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Phosphotyrosine-containing proteins in *Dictyostelium discoideum*

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Phosphotyrosine-containing proteins in *Dictyostelium discoideum* were detected by immunoblot analysis and immunoprecipitation using a monoclonal anti-phosphotyrosine antibody. The iodinated antibody recognized on bots a cluster of 205–220 kDa polypeptides and bands of 107 and 60 kDa. The 107 and 60 kDa polypeptides and, in addition, a 82 kDa one became phosphorylated on tyrosine when the immunoprecipitate was incubated with [γ-^{32}P]ATP. In preparations from differentiating cells the intensity of the label was increased in the 60 kDa band and decreased in the 107 and 205–220 kDa bands.

Phosphotyrosine antibody; Protein-tyrosine kinase; *Dictyostelium discoideum*

1. INTRODUCTION

Phosphorylation of tyrosine residues is catalysed by retroviral protein-tyrosine kinases (PTKs) and their cellular homologues, and by growth factor receptor-associated PTKs [1,2]. These enzymes have been studied in detail in vertebrates. A kinase associated with pp60^{c-src}, which is the cellular homologue of the oncogene v-src of Rous sarcoma virus, exists in many or all Metazoans including sponges [3]. In *Drosophila*, DNA sequences corresponding to PTK genes have been recognized [4]. The kinase has not been detected in unicellular eukaryotes and in plant cells. It has been suggested that certain PTKs, in particular the pp60$^{c\text{-}src}$ kinase, are phylogenetically old proteins that evolved early in the phylogenesis of multicellular organisms as regulators of cell proliferation and differentiation [3]. In this paper we investigate tyrosine phosphorylation in an organism that does not belong to the Metazoans. *Dictyostelium discoideum* is a lower eukaryote which is unicellular during growth and develops upon starvation into a multicellular state by cell aggregation.

Presence of phosphotyrosine in *D. discoideum* proteins has been detected in previous experiments by in vivo [^{32}P]phosphate incorporation (W. Huttner, personal communication). Here we report on the presence in *D. discoideum* of distinct proteins phosphorylated on tyrosine. Changes during development are shown. The proteins are characterized by immunoprecipitation using a monoclonal anti-phosphotyrosine antibody and by in vitro tyrosine phosphorylation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Phosphoamino acids and sodium orthovanadate were purchased from Sigma (München), *Streptococcus aureus* (Pansorbin cells) and rabbit anti-mouse Ig serum from Calbiochem (Frankfurt), cellulose-coated plastic sheets (Polygram Cel 400) from Macherey und Nagel (Düren), and [γ-^{32}P]ATP (5000 Ci/mmol) from Amersham Buchler (Braunschweig). The hybridoma cell line producing anti-phosphotyrosine mAb was kindly provided by Dr Axel Ullrich, Martinsried, re-cloned by us, and a strongly IgG 2a producing clone, 5E2-1-10, was selected.

2.2. *Growth and fractionation of cells*

Dictyostelium discoideum strain AX2-214 was grown axenically in nutrient medium (Ashworth) as described [5] up to cell densities of 3–5 × 10^6 cells/ml. For development, cells were kept in 17 mM phosphate buffer, pH 6.0, at a density of 1 × 10^7 cells/ml. Cells were sedimented and washed once with the phosphate buffer containing 1% Trasylol (aprotinin) and 100 μM sodium vanadate.

Purified plasma membranes were prepared by a two-phase dextran/polyethyleneglycol procedure of Brunette and Till [6] according to Stadler et al. [7].

A detergent soluble fraction of *D. discoideum* proteins was obtained by centrifugation of cells on a step gradient. About 5 × 10^8 cells were suspended in 2 ml phosphate buffer and applied on two layers of sucrose in 20 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 5 mM benzamidine, 1% Trasylol, 250 μM vanadate. The 10 ml bottom layer contained 0.44 M sucrose, the 5 ml top layer 0.32 M sucrose plus 0.5% Triton X-100. Centrifugation was for 10 min in a swing-out rotor at 1000 × g in the cold. The upper 2 ml of the 0.32 M sucrose/Triton X-100 layer were collected; the pellet containing crude nuclei was discarded.

