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Antibodies to fluorylsulfonylbenzoyladenosine permit identification of protein kinases

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Antibodies were raised to fluorylsulfonylbenzoyladenosine (FSBA) coupled to carrier proteins. Affinity purified sera are shown to specifically detect protein kinase C-β_1 treated with FSBA. Immunodetection is specific to modification at the ATP binding site as judged by the ability of ATP to block subsequent detection. The antisera are shown to react with a number of U937 cell proteins fractionated on Mono Q and treated with FSBA; the majority of these immunoreactions are prevented by inclusion of ATP in the FSBA treatments. One of these is positively identified as PKC-β_1.

Fluorylsulfonylbenzoyladenosine; Kinase; Antibody

1. INTRODUCTION

The protein kinase superfamily currently numbers more than 200 and has been predicted to exceed 1000 [1]. These proteins provide a powerful regulatory mechanism that spans the breadth of cellular functions and are implicated in many pathological states.

Identification of protein kinases has been achieved both through conventional purification [2] and through various cloning strategies, including homology screening [3], expression cloning [4] and PCR [5]. While cDNA cloning has provided a rapid increase in our appreciation of the size of this family, the defined protein kinases are slow to be attributed roles in the absence of defined substrates. By contrast, purifications are often based upon the phosphorylation of particular substrates and as such, provide an immediate insight into function. Such procedures, however, are often limited by the low concentrations of protein kinases and a need to be able to unequivocally assign catalytic function to a particular polypeptide within a purified preparation. Thus protein kinase activities can, on occasion, be recovered through renaturation following SDS-PAGE; this property can even be employed in screening for kinases [6]. Selective labelling of protein kinases has also been carried out with the reactive ATP analogue fluorylsulfonylbenzoyladenosine (FSBA). This reagent will react with primary amino groups, i.e. with lysine residues within the polypeptide, and has been shown to react with an invariant lysine residue present within the ATP binding site of the protein kinase superfamily [7].

In order to facilitate identification of protein kinases in otherwise more or less complex mixtures of proteins, antibodies to FSBA have been raised and characterized and are shown here to identify specifically a known protein kinase in crude fractionated mixtures. These reagents should prove very powerful in the identification of novel protein kinases and in their immunoaffinity purification.

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2. MATERIALS AND METHODS

2.1. *Materials*

Keyhole limpet haemocyanin (KLH) was obtained from Calbiochem. Chromatographic materials were from Pharmacia; [γ-^{32}P]ATP and ECL detection reagents were obtained from Amersham International. Other biochemical reagents, including FSBA, were obtained from Sigma.

2.2. *Preparation and purification of antisera to FSBA*

FSBA was coupled to KLH (5 mg/ml), lysine-rich histone (Histone III-S) (5 mg/ml) or polysine (5 mg/ml) in 10 mM sodium borate pH 9.5 at 4°C overnight. The coupled proteins were dialysed exhaustively against phosphate buffered saline prior to immunization.

Serum samples were initially screened by ELISA against each of the three FSBA-coupled protein preparations. The serum showing the highest specific titre (see section 3) was affinity-purified on FSBA-coupled to polylysine-Sepharose prepared as described above for the immunogens.

2.3. *Preparation and fractionation of U937 cell extracts*

U937 cells were cultured in RPMI medium with 10% foetal calf serum in 5% CO_2 at 37°C to a density of 1.5×10^6 cells/ml. Cells (3×10^7) were harvested by centrifugation (1000 rpm for 3 min) washed once in phosphate-buffered saline (at 4°C) and the cell pellet extracted at 4°C, in 1 ml of 1% (v/v) Triton X-100, 20 mM Tris-HCl pH 7.5, 5 mM EGTA, 5 mM EDTA, 10 mM benzamidine, 0.1 mg/ml leupeptin and 50 μg/ml phenylmethylsulfonylfluoride. The extract was cleared by centrifugation in a microfuge at 4°C. The supernatant was loaded onto a 1 ml Mono Q column and eluted at 0.5 ml/min with a 10 ml linear gradient of 0 to 1.0 M NaCl in 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.1% (v/v) Triton X-100, collecting 0.5 ml fractions.

