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Antipeptide antibodies directed against the C-terminus of protein kinase Cζ (PKCζ) react with a Ca^{2+}- and TPA-sensitive PKC in HT-29 human intestinal epithelial cells

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Abstract

We have studied the PKC isoforms present in HT-29 M6 colon cancer cells, the differentiation of which to mucus-secreting cells is blocked by TPA. In addition to a major 72 kDa band, a 77 kDa PKC isoform was recognized by two different antibodies raised against a C-terminus-specific peptide for the TPA-insensitive isoform, PKCζ. By different criteria (association to the membrane, down-regulation, PKC activity in immunoprecipitates) we conclude that, contrary to the 72 kDa band, the 77 kDa band corresponds to a Ca^{2+}- and TPA-sensitive PKC. These results suggest that antipeptide antibodies directed against the C-terminus of PKCζ react in human cells with a member of the conventional PKC subfamily besides PKCζ. Therefore, the data indicating that PKCζ is sensitive to different agents in various cell lines should be carefully re-evaluated.

Key words: Protein kinase C; Protein kinase C isozyme; Antipeptide antibody; HT-29 cell

1. Introduction

Protein kinase C (PKC) plays a crucial role in the signal transduction mechanisms activated by growth factors, hormones and neurotransmitters [1,2]. Several members of this extended family have been described so far; these protein kinases have been classified in to three different subfamilies [1,2]. The conventional PKC subfamily (cPKC) comprises four members α, βI, βII and γ; these isoforms require Ca^{2+}, phospholipid and diacylglycerol or phorbol ester for activation. The novel PKC subfamily (nPKC), composed of isoforms δ, ε, η and θ, lacks the Ca^{2+}-binding domain and the activity of its members is independent of Ca^{2+} [1–3]. Recently, two other PKC isoforms, ζ and ι, have been gathered into a new subfamily, atypical PKCs (aPKC), in accordance with their insensitivity to diacylglycerol, the natural activator of the other PKCs [1,4].

Antibodies raised against peptides specific for the different isoforms of PKC have been used as a powerful tool to examine the presence of the members of the PKC family in different cells and tissues. Among them, the presence of aPKCζ has received a growing interest since this PKC isoform has been shown to be involved in mitogenesis in several systems [5,6]. C-Terminus-directed antipeptide antibodies have been widely used to study the distribution of this PKC by many groups in human [7–9] and other species [5,10–17]. In this work we demonstrate that these antipeptide antibodies are not specific and also react with a Ca^{2+}- and TPA-sensitive PKC in human intestinal epithelial cells.

2. Materials and methods

2.1. Materials

Phosphatidylserine (PS), 1-oleoyl-2-acetyl-glycerol, histone IIIS, myelin basic protein (MBP), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and phenyl-methyl sulphonyl fluoride (PMSF) were supplied by Sigma (St. Louis, MO). The PKC inhibitor GF109203X was kindly provided by Dr. J. Moscat (CSIC, Madrid). Leupeptin was from Boehringer (Mannheim, Germany). Protein A-agarose was from ICN Biochemicals (Cleveland, OH). DEAE-Sephacel was from Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP was purchased from New England Nuclear. Prestained SDS-PAGE molecular weight (M_w) markers were from Bio-Rad. All the other chemicals were commercial products of the highest grade available.

2.2. Antibodies

The following antibodies were used in our study. Anti-cPKC, antipeptide antibody from Gibco/BRL (Gaithersburg, MD), raised against peptide ILKKDVIVQDDDVD, common to cPKCα (amino acid residues 369–382), cPKCβ (372–385) and cPKCγ (381–394). Anti-cPKCα, monoclonal antibody (mAb) MC5 (Amersham), mapped to residues 312–323 (kindly provided by Dr. D. Colomer, Hospital Clínic, Barcelona). Anti-nPKCε, antipeptide antibody from Gibco, raised against peptide DGFSYFGEDLMP (amino acid residues 726–737). Anti-aPKCζ, antipeptide antibody from Gibco, raised against peptide GFEYINPLLLSAEESV (amino acid residues 577–592). A polyclonal antibody was prepared in our laboratory by immunizing rabbits with this same aPKCζ-specific peptide (synthetized by Dr. D. Andreu, Departament de Química Orgànica, Universitat de Barcelona) coupled to keyhole limpet hemocyanine (KLH) by *m*-maleimido benzoic acid-

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hydroxysuccinimide ester (MBS). Serum was collected according to standard protocols and used at a 1:1,000 dilution, without further purification.

