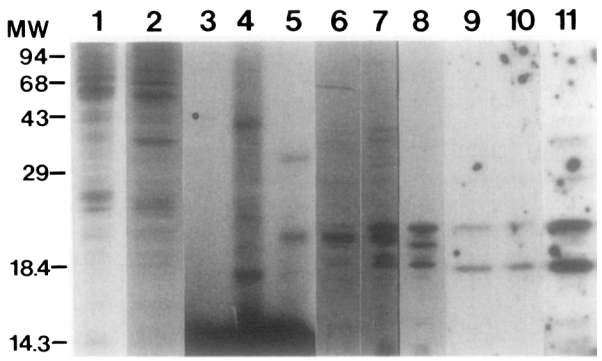

FIG. 1. Comparison of the electrophoretic mobilities of proteins of M. anisopliae detected by ADP-ribosylation, [^{32}P]GTP binding and immunological reactivity. The lanes are as follows: Coomassie blue visualization of proteins in a crude extract (Lane 1) and in isolated plasma membranes (Lane 2). ADP-ribosylation of membrane proteins catalyzed by botulinum neurotoxin D (the position of the single very faint band is indicated by a dot) (Lane 3); cholera toxin (Lane 4); pertussis, toxin (Lane 5). Western blot analysis of the immunological crossreactivity of proteins detected in crude extract (Lane 6) and plasma membranes (Lanes 7 and 8) using R5,6 at 1:200 dilution (Lanes 6 and 7) and anti-ras p21, pan serum at 1:1,000 dilution (Lane 8). Binding of [α-^{32}P] GTP to proteins in a crude extract (Lanes 9 and 11) and membranes (Lane 10). Autoradiographs were incubated for 12 h (Lanes 9 and 10) or 48 h (Lanes 3, 4, 5 and 11).

18.6 and 24 kDa proteins and two minor components (18.2 and 18.8 kDa) showed strong cross-reactivity with the R5,6 antiserum indicating that these proteins are antigenically related to both mammalian G-proteins and ras-proteins. The 18.2 kDa band also co-migrated with a cholera toxin substrate. In higher eukaryotes, G-proteins with large Mr values and ras gene products with comparatively low Mr values (ca. 20 kDa) are distinct and antigenically unrelated (1, 11, 12). It is unlikely that the cross-reactivity represents non-specific binding to common species of protein as high molecular weight proteins predominant in the plasma membrane and the immunoreactive proteins are barely detectable by coomassie blue staining (Fig. 1, lanes 1 and 2). If further studies confirm that the co-migrating bands represent the same proteins then this suggests that multiple coupling proteins in higher eukaryotes may have evolved from a single progenitor which is conserved in Metarhizium. From a comparison of the level of R5,6 immunoreactive bands in membranes and crude extract it is clear that the 18.6 and 24 kDa bands are enriched in the membrane fraction compared to the whole homogenate (Fig. 1, lanes 6 and 7). Ras immunoreactive bands (18.6 and 24 kDa) were also enriched in the membrane fraction (data not shown).

The mobilities of the toxin substrates and G-proteins were also compared by SDS-PAGE electrophoresis. The two major [α-^{32}P]GTP binding proteins, the ras-immunoreactive proteins and the high Mr, R5,6 reactive proteins (31, 32.2, 35.6, 38.2, and 53.5 kDa, Fig. 1, lanes 6 and 7) were not labelled by pertussis, cholera or botulinum D toxins. Metarhizium membranes contained separate substrates to toxins in contrast to Dictyostelium discoideum in which a transducin-like 39 kDa protein is labelled in response to both cholera and pertussis toxins (13). The α subunit of Gs, the G-protein that interacts with stimulatory hormone receptors, is identified in mammalian cells by an ADP-ribosylation catalyzed by cholera toxin. Two bands (45 and 52 kDa) have been identified with SDS-PAGE (14, 15). Likewise, two major cholera substrates (18.2 and 40 kDa) were identified in Metarhizium membranes, although cholera toxin has little effect on Metarhizium adenylate cyclase (unpublished data). ADP-ribosylation of the 41 kDa α-subunit of Gi, the G-protein that interacts with mammalian inhibitory hormone receptors, occurs in response to pertussis toxin (14, 15). A pertussis toxin substrate in Metarhizium membranes (lane 5) co-migrated with a major 23 kDa band immunologically distinct from ras proteins but recognized by R5,6 prepared against bovine brain Gi. This protein is the major band in a crude extract (Fig. 1, lane 6). Botulinum toxin ADP-ribosylates a 21 kDa protein in mammalian membranes (16) clearly distinguishable from the single 43 kDa protein ribosylated in Metarhizium membranes.

We conclude from these results that plasma membranes of Metarhizium contain a multiplicity of GTP-binding proteins in at least six separate families, namely Gn proteins, G/ras proteins, ras-proteins, cholera toxin G-protein substrates, pertussis toxin G-protein substrates and a botulinum toxin G-protein substrate. Although the individual functions of these proteins are unknown, their localization on the plasma membrane implies an involvement in transmembrane signalling reactions, the only role so far ascribed to them in mammalian systems (1). The plasma membrane adenylate cyclase of Metarhizium is regulated by as yet unidentified GTP-binding proteins (unpublished data) and other possible functions are also under investigation.

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