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Abstract

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Assay method and localization of GTP binding proteins in N. crassa

Neurospora crassa is a unique lower eukaryote which has the ability to receive signals from light and from plant growth regulators such as gibberellin, gibberellic acid and an auxin, 2,4-dichlorophenoxyacetic acid (K. Hasunuma et al. 1986. Fungal Genetics Newsl. 33:25-26). Light and plant growth regulators are external signals (first messengers) that may be transduced to changes in the intracellular concentration of second messengers, such as cyclic 3',5'.-AMP, cyclic 3',5'-GMP, inositol 1,4,5-triphosphate, diacyl glycerol and cytosolic free calcium, [Ca^2+]. We have detected changes in the concentration of cyclic 3',5'- AMP and cyclic 3',5'-GMP by light (K. Hasunuma et al. 1987. Curr. Genet. 11: in press), and have postulated the involvement of GTP-binding proteins (G.B. Rosenberg and M.L. Pall 1983. Arch. Biochem. Biophys. 221:243-253) in signal transduction from photoreceptors as in a bovine system (N. Bennet and Y. DuPont 1985. J. Biol. Chem. 260:4156-4168) and from receptors of hormones (H. Kurose et al. 1986. J. Biol. Chem. 261:6423-6428).

In the present study, we tested directly for the presence of GTP-binding proteins in Neurospora. Seven-day-old conidia from wild-type strain 74-OR23-1A, grown on glycerol complete medium, were inoculated at a density of 1 x 10^6 into 100 ml of Fries minimal medium in 1000 ml Roux flasks. They were incubated at 25° C for 12 h in darkness, 12 h under white fluorescent light (3.9 W/m^2) and 10 h in darkness. We have previously shown that light-dark entrainment of this kind causes the mycelia to become very sensitive to light such that white light leads to an abrupt decrease in the concentrations of cyclic 3',5'--AMP and cyclic 3',5'-GMP (K. Hasunuma et al. 1987. Curr. Genet. 11: in press). Following entrainment, the mycelial culture was harvested on filter paper under red safelight and stored at -80°C until preparation of crude extract

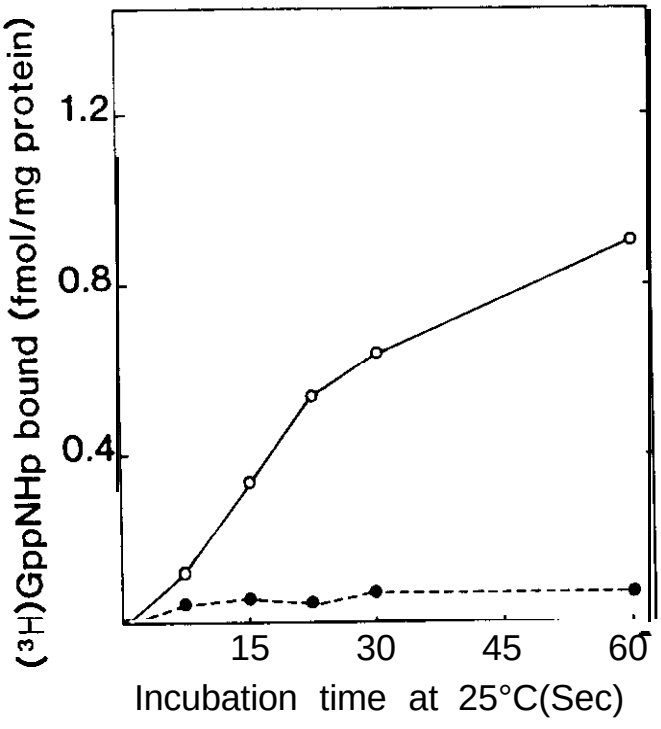

Fig. 1. The binding of [^3 H]Gpp(NH)p to GTP binding proteins in membrane and soluble fractions. Membrane fraction (--O--) and soluble fraction (--*--).