2.3. Cell culture

The HT-29 M6 cell line, originally described and characterized with the name HT-29 (10^{-6} methotrexate) [18] was provided by Dr. Alain Zweibaum (INSERM, Villejuif, France). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco) as previously described [19].

2.4. Preparation of cytosol and membrane cell fractions

Cells were washed twice in buffer A (25 mM Tris HCl, pH 7.6, 1 mM EGTA, 10 mM NaCl) and homogenized on ice in a Douncer in this buffer containing 1 mM PMSF and 25 μg/ml leupeptin. The homogenate was centrifugated at 15,000 × *g* for 30 min at 4°C; the supernatant constituted the soluble cytosolic fraction. The pellet was resuspended in buffer A-Triton (buffer A plus PMSF and leupeptin and made 1% in Triton X-100) by ten strokes in a Douncer homogenizer and allowed to stand on ice for 30 min. After centrifugation under the same conditions as before, the supernatant constituted the detergent-extracted, membrane fraction. Occasionally, total extracts were prepared directly in buffer A plus Triton. In the cases mentioned, these fractions (total homogenate, cytosol or membrane) were purified by chromatography on DEAE-Sephacel: fractions were applied to a DEAE-Sephacel column pre-equilibrated in buffer A and PKCs were eluted in this same buffer made 150 mM in NaCl.

To separate the 77 and 72 kDa bands an extract was prepared under the following conditions. Cells were washed twice with buffer B (25 mM Tris-HCl, pH 7.6, 10 mM NaCl, 3 mM $CaCl_2$) and homogenized in 25 ml of buffer B containing 1 mM PMSF and 25 μg/ml leupeptin by 50 strokes in a Douncer. After 30 min on ice, the homogenate was centrifugated for 30 min at 15,000 × *g*. The pellet was washed once with the same buffer and spun under the same conditions. After this second centrifugation, the pellet was resuspended in 10 ml of buffer C (25 mM Tris-HCl, pH 7.6, 10 mM NaCl, 4 mM EGTA) plus PMSF and leupeptin by ten strokes in a Douncer, and allowed to stand on ice for 30 min. After centrifugation (same conditions as before), the supernatant constituted the EGTA wash fraction. This fraction was usually purified by chromatography on DEAE-Sephacel as mentioned above.

2.5. PKC activity

PKC activity was assayed under the conditions described [20] using lysine-rich histone (histone III-S) or MBP (0.5 mg/ml in both cases) as substrates and immunoprecipitates as the source of the enzyme. One unit of activity was defined as one pmol of phosphate incorporated into the substrate per minute. Autophosphorylation reactions were carried out under the same protein kinase conditions except that exogenous substrates were omitted and ATP was used at 2 μM with a specific activity of 5×10^4 cpm/pmol. Phosphorylation of the 77 kDa band was detected after SDS-PAGE in 10% gels and autoradiography.

2.6. Other methods

Western blot analysis was performed exactly as described [19]. Protein concentration was determined by the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as standard.

