Direct photoaffinity labeling of soluble GTP-binding proteins in *Dictyostelium discoideum*

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Major cytoplasmic GTP-binding proteins in *Dictyostelium discoideum* were identified by direct photoaffinity labeling with [α-^{32}P]GTP. Three actin-binding proteins and a protein with an apparent molecular mass of 24 kDa (p24) could be labeled with [α-^{32}P]GTP. p24 binds to DEAE-cellulose, behaves like a monomer during gel filtration and was purified to homogeneity by GTP-affinity chromatography. In comparison to other nucleotide triphosphates the binding of GTP to p24 is highly specific.

Actin-binding protein (Dictyostelium) *Photoaffinity labeling* *Nucleotide-binding protein*

1. INTRODUCTION

The binding of nucleotides causes a conformational change of certain proteins followed by an alteration in their function. Several of the nucleotide-binding proteins are membrane-associated, e.g. the G-proteins which stimulate (G_s) or inhibit (G_i) adenylate cyclase [1,2], or transducin which stimulates a cGMP-dependent phosphodiesterase [3]. All 3 proteins require GTP for activation. Other membrane-bound GTP-binding proteins, which show an apparent molecular mass of 21 kDa, are encoded by normal and transforming *ras* genes [4]. Among soluble GTP-binding proteins are polypeptide elongation and initiation factors [5] and several cytoskeletal proteins are also known to bind nucleotides like tubulin [6,7] and actin [8] or the actin polymerization regulating protein cap42 from *Physarum polycephalum* [9]. In these cases the exact function of nucleotide binding is not understood.

In *Dictyostelium discoideum,* nucleotides play an important role in development and signal transduction [10]. For identification of the major cytoplasmic GTP-binding proteins in *D. discoideum,* we used direct photoaffinity labeling with [α-^{32}P]-GTP. The F-actin crosslinking proteins α-actinin [11,12], the 120 kDa gelation factor [13] and a 17 kDa actin-binding protein [14] could be identified as nucleotide-binding proteins. The most prominent GTP-binding protein, which shows an apparent molecular mass of 24 kDa after SDS-polyacrylamide gel electrophoresis SDS-PAGE, was purified using GTP-affinity chromatography.

2. MATERIALS AND METHODS

2.1. *Cell culture*

D. discoideum strain AX2-214 was grown axenically in fermenters at 23°C [15], starved in 17 mM Soerensen phosphate buffer, pH 6.0, and stimulated with pulses of cyclic AMP to enhance the development to aggregation competence [16,17].

2.2. *Direct photoaffinity labeling*

For direct photoaffinity labeling protein solutions (usually 1 mg/ml) were preincubated for 30 min at 0°C with [α-^{32}P]GTP (New England Nuclear) in 60 μl TEDA buffer and then irradiated with UV light (254 nm) for 45 min [18,19]. [α-^{32}P]-GTP was used at the highest possible specific activity (750–800 Ci/mmol); 3–5 μCi per assay proved to be sufficient. After irradiation the samples were mixed with SDS sample buffer, incubated for 5

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min in a boiling water bath and subjected to SDS-PAGE [20] (13% acrylamide) and autoradiography on Kodak XAR-5 X-ray films.

2.3. *Preparation of actin-binding proteins*

D. discoideum α-actinin, the 120 kDa gelation factor and severin were purified essentially as described [11-14,22]. The 17 kDa membrane-associated protein was also found in the soluble fraction. This protein eluted from DEAE-cellulose at 80 mM NaCl; after precipitation with ammonium sulfate (40–60%) the protein was purified as a monomer on a gel filtration column (G-150, 2.7×114 cm).

2.4. *Preparation of protein p24*

D. discoideum cells were harvested, opened with a Parr bomb and subcellular fractions obtained essentially as in [14]. After centrifugation at $100\,000 \times g$ for 3 h the supernatant was adjusted to pH 7.5 and loaded onto a DEAE-column (DE-52, 5×40 cm, equilibrated in DEAE buffer). Bound proteins were eluted with a linear salt gradient (2×1000 ml, 0–300 mM NaCl). Five different pools were tested for the presence of GTP-binding proteins (see fig.2A). At a conductivity of 4.7–5.5 mS most of the 24 kDa protein was eluted, as tested by direct photoaffinity labeling. After a 40–60% ammonium sulfate cut the pellet was dissolved in a minimal amount of IDENA buffer and loaded onto a gel filtration column (G-150, 2.7×114 cm). Fractions containing the 24 kDa protein (see fig.2B) were pooled and applied to a GTP-affinity column (GTP-agarose, Sigma, 1.5–5 cm, equilibrated in IDENA buffer). The column was washed with 3 vols buffer and then incubated for 30 min with one column volume of buffer containing 1 mM GTP. The 24 kDa protein was eluted with two additional column volumes of the same buffer.