2.4. *Reaction of proteins with FSBA*

Purified protein kinase C-β_1 (PKC-β_1) was prepared as described previously [8]. For reaction of PKC-β_1 with FSBA, the kinase was dialysed into 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.02% (v/v) Triton X-100 in order to remove dithiothreitol from the PKC storage buffer, which would otherwise react with FSBA. The dialysed PKC was then incubated with 10 mM Mg^{2+} and 1 mM FSBA (added from a 50 mM stock made up in DMSO) or vehicle (DMSO), in the presence or absence of 1 mM ATP as indicated. Reactions were terminated by boiling samples in SDS-PAGE sample buffer [9] containing 150 mM β-mercaptoethanol to quench the FSBA.

Reaction of fractionated U937 cell proteins with FSBA was carried out in a trial volume of 250 μl by addition of 10 mM Mg^{2+} and 1 mM FSBA to 195 μl of each fraction in the presence or absence of 1 mM ATP as indicated. Reactions were allowed to proceed at 30°C for 20 min and then terminated by boiling in SDS-PAGE sample buffer.

2.5. *Western blotting and immunoprecipitation*

Proteins were separated on 10% polyacrylamide gels according to Laemmli [9] and transferred to PVDF membranes and blocked as described previously [10]. Blots were incubated with antisera overnight at 4°C at the following dilutions: anti-FSBA 1/400; anti-PKC-β_1 1/2000 [10]. Immunoreaction was visualized by ECL. Immunoprecipitation was carried out by 10-fold dilution of protein samples stored in SDS-PAGE sample buffer, in phosphate buffered saline containing 1% Triton X-100, followed by incubation overnight at 4°C with 10 μl of the PKC-β_1 antiserum. Immunocomplexes were recovered on Protein A-agarose, washed three times in phosphate buffered saline containing 0.2% Tween-20 and eluted by boiling in SDS-PAGE sample buffer. Proteins were then separated on a 10% polyacrylamide gel and subjected to Western blotting as described above.

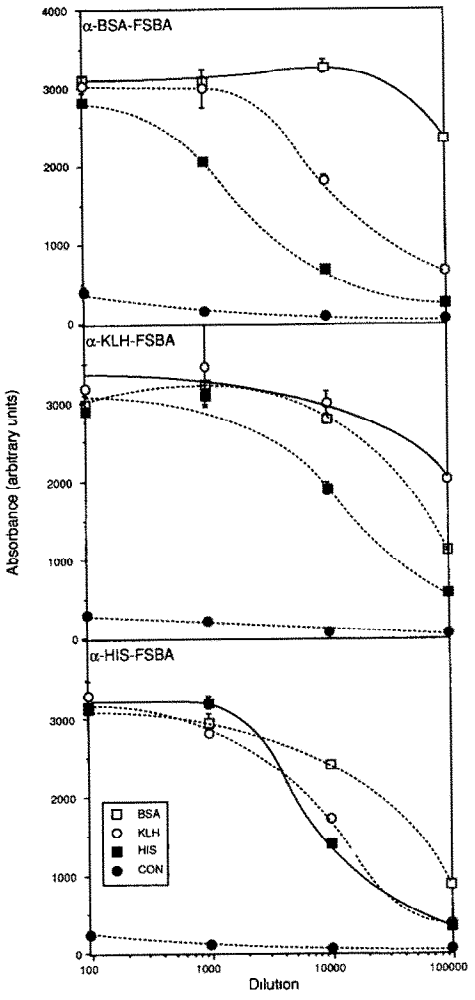

Fig. 1. Immunoreactivity of anti-FSBA sera. Sera raised against FSBA coupled to BSA (α-BSA-FSBA, top panel), KLH (α-KLH-FSBA, middle panel) and histone (α-His-FSBA, bottom panel) were titred by ELISA against their respective immunogens, the heterologous immunogens, or control wells. Immunoreaction against BSA-FSBA (□-□), KLH-FSBA (○-○), His-FSBA (■-■) and control (●-●) is shown as indicated in the inset.