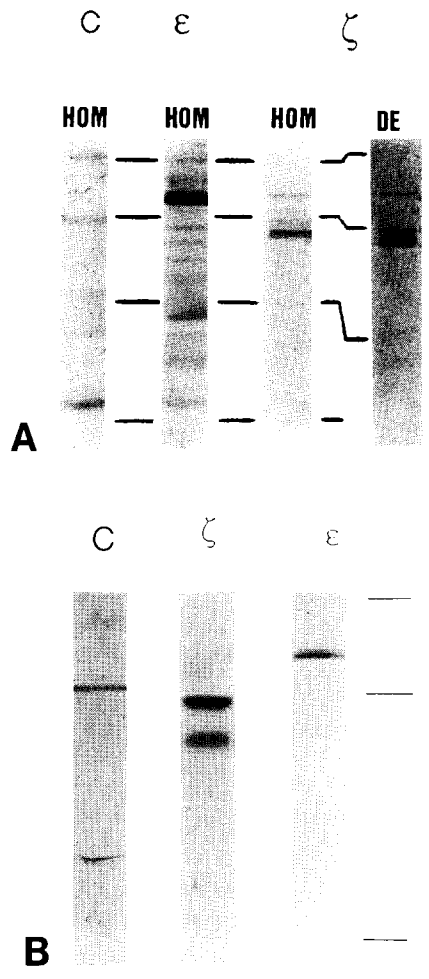

Fig. 1. Western blot analysis of HT-29 M6 PKC isoforms. Cells were homogenized in buffer A plus 1% Triton X-100, 1 mM PMSF, 25 μg/ml leupeptin as described in section 2. (A) Total cell homogenates (300 μg of protein) (HOM) or PKCs purified by DEAE-Sephacel chromatography (13 μg) (DE) were analyzed by SDS-PAGE in 10% polyacrylamide gels and immunoblotted with antibodies specific for cPKCs, aPKCζ and nPKCε as described. The bars indicate the position of the 116, 80, 49 and 32 kDa molecular weight markers. (B) DEAE-Sephacel-purified PKC fraction (DE-150) (15 μg) was subjected to SDS-PAGE on 10% gels, over-running the gels until the pre-stained 49 kDa molecular weight marker was 1 cm from the bottom. Proteins were transfered and analyzed with the antibodies against the different isoforms of PKC as mentioned. Only the 116, 80 and 49 kDa molecular weight markers are shown.

3. Results

The phorbol ester TPA has been shown to produce remarkable morphological changes in HT-29 M6 cells, inhibiting their differentiation to a mucus-secretive phenotype [19] and altering cell-cell contacts [21]. As a first step to characterize the mechanism by which TPA exerts these effects, we have studied the presence of the different PKCs in HT-29 M6 cells, using antibodies raised against synthetic isoform-specific peptides. These antibodies, purchased from Gibco, have been used previously in similar studies by several groups in different cellular systems [7–17]. The analysis by immunoblot of total cell extracts showed that HT-29 M6 cells contain abundant nPKCε and aPKCζ, and low levels of cPKC (Fig. 1A). The antipeptide antibody used in this first assay did not distinguish between the different isoforms of this subfamily; however, cPKCα was detected when blotting with the specific mAb MC5 (not shown). The immunoblot was repeated with PKCs partially purified by chromatography on DEAE-Sephacel. Results obtained with this fraction were similar to those obtained with extracts concerning cPKC or nPKCε, however, the pres-

ence of a band of slightly higher molecular weight than the major PKCζ form was clearly observed in this fraction (Fig. 1A). This band was faint in total homogenates but was enriched during this purification step. In order to better separate the two bands, SDS-PAGE was performed over-running the 10% polyacrylamide gels. The two bands observed with the anti-aPKCζ antibody were clearly separated (Fig. 1B); their estimated molecular weights corresponded to 77 and 72 kDa.

The response of the different PKC isoforms to TPA addition was examined. cPKC and PKCε were rapidly degraded after TPA stimulation of HT-29 M6 cells (not shown). The two bands detected with the aPKCζ antiserum responded differently to TPA; while the 72 kDa form was not affected, the 77 kDa form was almost completely down-regulated after 24 h of incubation with this phorbol ester (Fig. 2A). The effect of TPA on the cellular distribution of these forms was also studied. When cell extracts were prepared in the presence of EGTA, both forms were mainly detected in the cytosol fraction (Fig. 2B). A similar distribution of aPKCζ has been observed in other systems [11,14,22]. TPA induced a rapid and complete translocation of the 77 kDa form, but not the 72 kDa form, from the soluble to the detergent-solubilized fraction, presumably membranes (Fig. 2B). The same results were obtained in three independent experiments. The translocation of the 77 kDa was complete in 15 min; no changes in the total cellular levels of this form were detected within 6 h of TPA addition (data not shown).

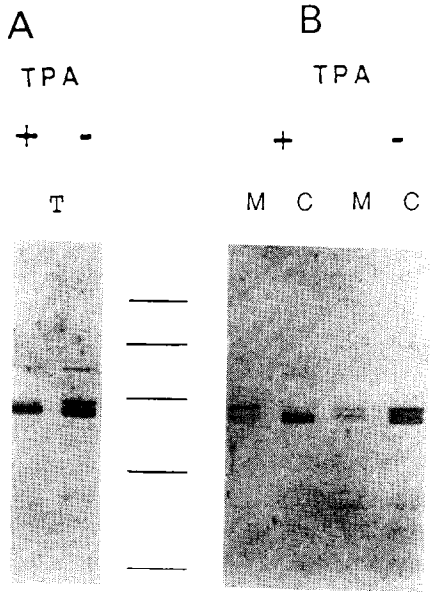

Fig. 2. The 77 kDa PKC is sensitive to TPA. HT-29 M6 cells were grown in complete DME medium supplemented with 10% FBS. Cells were incubated with TPA (400 nM) or the vehicle alone (DMSO) for 24 h (panel A) or 1 h (panel B). Cells were harvested and total cellular homogenates (T) (panel A) or cytosolic (C) and membrane (M) fractions (panel B) were prepared as described in section 2. After purification by DEAE-Sephacel chromatography, PKC forms were analyzed as mentioned in Fig. 1 with the antibody raised against the C-terminus of PKCζ. The bars indicate the position of the 205, 116, 80, 49 and 32 kDa pre-stained molecular weight markers. This figure shows the results of a representative experiment of a total of five performed.