2.5. *Immunoblots*

Whole cell homogenates from *D. discoideum* and protein samples enriched in p24 were subjected to SDS-PAGE and blotted onto nitrocellulose filters (BA 85, Schleicher and Schuell) [21]. The nitrocellulose was saturated in NCP buffer and incubated for 10–14 h at room temperature with polyclonal antibodies raised in rabbits against *D. discoideum* ras protein. The serum was diluted

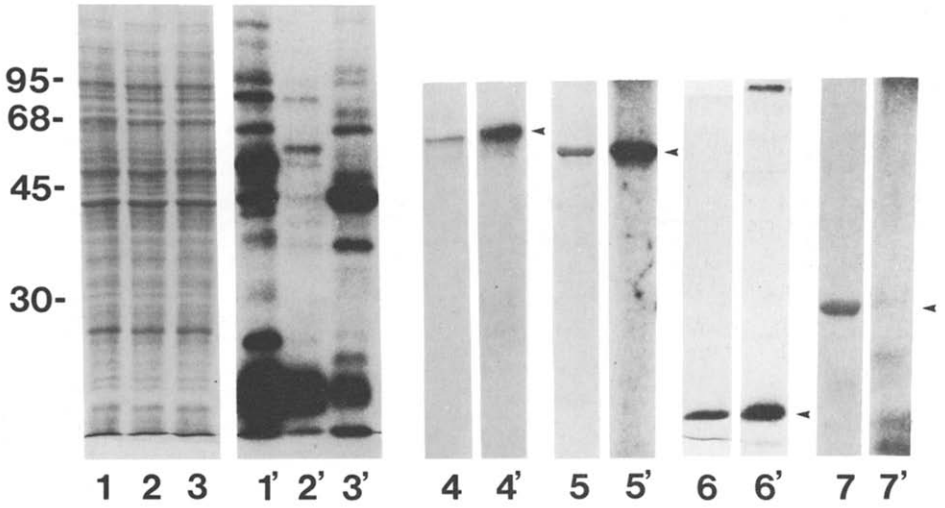

Fig.1. Detection of nucleotide-binding proteins of *D. discoideum*, using a soluble fraction from homogenized cells or purified actin-binding proteins. Equal amounts of the soluble fractions were incubated with [α-^{32}P]GTP (lanes 1/1'), [α-^{32}P]GTP plus an 850-fold excess of unlabeled GTP (lanes 2/2') and [α-^{32}P]ATP (lanes 3/3'), irradiated with UV light and separated on SDS-PAGE. Four purified actin-binding proteins are shown (arrowheads): 120 kDa gelation factor (lanes 4/4'), α-actinin (lanes 5/5'), 17 kDa protein (lanes 6/6') and severin (lanes 7/7') after direct photoaffinity labeling with [α-^{32}P]GTP and SDS-PAGE. Lanes 1–7 show the Coomassie blue staining patterns and lanes 1'–7' the corresponding autoradiograms.

from 1:200 up to 1:50 with NCP buffer. After washing the nitrocellulose was overlaid for 4 h with ^{125}I-labeled goat-anti-rabbit IgG or ^{125}I-labeled protein A (5×10^5 cpm/ml), washed and autoradiographed on Kodak XAR-5 X-ray film.

2.6. *Buffers*

DEAE buffer: 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EGTA, 1 mM benzamidine. 0.5 mM PMSF, 0.02% NaN_3. TEDA buffer: 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EGTA, 0.02% NaN_3. IDENA buffer: 10 mM imidazole (pH 7.2), 1 mM DTT, 1 mM EGTA, 0.15 M NaCl, 0.02% NaN_3. NCP buffer: 10 mM Tris-HCl (pH 7.8), 0.15 M NaCl, 0.02% NaN_3, 0.05% Tween 20.

3. RESULTS AND DISCUSSION

Soluble GTP- and ATP-binding proteins from *D. discoideum* are shown in fig.1. Aliquots from