2.3. *Immunoprecipitation*

Aliquots of the detergent soluble fraction (usually 100 μl) were incubated with 10 μg mAb 5E2 for 60 min on ice. 10 μl Pansorbin cells pre-coated with rabbit anti-mouse Ig Ab were added and the mixtures kept on ice for 30 min. Cells were then sedimented in an Eppendorf centrifuge and washed twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM DTE, 100 μM vanadate, 1% Trasylol, and once with 20 mM Tris-HCl, pH

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7.5, 150 mM NaCl, 1 mM dithioerythrol, 100 mM vanadate, 1% Trasylol.

To label immunoprecipitates, the pellets were resuspended in 20 mM Hepes, pH 7.0, 5 mM $MnCl_2$, and incubated for 5 min at 25°C in the presence of 10 μCi [^{32}P]ATP. The reaction was stopped with 40 μl SDS sample buffer. Mixtures were heated to 55°C for 1 h and separated by SDS-PAGE.

2.4. *Analytical procedures*

Whole cells or purified membranes were solubilized in SDS sample buffer; soluble fractions were mixed with 2 vols of the same buffer. All samples were incubated at 55°C for 1 h and separated on 5–20% SDS-polyacrylamide gels [8]. Gels were stained with Coomassie blue and dried, or proteins were blotted onto nitrocellulose membranes [9] for detection of phosphotyrosine proteins using ^{125}I-labelled mAb 5E2-1-10 (0.5 μCi/ml).

For phosphoamino acid analysis labelled bands were cut out from the dried gel. Proteins were extracted and hydrolysed in 6 N HCl. The phosphate-labelled phosphoamino acids were separated on cellulose thin-layer sheets by electrophoresis at pH 3.5 [10].

3. RESULTS

After separation of total proteins from growth-phase cells of *D. discoideum* by SDS-PAGE, few species were recognized by the iodinated anti-phosphotyrosine mAb (Fig. 1a). These were proteins forming a cluster in the 205–220 kDa region, a 107 kDa and a 60 kDa species. Labelling of the 60 kDa band was of low intensity in the growth-phase cells. Cofractionation of the 205 kDa species with plasma membranes enriched by the dextran/polyethyleneglycol method is shown in Fig. 1b. When cells were incubated for 15 min with 30 μM phenylarsine oxide (PAO), which enhances reactivity of phosphotyrosine-containing proteins with antibody [11], an additional band larger than 220 kDa was labelled (data not shown).

During development of starving cells in suspension changes in the pattern of antibody labelled proteins were observed (Fig. 2). Labelling of the 205/220 and 107 kDa species was detected throughout early development up to the 6 h stage of aggregation competent cells, and was strongly reduced at the 22 h stage when tight aggregates had been formed. Labelling of the 60 kDa band which was low in growth-phase cells increased during the 6 h period and remained high after 22 h.

To determine the specificity of labelling, immunoblots were labelled with [^{125}I]mAb 5E2 in the presence of 1 mM of phosphoamino acids. Fig. 3a–d shows that only phosphotyrosine competed for labelling of the 205/220, 107 and 60 kDa bands, while phosphoserine and phosphothreonine showed no inhibitory effect within the limits of variation.

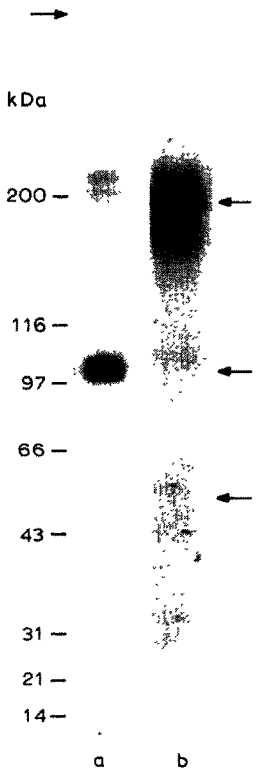

Fig. 1. Detection of phosphotyrosine-containing proteins. Proteins from 1 × 10^6 AX2 growth-phase cells (a) or enriched plasma membranes (b) were solubilized and separated by SDS-PAGE. Following transfer to nitrocellulose membrane, proteins were labelled with [^{125}I]anti-phosphotyrosine antibody. Molecular masses of marker proteins are shown at left. Arrowheads show positions of major labelled proteins.