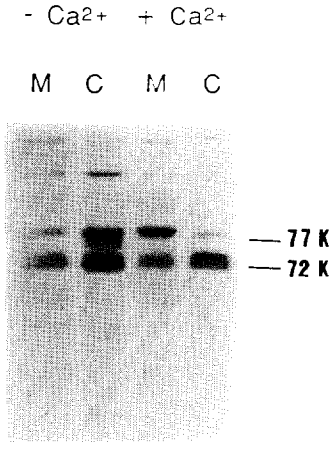

Fig. 3. The 77 kDa PKC form associates with the membrane in the presence of Ca^{2+}. Cells were homogenized either in the presence of Ca^{2+} (buffer B plus 1 mM PMSF and 25 μg/ml leupeptin) or in the presence of EGTA (buffer A plus these protease inhibitors) with a Douncer and centrifuged as described; the supernatant constituted the cytosolic fraction (C). The pellet was extracted with buffers A or B (plus protease inhibitors) supplemented with 1% Triton X-100; after centrifugation the supernatant constituted the membrane fraction (M). After chromatography in DEAE-Sephacel, the presence of the 77 kDa band was analyzed as mentioned.

The characteristics of this TPA-sensitive PKCζ form were studied in greater detail. First, its association with the membrane fraction was examined. As already mentioned, when cell extracts were prepared in the presence of EGTA, both the 72 and 77 kDa PKC forms were detected mainly in the cytosol fraction. However, when extracts were obtained in the presence of Ca^{2+} (3 mM), the 77 form kDa was predominantly associated to the Triton-solubilized membrane fraction (Fig. 3). This stronger association to the membrane in the presence of Ca^{2+} suggested that the antibody raised against the C-terminus of aPKCζ was recognizing a Ca^{2+}-dependent PKC isoform as well.

To further study the characteristics of the 77 kDa enzyme, PKC kinase activity was determined in immunoprecipitates obtained with the antipeptide antibody from fractions devoided of 72 kDa aPKCζ. This fraction (EGTA wash) was prepared by taking advantage of the different associations to the membrane of both PKCs (see above). After chromatography in DEAE-Sephacel, the 77 kDa PKC form was immunoprecipitated with the C-terminus aPKCζ antipeptide antibody. PKC activity was observed when MBP was used as the substrate (Fig. 4A); this PKC phosphorylated histone H1 with much lower affinity (not shown). Maximal activity was observed in the presence of PS, TPA and Ca^{2+} (Fig. 4A); this ion stimulated the PKC activity in these conditions (plus PS and TPA) from 40 to 100% in different experi-