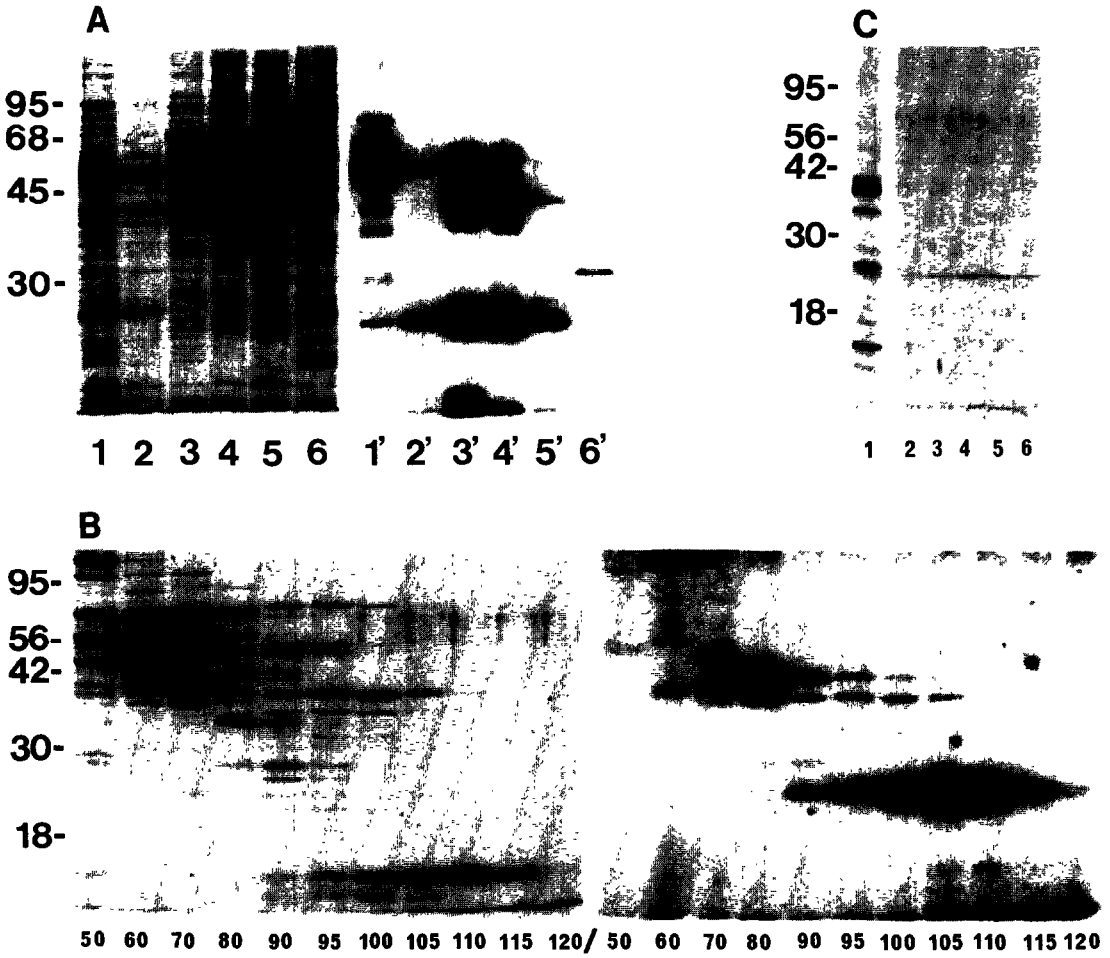

Fig.2. SDS-PAGE and autoradiography of protein fractions after separation on a DEAE-column and direct photoaffinity labeling with [α-^{32}P]GTP. (A) Left panel (lanes 1–6): Coomassie blue stain; right panel (lanes 1′–6′): corresponding autoradiogram. Proteins were eluted from the column at a conductivity of 0.9 mS (flow through fraction, lanes 1/1′), 1.3–2.7 mS (lanes 2/2′), 2.8–4.6 mS (lanes 3/3′), 4.7–5.5 mS (lanes 4/4′), 5.6–7.7 (lanes 5/5′), or 7.7–11.3 (lanes 6/6′). (B) p24 further purified by gel filtration. Proteins (fractions 50–120; fraction volume, 5.0 ml) eluted from a G-150 column were irradiated in the presence of [α-^{32}P]GTP, separated on SDS-PAGE (Coomassie stain, left) and subjected to autoradiography (right). (C) Coomassie blue staining of fractions recovered from a GTP-agarose column. Flow through (lane 1), peak fractions containing purified 24 kDa protein obtained by elution with 1 mM GTP (lanes 2–6).

the soluble fraction were incubated with [α-^{32}P]-GTP with and without an excess of unlabeled GTP or [α-^{32}P]ATP of the same specific activity as the [α-^{32}P]GTP. Three classes of nucleotide-binding proteins could be distinguished:

(i) proteins which bind specifically GTP or ATP (see fig.1, lanes 1′,3′; apparent molecular masses 80, 56 and 24 kDa, binding to GTP; 38 kDa, binding to ATP;

(ii) proteins which bind both GTP and ATP with similar affinity (see fig.1, lanes 1′,3′; apparent molecular mass 65 kDa);

(iii) proteins which bind preferentially GTP or ATP (see fig.1, lanes 1′,3′; apparent molecular mass 42, 18–21 kDa).

