A monoclonal antibody recognizes a phosphorylated epitope shared by proteins of the cell nucleus and the erythrocyte membrane skeleton

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Monoclonal antibody 3C5 recognizes a family of proteins in the nuclei of cultured cells [(1985) Eur. J. Cell Biol. 38, 344]. This antibody has now been shown to recognize equivalent proteins in liver nuclei and in the Triton-insoluble fraction of tissue extracts. In human erythrocytes the antibody recognized a single protein, present in the membrane skeleton fraction and with the molecular mass and extraction properties of β-spectrin. The epitope recognized by 3C5 was destroyed by alkaline phosphatase. We conclude that this antibody recognizes a phosphorylation site shared by nuclear proteins and a protein of the erythrocyte membrane skeleton, probably β-spectrin.

Phosphorylation site *Nuclear protein* *Spectrin* *Monoclonal antibody* *Protein epitope* *Alkaline phosphatase*

1. INTRODUCTION

Monoclonal antibodies to nuclear protein antigens are now increasingly used for analysis of cell cycle-related and developmentally related changes in nuclear structure and composition [1]. By a combination of immunomicroscopical techniques and immunostaining methods for detection of protein antigens separated by electrophoresis target antigens can be precisely localized and characterized.

We recently described a monoclonal antibody which, by indirect immunofluorescence microscopy, stained the nuclei of a variety of cultured cell types with a characteristic speckled pattern and which showed striking, cell cycle-related changes in the distribution and intensity of staining [2]. This antibody recognized a family of proteins with subunit molecular masses ranging from 23 to 130 kDa, all of which were predominantly or exclusively nuclear. Several possible explanations for this multiple specificity were eliminated, including non-specific cross reaction, in vitro proteolysis and a common carbohydrate moiety. In this report we show that these proteins are present in various rodent tissues and provide evidence that antibody 3C5 recognizes a phosphorylation site shared by a family of nuclear proteins and by β-spectrin from human erythrocytes.

2. MATERIALS AND METHODS

2.1. *Electrophoresis, protein blotting and immunostaining*

Electrophoresis in SDS-containing polyacrylamide gels was carried out as described by Laemmli [3]. Proteins were electrophoretically transferred onto nitrocellulose filters (Schleicher and Schuell BA85) by the procedure of Towbin et al. [4] and immunostained as described [2].

2.2. *Preparation of tissue extracts*

All procedures were carried out at 0–4°C. Tissue pieces (0.5 g wet wt) from Balb/c mice were minced in 10 vols (w/v) of TPT buffer (10 mM Tris/HCl, pH 7.5, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)) and homogenized

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for 2 min at 3/4 speed with an MSE metal-blade homogenizer. Homogenates were filtered through gauze and centrifuged at 100 000 × *g* for 60 min. Pellets were raised in 5 ml TPT buffer containing 0.5% Triton X-100 and dispersed in an all-glass Wheaton tissue grinder ('A' pestle, Jencon Scientific, Bedfordshire, England). After 10 min on ice samples were centrifuged at 100 000 × *g* for 60 min. Pellets were extracted with 2 ml of 8 M urea for 5 min at 100°C. Extracts were centrifuged at 25 500 × *g* for 60 min and the supernatants stored at -80°C. Prior to electrophoresis 1/4 vol. SDS-dissociating buffer was added (1% SDS, 1% 2-mercaptoethanol in 0.1 M Tris, 1 mM Na_2 EDTA, pH 7.2) and the samples heated at 100°C for 5 min.

Human erythrocyte membranes were prepared and extracted according to Bennett and Stenbuck [5]. Samples were dialysed overnight and solubilized in SDS-dissociating buffer.

Rat liver nuclei were prepared by the method of Karn and Allfrey [6].