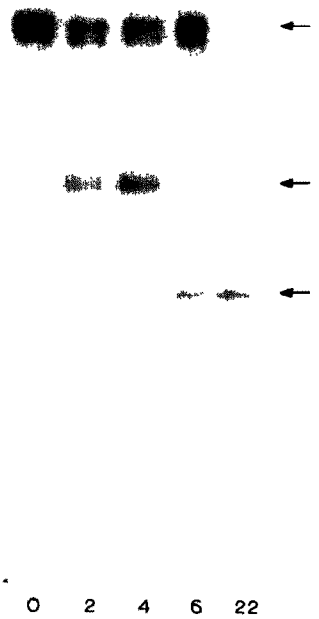

Fig. 2. Phosphotyrosine-containing proteins in development. Aliquots of starving AX2 cells in solution were removed at 0, 2, 4, 6 and 22 h. Cell proteins were separated and analysed for phosphotyrosine as shown in the legend to Fig. 1.

The mAb 5E2 was then used to precipitate phosphotyrosine-containing proteins to investigate their phosphorylation in the precipitate (Fig. 4). Proteins from a Triton X-100 extract of growth-phase cells were precipitated and the washed pellets incubated with [^{32}P]ATP. Subsequently the precipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes and autoradiographed for ^{32}P. Precipitated proteins not labelled with ^{32}P-ATP were blotted in parallel for labelling with mAb 5E2. The antibody recognized in the precipitation the same proteins as in the homogenate, indicating that the 205/220, 107 kDa, as well as the 60 kDa polypeptides were precipitated (Fig. 4a). ^{32}P was strongly incorporated into the 107 kDa band, and also in a 150 kDa band which was not recognized by the antibody (Fig. 4b). The 60 kDa band was weaker but nevertheless clearly labelled with ^{32}P. About to the same extent ^{32}P was incorporated into a 82 kDa polypeptide that was not significantly recognized by the antibody. No or only trace amounts of ^{32}P were found in the 205/220 kDa doublet strongly recognized by the antibody.

The ^{32}P-labelled bands marked 1–4 in Fig. 4b were cut out from a gel for hydrolysis of the proteins and analysis of phosphoamino acids by thin-layer electrophoresis. Only [^{32}P]phosphothreonine was found in the 150 kDa band (Fig. 5, lane 1). A strong signal of phosphotyrosine was discovered in the 107 kDa band together with a phosphothreonine signal which might be due to contamination with a second protein (Fig. 5, lane 2). Weak but unequivocal ^{32}P-label in phosphotyrosine was also found in the 82 and 60 kDa bands (Fig. 5, lanes 3 and 4).

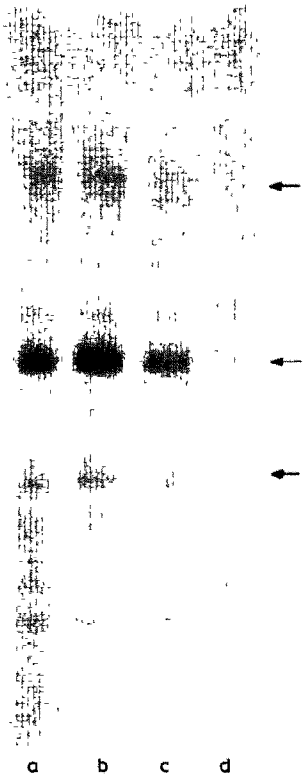

Fig. 3. Specificity of labelling of proteins with anti-phosphotyrosine antibody. Identical lanes of blotted proteins from growth-phase cells were incubated with the iodinated antibody alone (a), or in the presence of 1 mM phosphoserine (b), 1 mM phosphothreonine (c), 1 mM phosphotyrosine (d). Other conditions of SDS-PAGE and immunoblot analysis were as shown in the legend to Fig. 1.

