Assay of protein kinase C with an *N*-bromosuccinimide-cleavage fragment of histone H1

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N-Bromosuccinimide cleavage of a lysine-rich histone fraction (histone III-S) yields a peptide substrate, purified by reverse-phase h.p.l.c., for the Ca^{2+} + phospholipid-dependent protein kinase (protein kinase C). This substrate displays no reactivity with the cyclic AMP-dependent protein kinase, and may prove useful for the detection of protein kinase C activity in crude tissue extracts.

INTRODUCTION

The Ca^{2+} + phospholipid-dependent protein kinase (protein kinase C) is receiving increasing attention as an important modulator of intracellular metabolism and cell growth (Nishizuka, 1984). This protein kinase, like the cyclic AMP-dependent protein kinase, has broad substrate specificity. *In vitro* it has been shown to phosphorylate several histone fractions, protamine and myelin basic protein, as well as an increasing number of other intracellular proteins (Wise *et al.*, 1982; Kikkawa *et al.*, 1982; Nishizuka, 1984). Histone H1 has been most often employed as a substrate to monitor the activity of protein kinase C; many investigators have used the commercially available histone III-S fraction, which is enriched in lysine-rich histones, especially histone H1. Because of overlapping substrate specificity of various protein kinases, histone H1 is not a specific substrate for protein kinase C; one must depend on the demonstration of Ca^{2+} + phospholipid-dependence to identify protein kinase C activity. This may present some problems in the measurement of this kinase in crude tissue extracts, which might contain phospholipid and the activator of the kinase, diacylglycerol, which lowers the apparent K_a for Ca^{2+} (Kishimoto *et al.*, 1980).

Protein kinase C and the cyclic AMP-dependent protein kinase are abundant kinases in a number of tissues. Histone H1 is phosphorylated at significant rates by both of these kinases; the kinase activities against histone H1 may be distinguished, however, by examination of the sites of phosphorylation. The cyclic AMP-dependent protein kinase preferentially phosphorylates serine residues in the *N*-terminus of histone H1, whereas protein kinase C phosphorylates serine and threonine residues in the *C*-terminus (Isawa *et al.*, 1980). This observation suggests that a more specific assay for protein kinase C might be developed with a *C*-terminal peptide of H1. Such an approach was taken by O'Brian *et al.* (1984), using a synthetic nonapeptide encompassing the phosphorylation site sequence on histone H1. However, this substrate displayed relatively poor substrate kinetics as compared with native histone ($K_{m(\text{app.})}$ 130 μM) and is expensive to prepare.

The present study describes the easy preparation of a readily available substrate for protein kinase C by *N*-bromosuccinimide digestion of lysine-rich histones.

This substrate is not phosphorylated at significant rates by the cyclic AMP-dependent protein kinase, and may prove useful in the measurement of protein kinase C activity in crude tissue extracts.

MATERIALS AND METHODS

Materials

Protein kinase C was isolated from rat brain up to and including the gel-filtration step (Kikkawa *et al.*, 1982). *N*-Bromosuccinimide, *L*- α -phosphatidyl-L-serine from bovine brain, the catalytic subunit of the cyclic AMP-dependent protein kinase, histone (type III-S) from calf thymus and ATP from horse muscle were purchased from Sigma Chemical Co. [γ - ^{32}P]ATP was obtained from ICN Radiochemicals. Adipocytes were prepared by collagenase digestion of epididymal fat-pads from Sprague–Dawley rats obtained from Charles River Breeding Laboratories.

Protein kinase assays

Protein kinase C was assayed against histone III-S (1.25 mg/ml) or the *N*-bromosuccinimide-cleavage peptide (1.0 mg/ml) in the presence or absence of phosphatidylserine (80 $\mu\text{g/ml}$) and Ca^{2+} (1 mM) (Parker *et al.*, 1984). Preliminary experiments revealed that ^{32}P incorporation into the peptide was best quantified by precipitation of portions of the reaction mixture on to phosphocellulose paper followed by immersion in 20% (w/v) trichloroacetic acid/100% (w/v) silicotungstic acid (4:1, v/v). The papers were then washed extensively in 10% trichloroacetic acid before being dried and quantification of incorporation by Čerenkov radiation in water. Significantly lower incorporation was noted either by omission of the silicotungstic acid or by direct precipitation of the reaction mixture in the presence of carrier bovine serum albumin by trichloroacetic acid/silicotungstic acid. The cyclic AMP-dependent protein kinase was assayed under identical conditions except for the omission of phospholipid and Ca^{2+} .