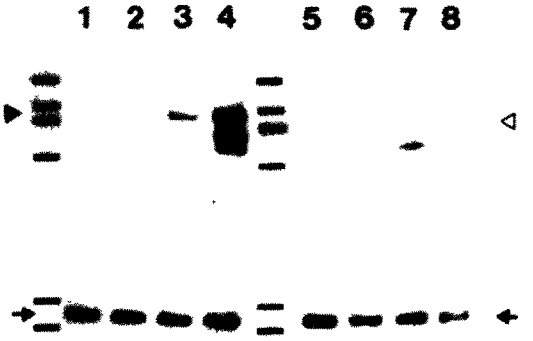

Fig. 2. Time dependent reaction of PKC-β_1. PKC-β_1 was reacted with FSBA as described in section 2. Reactions were carried out in the absence (lanes 1–4) or presence (lanes 5–8) of 1 mm ATP, for 0 (lanes 1,5), 10 (lanes 2,6), 20 (lanes 3,7), 40 (lanes 4,8) min. Reacted protein was visualized by Western blotting and affinity purified anti-FSBA sera (upper panel) and total PKC-β_1 visualized with an anti-PKC-β_1 serum (lower panel). The upper panel shows reactivity with the 80 kDa PKC-β_1 (filled arrowhead) with no equivalent reactivity when ATP is included (open arrowhead). The arrows in the lower panel indicate the 80 kDa PKC-β_1 migrating between the phosphorylase and BSA molecular weight markers (as in the upper panel).

3. RESULTS AND DISCUSSION

3.1. *Characterization of anti-FSBA sera*

Sera obtained following immunization with FSBA-coupled to KLH, histone or polylysine were screened by ELISA against the homologous and heterologous FSBA-coupled proteins (Fig. 1). It is evident that in each case there is a specific immunoreaction as judged by binding to the heterologous FSBA-coupled proteins. As would be expected, the titre is highest against the homologous FSBA-coupled protein reflecting the antigenicity of the carrier protein itself. The sera obtained from the KLH-FSBA immunization displayed the highest specific titre and was employed for further purification and characterization. It should be noted that this is not necessarily the optimum antigen since this set of data is derived from a single series of immunizations.

The KLH-FSBA antiserum was affinity-purified on FSBA-reacted polylysine-Sepharose in order to obtain an FSBA-specific antibody preparation (see section 2). This anti-FSBA serum was then tested for immunoreaction with PKC-β_1 that had been pretreated with FSBA in the presence or absence of ATP for varying times (Fig. 2). It is evident that the antiserum specifically recognises the 80 kDa PKC-β_1 polypeptide and that this

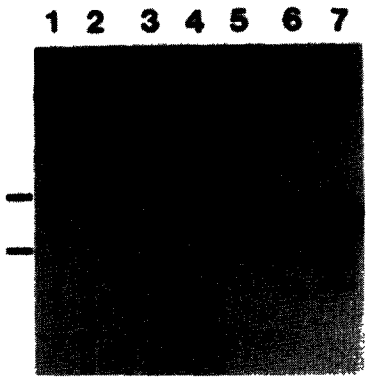

Fig. 3. Sensitivity of the anti-FSBA serum. FSBA-reacted PKC-β_1 was serially diluted and then subjected to Western blotting with the affinity purified anti-FSBA serum. The amount of PKC-β_1 in lanes 1–7 is in ng: 290, 145, 73, 36, 18, 9, 4.5. The original figure shows detection at 9 ng. The purified PKC-β_1 employed here shows two bands on staining; these appear to be differentially phosphorylated forms. Both species react with FSBA and can be detected with the anti-FSBA serum.