Since tubulin (56 kDa) and actin (42 kDa) are known to bind nucleotides [6–8], the labels found in these molecular mass ranges probably represent these two proteins. Following the purification protocol for actin-binding proteins in *D. discoideum*, we were able to identify the crosslinking protein α-actinin [11,12], the 120 kDa gelation factor [13] and the 17 kDa actin-binding protein [14] as GTP-binding proteins (fig.1, lanes 4/4′, 5/5′, 6/6′, respectively). Under identical conditions, severin, an F-actin severing protein [22], could not be labeled (fig.1, lanes 7/7′). A more detailed characterization of the interaction between certain actin-binding proteins and nucleotides is in progress.

Additional GTP-binding proteins were detected by fractionating the soluble extract on DEAE-cellulose. Proteins eluting from the column were pooled as indicated in fig.2 and assayed for GTP-binding. As with the unfractionated soluble fraction, the most prominently labeled band was that of a 24 kDa protein. To determine whether its high GTP-binding efficiency is due to the abundance of the protein or to its high specific binding activity for GTP, we have purified this protein.

Most of the GTP-binding 24 kDa protein precipitates in the range 40–60% ammonium sulfate. On a gel filtration column this protein behaved like a monomer (fig.2B). From a GTP-affinity column a single protein of apparent molecular mass 24 kDa eluted with 1 mM GTP (fig.2C). After this final purification step it was no longer possible to label the protein with [α-^{32}P]GTP. Extensive dialysis over several days against nucleotide-free buffer restored only part of the [α-^{32}P]GTP-binding capacity. Therefore, we assume that the GTP-binding site was saturated by the high concentration of unlabeled GTP which was needed for elution and that the exchange rate of the GTP was low.

Because of the inefficient GTP labeling of affinity-purified 24 kDa protein, we used partially purified p24 to test the specificity of nucleotide binding. [α-^{32}P]GTP was applied after preincubation of the protein with a 2400-fold excess of unlabeled GTP, ATP, UTP or CTP. After incubation for 15 min with unlabeled nucleotides, [α-^{32}P]-GTP was added, and after another 30 min the photoaffinity labeling was started. As shown in fig.3 the protein was completely saturated by preincubation with cold GTP (lane 1) whereas ATP, UTP and CTP (lanes 2–4) did not substantially compete for the GTP-binding site. In similar experiments with an excess of cold GTP, GDP, GMP, cGMP, guanosine, pyrophosphate or phosphate only GTP or GDP competed for the GTP-binding site of p24.

Fractions containing partially purified 24 kDa protein showed a strong photoaffinity label (fig.3) but only an extremely weak staining of the protein with Coomassie blue or silver. This indicates that

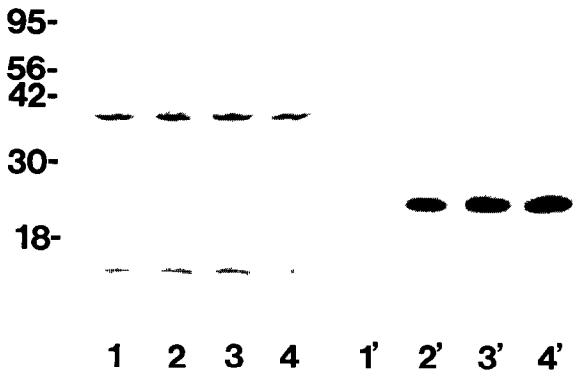

Fig.3. Specificity of p24 for binding of GTP. The samples were preincubated with a 2400-fold excess of unlabeled GTP (lanes 1/1′), ATP (lanes 2/2′), UTP (lanes 3/3′) and CTP (lanes 4/4′) before being subjected to photoaffinity labeling with [α-^{32}P]GTP (Coomassie blue stain, left; autoradiogram, right).

the intense labeling of the 24 kDa band was due to either a high affinity of the protein for GTP or multiple GTP-binding sites rather than to its abundance. The possibility that p24 is identical with the highly conserved GTP-binding elongation factor EF-1α [5,23,24] can be excluded by the differences in the molecular mass. It is conceivable that the GTP-binding activity of proteins with an apparent molecular mass at around 50 kDa (fig.2A, lanes 3′,4′) is due to the direct photoaffinity labeling of the elongation factor EF-1α.

Recently, a protein was detected in *D. discoideum* which belongs to the group of ras proteins [25,26]. Using a polyclonal antiserum raised against a part of the *D. discoideum* ras protein [25] we could not detect any significant crossreaction with p24. The comparison of the amino acid compositions as well as preliminary sequency data obtained from p24 show numerous differences between the ras protein from *D. discoideum* and p24. Together with the identification of p24 as a soluble, cytoplasmic protein and the failure of the binding to a ras protein-specific antibody, we suggest that p24 is not the ras protein of *D. discoideum*. The low abundance of p24 may indicate that this GTP-binding protein has a regulatory function.

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