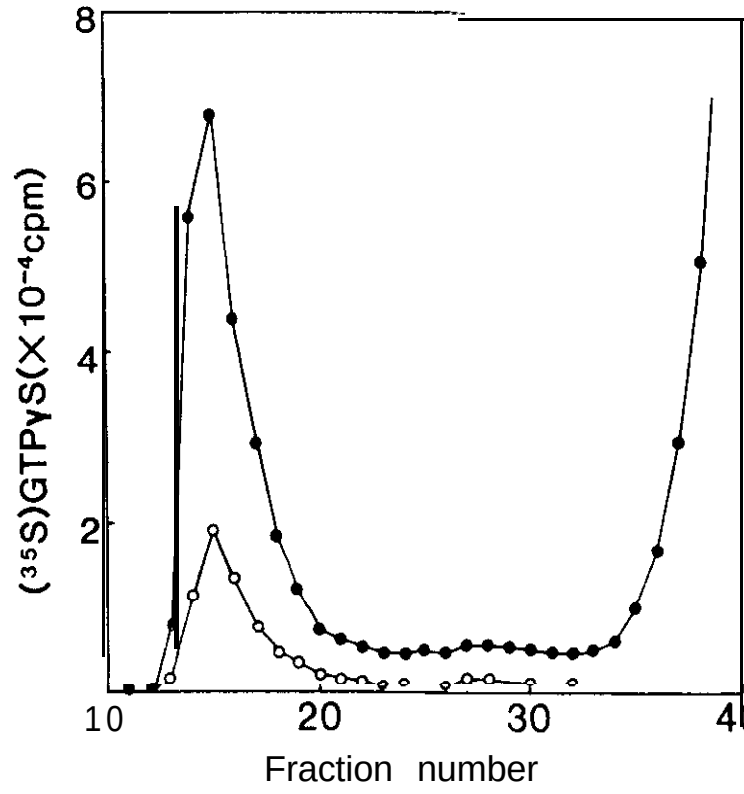

Fig. 2. Gel filtration through a Sephadex G-100 column of crude extract containing membrane components binding [^35 S]GTPYS. Radioactivity counting in 0.4 ml of each fraction (--*--) and those retained on membrane filter after washing by the buffer (--O--)

The following procedures were carried out under red safelight at 0 to 4° C. The mycelia was macerated in 2.5 vol of an extraction buffer containing 25 mM PIPES, pH 6.4, 0.25 mM EDTA, 1 mM MgCl2, 0.5 mM diazoacetyl-DL-norleucine methyl ester, 1 mM phenyl methylsulfonyl fluoride and 0.01 mM pepstatin A using an ice chilled mortar and pestle. Tissue was further homogenized three times in an ice chilled Teflon-glass homogenizer. The homogenate was centrifuged at 15,000 x g for 20 min to yield a supernatant fraction (crude extract; 5.27 mg protein/ml) which was further centrifuged at 105,000 x g for 1 h to yield a supernatant (soluble fraction; 3.03 mg protein/ml) and a pellet which was dissolved in the buffer (membrane fraction; 1.10 mg protein/ml). All fractions were stored at -80° C.

The binding of [^35 S] GTPYS (Guanosine 5'[Y-thio]triphosphate, [^35 S]-; 1.013 Ci/mmol, NEG-030H) and [^3H]Gpp(NH)p (ß,Y-imido [8-^3 H]guanosine 5'-triphosphate; 7.7 Ci/mmol, TRK-467) was assayed as described elsewhere (K. Hasunuma and K. Funadera 1987. Biochem. Biophys. Res. Commun. 143:908-912). The reaction mixture (200 ul) contained 0.1 uCi of [^3 H]Gpp(NH)p, 20 mM PIPES, pH 6.4, 0.1 mM EDTA, 0.1 NaCl, 1.5 mM MgCl2 and 0.2 mM phenyl methylsulfonyl fluoride. The reaction was started by adding membrane or soluble fraction to reaction mixture that had been prewarmed to 25° C, and stopped by adding 400 ul of ice-cold quenching mixture containing 20 mM PIPES, pH 6.4, 0.1 Lubrol PX, 0.1 M NaCl and 25 mM MgCl2. The filter was washed 8 times with 2 ml of the same buffer, dried and the radioactivity was counted. Control experiments were performed by adding quenching mixture before the addition of membrane or soluble fraction, and the count was negligible.