immunoreaction is not observed in the sample treated in the presence of ATP (the lower band that appears on prolonged incubation is variably observed and is not competed by ATP). To control for any variation in processing or loading, the blots were stripped and reprobed with a PKC-β_1 antiserum (Fig. 2, lower panel). Reaction with serial dilutions of the FSBA-treated PKC-β_1 (Fig. 3) shows that the anti-FSBA serum can detect 9 ng of reacted protein (lane 2 is visible in the original), i.e. ~ 0.1 pmol of PKC. This is a minimum estimate since the actual stoichiometry of reaction could not be determined as under these reaction conditions, control (vehicle) incubations in the absence of FSBA also led to loss of activity (data not shown). Nevertheless, the detection limits with the anti-FSBA serum compares well with radiolabelled FSBA detection (the specific activity of commercially available FSBA is ~ 50 mCi/mmol).

3.2. *Identification of protein kinases in crude fractionated U937 cell extracts*

The specific reaction of the anti-FSBA serum with purified PKC-β_1 suggested that the serum would be suitable for immunodetection of kinases in complex mixtures. While in principle abundant proteins with available lysine residues may also react with FSBA and thus be detected, the ability of ATP to specifically block reaction at ATP-binding sites can be employed to identify protein kinases and perhaps other ATP-binding proteins. To test this, extracts from U937 cells were fractionated by anion exchange chromatography and individual fractions reacted with FSBA in the presence or absence of ATP (Fig. 4). A complex mixture of FSBA reacted proteins is observed; the majority of these are not detected or only poorly detected in the samples incubated in the presence of ATP suggesting that certain of these proteins may be protein kinases.

In order to provide evidence on the identity of one of these detected proteins, the same blots were stripped and reprobed with antisera to PKC-β_1. While backgrounds were enhanced due to the reprobing procedure, specific reaction with the appropriately sized protein was observed and found to comigrate with a major FSBA-reacted species (compare Figs. 4b and 5a).

Formal proof that the comigrating PKC-β_1 and the FSBA-reacted species were identical was obtained by immunoprecipitation of the kinase from the relevant fraction and subsequent immunoblot with the anti-FSBA serum. The immunoprecipitated kinase is seen to be detected by the anti-FSBA serum (Fig. 5b).

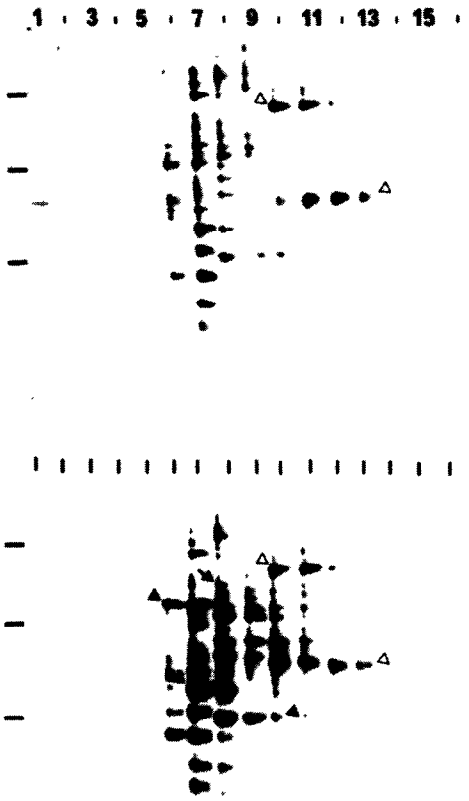

Fig. 4. FSBA-reacted proteins in fractionated U937 cell extracts. U937 cell extracts were prepared and separated on a Mono Q column as described in the section 2. Fractions were incubated with FSBA in the presence (upper panel) or absence (lower panel) of 1 mm ATP. Reacted proteins were visualized by Western blotting with affinity purified anti-FSBA serum. Open arrowheads indicate examples of proteins whose reaction with FSBA is not competed by ATP. Filled arrowheads indicate some of the proteins whose reaction with FSBA is substantially reduced in the presence of ATP. The arrow indicates the FSBA-reacted protein comigrating with PKC-β_1 (see Fig. 5). The molecular weight markers indicated are 97 kDa, 66.5 kDa and 45 kDa in descending order.