4. DISCUSSION

In the experiments described here we have identified phosphotyrosine-containing proteins as well as tyrosine kinase activity in the lower eukaryote *Dictyostelium discoideum*. The phosphotyrosine specific antibody mAb 5E2 precipitated not only all four major species of polypeptides that were recognized in immunoblots, but precipitated also strong tyrosine and threonine kinase activities. Substrate of the threonine kinase was a 150 kDa polypeptide not recognized by mAb 5E2, which was apparently tightly associated with a phosphotyrosine-containing protein bound by the an-

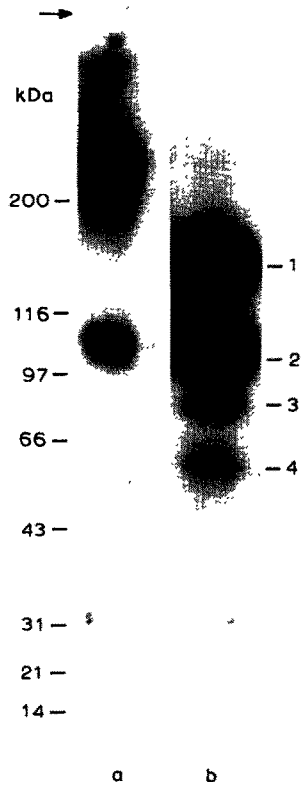

Fig. 4. Immunoprecipitation of phosphotyrosine-containing proteins. Phosphotyrosine proteins were immunoprecipitated from a Triton X-100 AX2 cell extract with the anti-phosphotyrosine antibody as described in section 2. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and analysed for phosphotyrosine using the iodinated antibody (a). In a parallel experiment, the precipitate was incubated with [^{32}P]ATP and the blot analysed for ^{32}P-labelled polypeptides (b). Numbers 1–4 at right mark major labelled bands in lane (b).

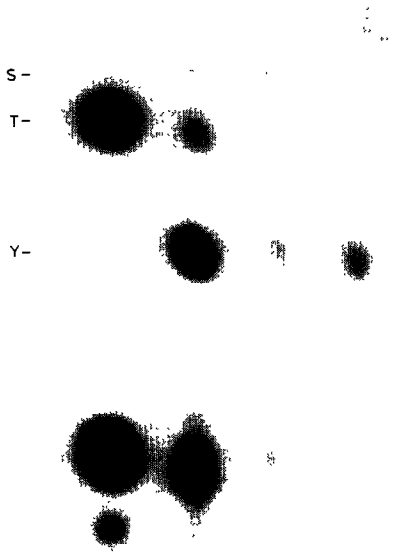

Fig. 5. Identification of phosphoamino acids. Proteins of the immunoprecipitate were phosphate-labelled as in the experiment in Fig. 4b, and were separated by SDS-PAGE. Bands corresponding to marked bands 1–4 on the blot were cut out from a gel. Proteins were eluted, hydrolysed and the phosphate-labelled phosphoamino acids identified by cellulose thin-layer electrophoresis at pH 3.5 in the presence of unlabelled phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). Numbers 1–4 below lanes correspond to bands 1–4.

tibody. It is possible that the threonine kinase phosphorylating the 150 kDa protein does itself contain phosphotyrosine and is therefore precipitated with the antibody. Three distinct polypeptides in the precipitate acted as substrates for tyrosine kinase. Studies with the renatured proteins [12] will show whether one or more of them is autophosphorylated in analogy to oncogene or receptor kinases [1,2].

Protein-tyrosine kinases of the $pp60^{c\text{-}src}$ type [3] have been discovered in many Metazoans. In *D. discoideum*, which is a microorganism on the borderline from unicellular to multicellular organisation, we have found a 60 kDa phosphotyrosine-containing protein which might have a relationship to c-src gene products. Other *D. discoideum* proteins recognized by phosphotyrosine specific antibody, particularly those forming bands in the 200–300 kDa region, might be receptor kinases involved in the control of proliferation and differentiation. Developmental regulation of the proteins recognized by mAb 5E2, or of the degree of their tyrosine phosphorylation, suggests their implication in these processes.

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