The results in Fig. 1 show that the membrane fraction efficiently bound [^3 H]Gpp(NH)p, while binding to the soluble fraction was poor. The result strongly suggests membrane localization of GTP-binding protein, although the possibility that there is a small amount of GTP-binding protein in the soluble fraction cannot be ignored.

With the above method, the efficiency of the retention of GTP-binding protein on the filter cannot be determined. The reaction mixture was expanded to 2 ml and 5 uCi of [^35 S]GTPYS was included. After incubation of the reaction mixture at 25° C for 5 min, 2 ml of 2-fold strength quenching mixture was added. It was loaded to a Sephadex G-100 column (2 x 32 cm) equilibrated with 20 mM Tris-HCl, pH 7.2, 0.1 M NaCl, 25 mM MgCl2 and 0.1% Lubrol PX and eluted with the same buffer into 2 ml fractions (Fig. 2). The results revealed an apparent peak of radioactivity at the void volume fractions (fractions 12-20) and a small peak at fractions 27-29. The same volume of each fraction was filtered on a Millipore HAWP membrane filter and washed with 2 ml of washing buffer 8 times. After counting the radioactivity in the filtrate using an Aquasol 2 scintillator it was possible to estimate the amount of [^35 S]GTPYS-binding protein retained on the filter. As shown in Fig. 2, approximately 30% of the [^35 S]GTPYS-binding proteins in void volume fractions and in smaller molecular weight fractions were retained on the membrane filter. From these results the localization of GTP-binding proteins in Neurospora was determined to be mainly in membranes and partly in the cytosol.

Table 1. Binding of [^3 H]Gpp(NH)p or [^35 S]GTPYS to proteins in crude extract of mycelia in the presence of various concentrations of ATP or GTP

ATP or GTP (uM)	Radioactivities retained on the filter (cpm)			
	[^3 H]Gpp(NH)p		[^35 S]GTPYS	
	ATP	GTP	ATP	GTP
0	3370^a	2950	21600	21900
0.001	3580	3190	22500	20100
0.01	3170	3010	22000	18200
0.1	3150	2330	21600	11100
1	**3660**	**959**	**24300**	**4740**
10	3750	156	20600	1690
100	2550	0	16100	0

The concentrations of [^3 H]Gpp(NH)p and [^35 S]GTPYS in the reaction mixtures were 2.5 nM and the input radioactivities were 193000 cpm for the former and 644000 for the latter. The reaction mixture was incubated at 25° C for 2 min.

a) From the radioactivities retained on the filters, the radioactivity in control experiment was subtracted. Each value was the mean of duplicate assays.

The binding of [^3 H]Gpp(NR)p and [^35 S]GTPYS to proteins was assessed for their capacity to bind them in the presence of GTP. In addition to the standard reaction mixture, crude extract containing membrane components and various concentrations of GTP or ATP were added, and the radioactivity retained on the filter was counted (Table 1). GTP but not ATP apparently inhibited the binding of [^3 H]Gpp(NH)p and [^35 S]GTPyS to proteins supporting the idea that these proteins were GTP-binding proteins. The efficiencies of binding of [^3 H]Gpp(NH)p and of [^35 S]GTPYS to GTP-binding proteins were similar, indicating both compounds to be equally useful to assay GTP-binding proteins. We are grateful to Mrs. M. Yazawa and Miss T. Imaizumi for excellent technical assistance.
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