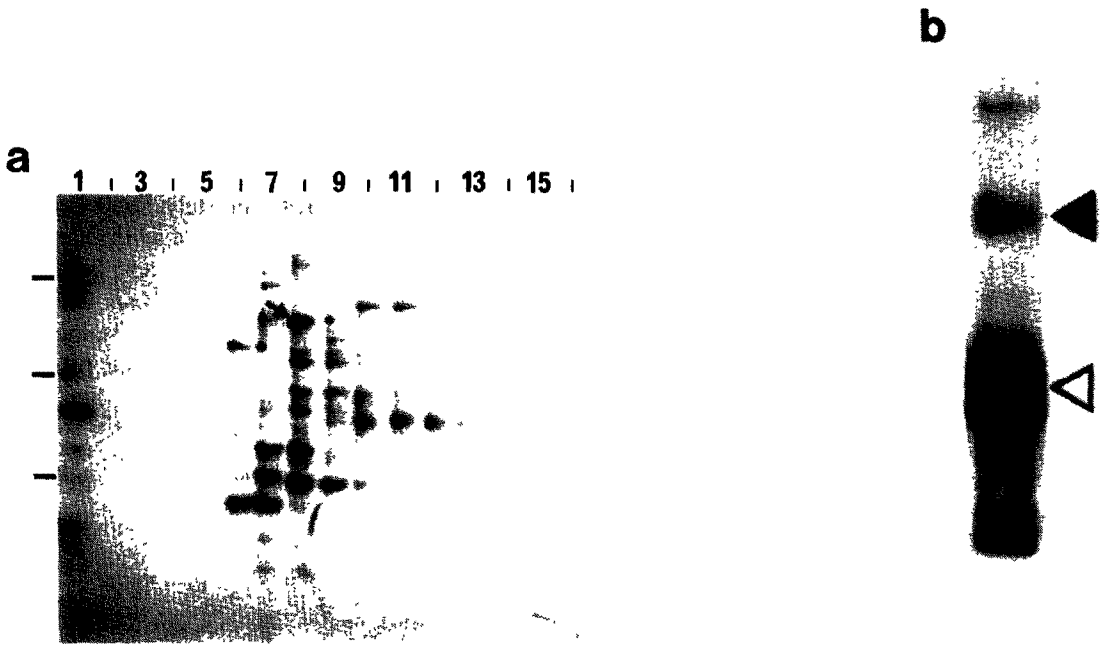

Fig. 5. Identification of FSBA-reactied PKC-β_1. (a) The Western blot from lower panel in Figure 4 was stripped of antibody (5 min in 10% acetic acid at 20°C) and reblotted to reveal PKC-β_1 (indicated by the arrow). The residual background immunoreaction permits this band to be lined up precisely with the FSBA-reacted protein indicated in Fig. 4, lower panel. (b) PKC-β_1 was immunoprecipitated from fraction 8 and then subjected to Western blotting with the affinity purified anti-FSBA serum. The reactive PKC-β_1 is indicated by the filled arrowhead. Immunoglobulin reacting with the second layer antirabbit-horseradish peroxidase is indicated by the open arrowhead.

3.3. *Conclusions*

The results described above provide an accessible procedure for the production of anti-FSBA antisera and demonstrate that these can be employed in the detection of protein kinases. Reaction of FSBA with other ATP-dependent proteins evidently occurs, e.g. reaction with FSBA has been employed recently in the identification of the catalytic subunit of the phosphatidylinositol-3′kinase [11]. Thus antisera to FSBA may prove useful in the identification of metabolite kinases in addition to protein kinases. The ability to follow rates of reaction with FSBA may prove useful in probing protein kinase ATP binding sites, their accessibility under various conditions and the action of inhibitors that may interact at these sites. Indeed, the non-isotopic detection of an FSBA-reacted kinase may provide a useful means of inhibitor screening. It is anticipated, however, that a major use of such reagents will be in the identification and immunoaffinity purification of novel partially purified protein kinases, permitting protein sequence information to be obtained.

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