2.3. *Alkaline phosphatase treatment*

Escherichia coli alkaline phosphatase in 2.5 M $(NH_4)_2SO_4$ was obtained from Sigma (Lot no. 52F-40362, 45 U/mg protein, 70 U/ml). Tissue extracts were incubated for 1–2 h at 37°C in the presence of 0.9–3.5 U/ml alkaline phosphatase, 1 mM PMSF, 0.1 mM TPCK, 0.1 TIU/ml aprotinin (Sigma), 10 mM Tris/HCl, pH 8.0. For most experiments 30–50 μl tissue extract (1–3 mg protein/ml) was incubated in a final volume of 200 μl. Where appropriate sodium phosphate buffer, pH 8.0, was added to a final concentration of 5–50 mM. The reaction was terminated by addition of 50 μl SDS-dissociating buffer and heated at 100°C for 5 min.

3. RESULTS

3.1. *Antibody 3C5 recognizes a family of proteins in rodent tissues*

In a variety of rodent tissues antibody 3C5 recognized a family of proteins equivalent to those previously detected [2] in cultured cells from various species (fig. 1). Tissue-specific differences were noted in the relative intensity of both the major bands and of several minor bands. All major bands recognized by 3C5 were present in purified rat liver nuclei (nuclei from other tissues have not yet been tested) and were found almost exclusively in the Triton-insoluble particulate fraction of all tissue extracts (not shown). These findings are in agreement with previous results obtained with various cultured cell types [2].

3.2. *Antibody 3C5 recognizes a single protein in human erythrocytes*

The proteins recognized by 3C5 in nucleated cells were absent from human erythrocytes. However, a single high-M_r protein band was detected by 3C5 in erythrocyte extracts and was found to be associated with the erythrocyte membrane fraction prepared by conventional procedures (fig. 2, lanes 1 and 2). This protein had the same mobility as the two closely adjacent bands designated 2 and 2.1 and representing two major proteins of the erythrocyte membrane skeleton, β-spectrin and ankyrin [7]. Like these proteins, the 3C5 antigen was not extracted by 0.5% Triton X-100 (not shown). The protein recognized by 3C5 was resistant to extraction by 1 M KCl (fig.2, lane 3), a procedure which extracts ankyrin and several other proteins associated with the erythrocyte membrane, but not spectrin [5]. In contrast, exposure to hypotonic conditions (0.3 mM phosphate), a procedure which efficiently extracts spectrin [7], solubilized the protein recognized by 3C5 (fig.2, lane 4).

3.3. *The epitope recognized by 3C5 is destroyed by alkaline phosphatase*

Pretreatment of tissue extracts with alkaline phosphatase invariably caused a significant reduction in the staining intensity of all bands with 3C5 (fig.3, lanes 1,3 and 5). In several experiments complete absence of staining was observed following exposure to alkaline phosphatase. This effect was abolished by inclusion of 5 mM phosphate in the reaction mixture (fig.3, lanes 2,4 and 6). Phosphate ions are known to inhibit the enzyme by a competitive mechanism with a K_i of less than 0.1 mM under the conditions used for these experiments [8]. These results, together with the lack of effect of the protease inhibitors PMSF, TPCK and aprotinin, argue strongly that destruction of the epitope is attributable to alkaline phosphatase itself and not to a contaminating protease.