N-Bromosuccinimide digestion of histone III-S and peptide isolation

N-Bromosuccinimide digestion was carried out by the method of Sherod *et al.* (1974). Histone III-S (2 mg/ml)

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was dissolved in 0.9 M-acetic acid and mixed with an equal volume of *N*-bromosuccinimide (100 µg/ml). The mixture was incubated at 30 °C for 10 min, and the reaction was stopped by the addition of an aqueous solution containing 10 mol of tyrosine/mol of *N*-bromosuccinimide. The reaction mixture was freeze-dried and the powder was redissolved in 0.1% trifluoroacetic acid. The peptide mixture was fractionated by h.p.l.c. on a Beckman 334 apparatus with a C_{18} reverse-phase column (Waters µBondapak C18). Histone III-S cleavage products were eluted with a linear gradient of acetonitrile in aq. 0.1% trifluoroacetic acid; after sample application, the column was washed for 5 min at 1 ml/min with 0.1% trifluoroacetic acid before the acetonitrile concentration

was increased at 1%/min. The C-terminal peptide was reproducibly eluted at 35–37% (v/v) acetonitrile; these fractions were pooled, and the acetonitrile and trifluoroacetic acid were removed by repetitive freeze-drying. In preliminary experiments the histone III-S was prelabelled by incubation with protein kinase C or the cyclic AMP-dependent protein kinase and [32 P]ATP before *N*-bromosuccinimide cleavage and peptide isolation. The purity of the peptide fractions obtained was assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) and radioautography. Peptide concentration was measured by the method of Lowry *et al.* (1951) and by determination of the A_{225}/A_{215} ratio (Waddell, 1956), which gave good agreement. Neither the original histone III-S mixture nor the isolated peptide showed reactivity in the protein assay procedure of Bradford (1976). A 5–10 mg yield of peptide has been routinely isolated beginning with 25 mg of histone III-S.

RESULTS AND DISCUSSION

Commercially available histone III-S is a mixture of lysine-rich histones, the most prominent of which are two major histone H1 species (Fig. 1, lane A), which migrate in this gel system with M_r values of 32000 and 30000. After cleavage with *N*-bromosuccinimide and purification by h.p.l.c., two peptides, of M_r 23000 and 21000, are observed (Fig. 1, lane B). These peptides are referred to below as $H1_{NBS}$. Isolation of these peptides (generated by *N*-bromosuccinimide cleavage of 32 P-histone III-S, labelled by incubation with protein kinase C and [32 P]ATP) by h.p.l.c. yields a nearly homogeneous preparation, corresponding to a major A_{215} and 32 P-radioactivity peak (Fig. 1, lanes B and C, and Fig. 2). It is likely that these

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel separation of histone III-S and *N*-bromosuccinimide-cleavage peptides

Histone III-S and peptides generated by *N*-bromosuccinimide digestion, after purification by h.p.l.c., were separated on an 18% polyacrylamide gel. Shown are the Coomassie Blue-stained histone III-S (lane A) and *N*-bromosuccinimide-generated fragments after purification by h.p.l.c. (lane B) and a radioautograph of 32 P-labelled peptides isolated by labelling of histone III-S followed by reaction with *N*-bromosuccinimide and purification by h.p.l.c. (lane C). Each lane contains 5 µg of protein. M_r markers are β -galactosidase (M_r 116000), phosphorylase b (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20000) and lysozyme (M_r 14300).

Fig. 2. H.p.l.c. separation of *N*-bromosuccinimide-cleavage peptides of histone III-S

Shown is a representative h.p.l.c. chromatogram (see the Materials and methods section) of the separation of 32 P-labelled peptides after labelling of histone III-S by incubation with protein kinase C with subsequent digestion with *N*-bromosuccinimide. The main chart depicts absorbance at 215 nm and the inset shows the eluted radioactivity (all radioactivity eluted in this peak). In the absence of *N*-bromosuccinimide digestion, all of the radioactivity was eluted at about 45% acetonitrile (result not shown).

Fig. 3. Phosphorylation of peptide H1_{NBS} by protein kinase C

Peptide H1_{NBS} was phosphorylated by incubation with protein kinase C (200 pmol units per reaction; 1 unit equals 1 μ mol of phosphate incorporated into histone III-S/min at 30 °C) in the presence of Ca²⁺ and phosphatidylserine (●), phosphatidylserine alone (○), Ca²⁺ alone (■) or no addition (□), as detailed in the Materials and methods section. Shown is pmol of ³²P incorporated as a function of assay time.

two peptides are C-terminal fragments of different H1 histones, both of which contain a phosphorylation site(s) for protein kinase C. The N-terminal peptide, preferentially labelled by the cyclic AMP-dependent protein kinase, is eluted at 47% acetonitrile (results not shown).