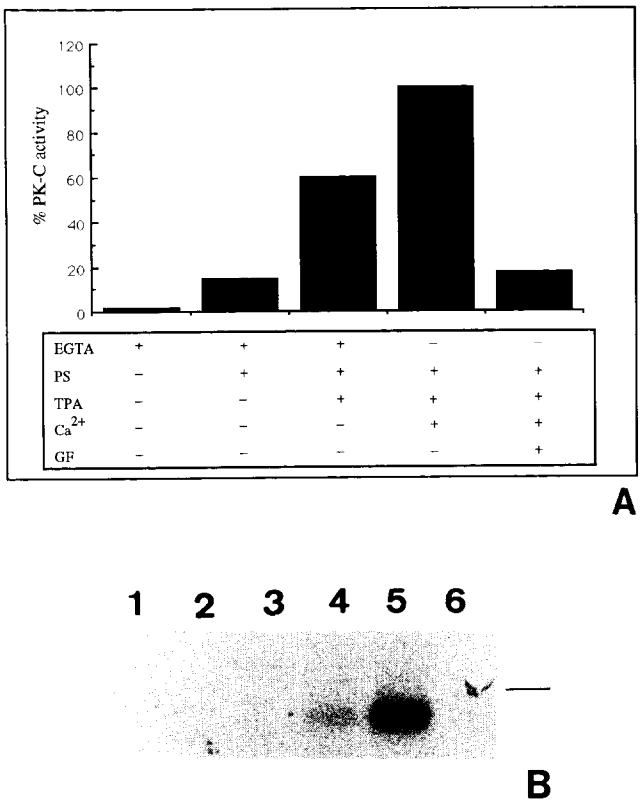

Fig. 4. Autophosphorylation and MBP kinase activity in 77 kDa PKC immunoprecipitates. 200 μl (150 μg) of EGTA wash fraction, purified by DEAE-Sephacel, was incubated with anti-PKCζ antibody (1:200) for 12 h at 4°C in the presence of leupeptin (25 μg/ml). Immunocomplexes were recovered with protein A-Sepharose, washed and assayed for the MBP kinase (A) or autophosphorylation (B) assays under the conditions indicated. Essentially identical results were obtained in three independent experiments. Panel A, lanes: 1, EGTA (1 mM); 2, EGTA and PS (80 μg/ml); 3, PS, EGTA and TPA (400 nM); 4, PS, TPA and Ca^{2+} (10 μM); and 5, PS, TPA, Ca^{2+} and GF (1 μM). Panel B, lanes: 1, EGTA; 2, EGTA and PS; 3, EGTA, PS and TPA; 4, PS, TPA and Ca^{2+} (10 μM); 5, PS, TPA and Ca^{2+} (50 μM); and 6, PS, TPA, Ca^{2+} (50 μM) and GF. To determine the free Ca^{2+} in the assay, an apparent association constant of 2×10^6 M^{-1} for the Ca^{2+}–EGTA complex was used.

ments. DAGs like 1-oleoyl-2-acetyl-glycerol stimulated the activity to a similar degree as TPA (not shown). The PKC-specific inhibitor GF [23] completely blocked this activity at a concentration of 0.5 μM (Fig. 4A). Similar results were obtained when the autophosphorylation of 77 kDa band was examined in immunoprecipitates. Autophosphorylation was even more dependent on the presence of Ca^{2+} than the MBP kinase activity and was also blocked by the inhibitor GF (Fig. 4B).

These results suggest that the PKCζ antipeptide antibody was reacting with a cPKC as well. In order to check that these results were not an artifact of a particular batch of the antibody, the Western blot analysis was repeated with two other different batches. No differences were observed; a 77 kDa band was always present in HT-29 M6 cells extracts. Moreover, an antibody directed against the C-terminus of PKCζ was prepared in our lab; the peptide corresponding to residues 577–592 from rat brain PKCζ was synthetized, coupled to KLH using MBS, and used to immunize rabbits. The antiserum obtained showed identical reactivity to Gibco's; in addition to a predominant 72 kDa band, it recognized and immunoprecipitated a 77 kDa band with Ca^{2+}- and TPA-dependent PKC activity (data not shown).

4. Discussion

In this article we show that antipeptide antibodies, prepared against a peptide corresponding to the C-terminus of aPKCζ, recognize, in addition to a 72 kDa band (presumingly aPKCζ), another band of 77 kDa in HT-29 M6 cells. This band corresponds to a cPKC since it is translocated and down-regulated after addition of TPA to the cells and, when immunoprecipitated by this antibody, presents a MBP kinase activity dependent on Ca^{2+}, phospholipid and TPA.

When the sequences of the different PKCs are compared, the aPKCζ C-terminus peptide (amino acids 577–592) was only observed in this isoform; no analogies with this peptide were found in other PKCs. For this reason this peptide was chosen to raise PKCζ-specific antibodies by Gibco and several authors, and used by many groups to determine the presence of this isoform in human [7–9] and other species [10–17]. Very recently, it has been reported that this antipeptide antibody also recognizes a newly described PKC, aPKCι [4]. This isoform is clearly different from the 77 kDa cPKC since it has a lower molecular weight and does not respond to TPA or Ca^{2+} [4].

Our results suggest that the use of these antibodies as the only tool to examine the presence of PKCζ in different cell systems could lead to erroneous conclusions. This finding is especially relevant because there is not a simple way to identify PKCζ among the different bands that show up on an immunoblot since (i) this PKC isoform does not respond to TPA by translocation or down-regulation like the rest of the PKCs, and (ii) the molecular weight of PKCζ has been shown to be substantially different in various cell systems, oscillating in a range from 68 to 80 kDa [3,7,14,16,24]. Additional analysis with other PKCζ-specific antibodies would be required in order to verify the presence of this isoform in different systems.

The identity of the cPKC isoform recognized by the antibody is still a matter of study. Previous experiments from our laboratory suggest that the 77 kDa band is different from the α or β cPKCs since its behaviour in columns of hydroxyapatite is not the same. A a more definitive answer to this question would require the isolation and sequencing of cDNAs coding for this PKC isoform.

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