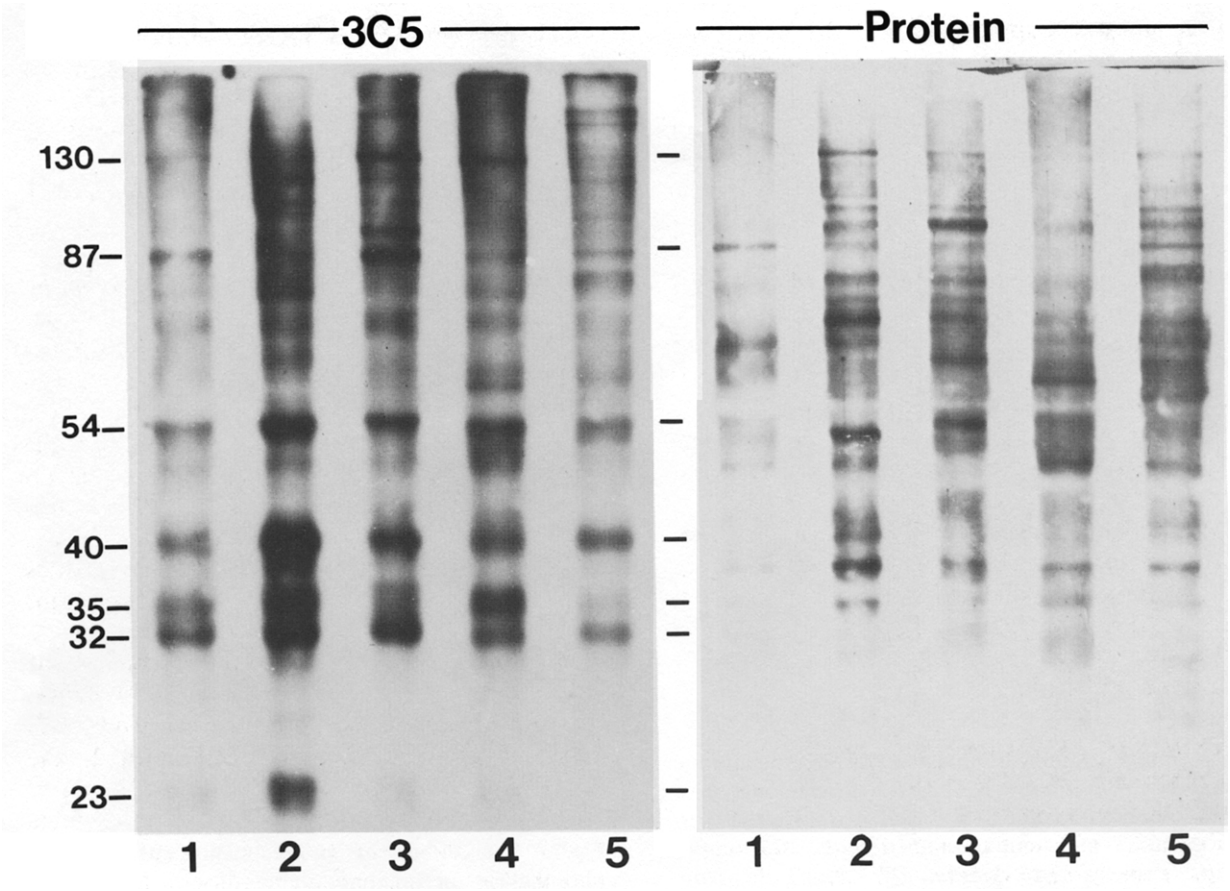

Fig.1. Immunostaining of protein antigens in extracts of mouse tissues with antibody 3C5. Urea extracts of Triton-insoluble material were run on a 10% polyacrylamide-SDS gel, transferred to nitrocellulose and immunostained with 3C5 and ^{125}I-labelled rabbit antibody to mouse immunoglobulin. Bound antibody was revealed by autoradiography (left panel). Proteins were detected by staining the same filter with ink (right panel) as described by Hancock and Tsang [11]. Lanes were loaded as follows to give about 60 μg soluble protein per lane: (1) kidney, (2) testis, (3) brain, (4) liver, (5) spleen. Apparent subunit molecular masses of shared, 3C5-stained bands are shown (kDa).

4. DISCUSSION

The results presented provide evidence that a phosphate group is an integral component of the epitope recognized by antibody 3C5 and that the family of proteins detected by this antibody have a common phosphorylation site. Thus, the variation in the relative staining intensity of the various protein bands from one tissue or cell type to another may represent tissue-specific differences in the level of phosphorylation of the epitope. The recognition of a phosphorylated epitope by monoclonal antibodies has been described by Davis et al. [9] who isolated two antibodies recognizing alkaline phosphatase-sensitive epitopes on a set of proteins with subunit molecular masses of 40–200 kDa. However, these proteins differ from those recognized by 3C5 in size, solubility and behaviour through the cell cycle.

If the preliminary identification of the protein recognized by 3C5 in erythrocytes as the β-chain of spectrin is confirmed by more detailed analysis this may provide a means of characterizing the epitope. The β-chain of spectrin can be phosphorylated at 4 residues (3 serines and 1 threonine) at the extreme C-terminal end of the molecule [10]. By identification and sequencing of tryptic peptides which react with 3C5 it may be possible to describe in detail the phosphorylation site shared by β-spectrin and by proteins of the cell nucleus.