Peptide H1_{NBS} phosphorylation by protein kinase C was characterized with respect to Ca²⁺ + phospholipid-dependence and to substrate kinetics. Although our partially purified protein kinase C does phosphorylate this peptide in the absence of Ca²⁺ and phospholipid, the initial rate of phosphorylation is increased 6–8-fold in their presence (Fig. 3). The K_m for ATP was 15 μ M. Reaction velocity was maximal at 10 mM-Mg²⁺ or 2 mM-Mn²⁺. However, the V_{max} in the presence of Mg²⁺ is twice that in the presence of Mn²⁺, and Mn²⁺ concentrations in excess of 2 mM were markedly inhibitory. These data suggest that Mg²⁺ is the preferred bivalent cation for this reaction (results not shown).

The apparent K_m for peptide H1_{NBS} was determined to be 222 μ g/ml (results not shown). On the basis of the reported M_r of 15000 for the C-terminal N-bromosuccinimide-cleavage fragment of histone H1, this corresponds to 14.8 μ M. Both intact and N-bromosuccinimide-cleaved histone H1 appear to run at anomalous M_r values in our gel system (Fig. 1). The rate of phosphorylation on peptide H1_{NBS} is biphasic with respect to peptide concentration, with progressive substrate inhibition at concentrations exceeding 1 mg/ml (results not shown).

Histone III-S is a better substrate for the cyclic AMP-dependent protein kinase than for protein kinase C. As shown in Fig. 4(a), the initial reaction velocity is 5–6-fold greater for the cyclic AMP-dependent protein kinase. However, protein kinase C phosphorylates histone III-S and peptide H1_{NBS} (measured at saturating concentrations for each) at nearly equal rates, whereas virtually no phosphorylation of peptide H1_{NBS} by the cyclic AMP-dependent protein kinase is detectable (Fig. 4b). Thus peptide H1_{NBS} is a specific reagent to distinguish these two kinase activities. Absolute specificity for protein kinase C cannot be claimed until the potential

Fig. 4. Phosphorylation of histone III-S and peptide H1_{NBS} by protein kinase C and the cyclic AMP-dependent protein kinase

Shown in (a) is a plot of the incorporation of ³²P (pmol) versus time for the phosphorylation of histone III-S by the cyclic AMP-dependent protein kinase (40 pmol unit/reaction, as defined by Sigma Chemical Co.) (●) or protein kinase C (200 pmol unit/reaction) (○). In panel (b) is plotted ³²P incorporation (pmol) on incubation with protein kinase C into histone III-S (○) and peptide H1_{NBS} (■) and on incubation with the cyclic AMP-dependent protein kinase into peptide H1_{NBS} (●).

Table 1. Measurement of protein kinase C activity in adipocyte extracts with peptide H1_{NBS}

Cytosolic extracts were prepared from isolated rat adipocytes by homogenization (1 g/ml) in medium containing sodium β -glycerophosphate (100 mM; pH 7.4), EDTA (2 mM), EGTA (2 mM), dithiothreitol (2 mM) and sucrose (0.25 M) followed by centrifugation at 100000 g for 1 h at 4 °C. A portion of the supernatant fraction (10–20 μ g of protein) was then assayed under linear rates for protein kinase activity, in the absence or presence of Ca²⁺ (1 mM), phosphatidylserine (PS; 80 μ g/ml) or dioleoin (DO; 8 μ g/ml), against peptide H1_{NBS}, as detailed in the Materials and methods section. Kinase activities from five preparations of adipocytes are expressed as pmol of ³²P incorporation/min per mg of extract protein (means $\pm$ S.D.) after subtraction of substrate-only and extract-only blank values.

Addition	Kinase activity
None	16.0 $\pm$ 6.2
PS	17.0 $\pm$ 6.0
PS + DO	16.0 $\pm$ 5.9
Ca ²⁺	26.4 $\pm$ 6.3
PS + Ca ²⁺	123.3 $\pm$ 31.0
PS + Ca ²⁺ + DO	135.7 $\pm$ 17.8

phosphorylation of this peptide by other kinases that react with histone H1 can be ascertained.

Peptide H1_{NBS} may prove to be useful for the measurement of protein kinase C activity in crude tissue extracts. We have found that nearly all of the histone H1 protein kinase activity in adipocyte cytosol, measured in the absence of Ca²⁺ + phospholipid, is directed at the *N*-terminal *N*-bromosuccinimide-cleavage peptide (results not shown). By using our *C*-terminal *N*-bromosuccinimide-cleavage peptide, protein kinase C activity in adipocyte cytosol can be readily identified (Table 1). No stimulation of peptide H1_{NBS} kinase activity by phospholipid or phospholipid + diolein was noted, but a 50% stimulation of kinase activity by Ca²⁺ was observed. Ca²⁺ + phospholipid with or without diolein lead to a further 5-fold increase in kinase activity. Further studies are required to characterize the kinases responsible for the phosphorylation of the peptide with no additions or Ca²⁺ addition alone to the kinase assay.

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