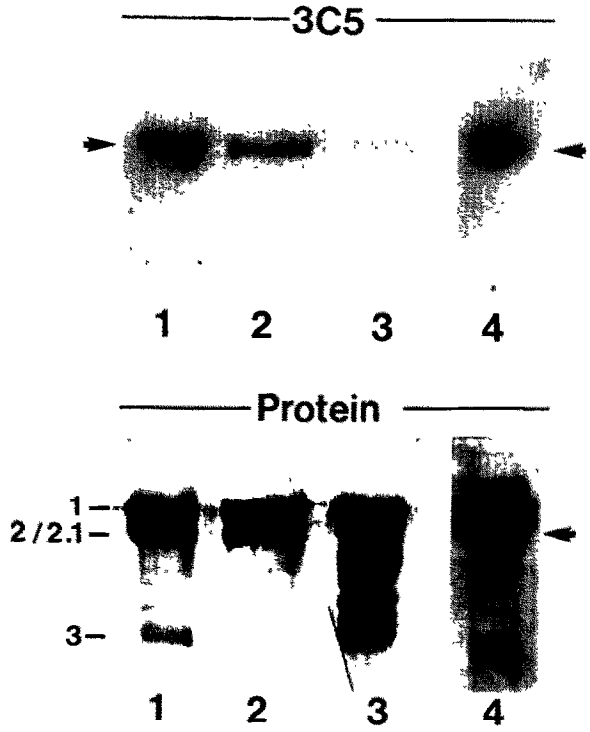

Fig.2. Immunostaining of human erythrocyte membrane proteins with antibody 3C5. Extracts were run on a 7.5% polyacrylamide-SDS gel, transferred to nitrocellulose and immunostained with 3C5 (upper panel). Proteins were detected by staining the same filters with ink (lower panel). For clarity only the upper section of each lane is shown. Lanes were loaded as follows: (1) erythrocyte membranes, (2) membranes treated with 1 M KCl, (3) proteins extracted by 1 M KCl, (4) proteins extracted by 0.3 mM phosphate. Lanes 1,2 and 3 are from the same gel.

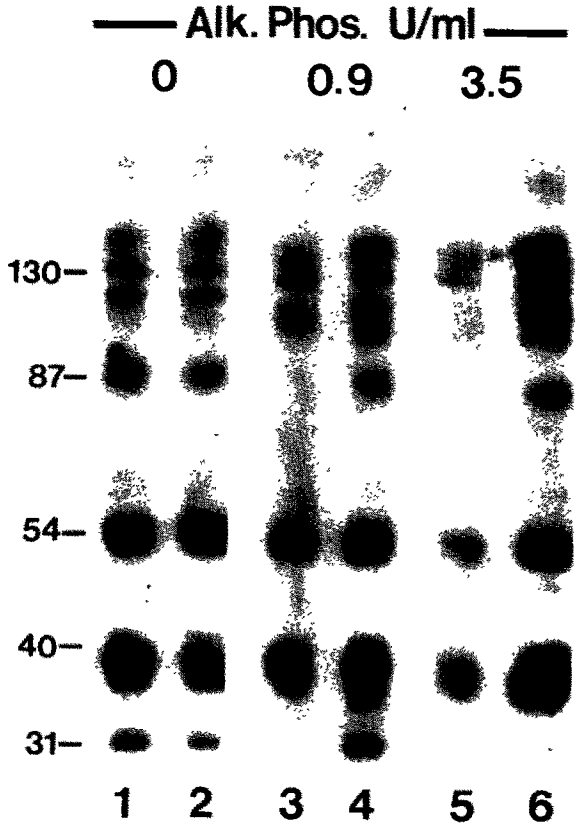

Fig.3. The effect of pretreatment with alkaline phosphatase on immunostaining of protein antigens with antibody 3C5. Extracts of mouse testis were treated with 0.9 or 3.5 U/ml alkaline phosphatase (lanes 3–6) or incubated in the absence of added enzyme (lanes 1,2). Preincubation was carried out in the presence (lanes 2,4 and 6) or absence (lanes 1,3 and 5) of 5 mM phosphate. Extracts were run on a 7.5% polyacrylamide-SDS gel.

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