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Stratifin, a keratinocyte specific 14-3-3 protein, harbors a pleckstrin homology (PH) domain and enhances protein kinase C activity

Elena Dellambra[1], Mauro Patrone[2], Bianca Sparatore[2], Armando Negri[3], Fabrizio Ceciliani[3], Sergio Bondanza[1], Francesco Molina[1], Fiorella Descalzi Cancedda[1,4] and Michele De Luca[1,*]

[1]National Cancer Institute/Advanced Biotechnology Center, Viale Benedetto XV no. 10, 16132, Genova, Italy
[2]Institute of Biological Chemistry, University of Genova, Genova, Italy
[3]Institute of Veterinary Physiology and Biochemistry, University of Milano, Milano, Italy
[4]International Institute of Genetics and Biophysics, Napoli, Italy

*Author for correspondence

SUMMARY

The intrinsic signal(s) responsible for the onset of human keratinocyte terminal differentiation is not yet fully understood. Evidence has been recently accumulated linking the phospholipase-mediated activation of protein kinase C to the coordinate changes in gene expression occurring during keratinocyte terminal differentiation. Here we report the purification of a keratinocyte-derived protein enhancing protein kinase C enzymatic activity. The stimulator eluted as a peak with estimated molecular mass of approximately 70 kDa, while analysis by SDS-PAGE showed a 30 kDa protein migrating as a distinct doublet, suggesting the formation of a 30 kDa homodimer. The amino acid sequence analysis allowed the unambigous identification of the protein kinase C stimulator as a mixture of the highly homologous σ (stratifin) and ζ isoforms of 14-3-3 proteins, which are homodimers of identical 30 kDa subunits. Mono Q anion exchange chromatography and immunoblot analysis further confirmed that stratifin enhances protein kinase C activity. Stratifin was originally sequenced from a human keratinocyte protein database, but its function was unknown. The pleckstrin homology domain has been recently related to protein translocation to the cell membrane as well as to functional interactions of intracellular proteins involved in signal transduction. We show here that stratifin (and 14-3-3 ζ) harbors a pleckstrin homology domain, and the consequent functional implications will be discussed.

Key words: keratinocyte, protein kinase C, PH domain, 14-3-3 protein, signal transduction, epidermis, melanocyte

INTRODUCTION

Several classes of growth factors and cytokines impinging on cells have their effects amplified by a complex network of protein phosphorylation and dephosphorylation leading to regulation of gene expression (Clemens et al., 1992). Among these pathways, glycerophospholipid breakdown is of particular relevance (for recent review, see Liscovitch and Cantley, 1994). Members of the phospholipase C (PLC) family, such as PLC-β, PLC-γ and PLC-δ, can be stimulated by the activation of G proteins (as for PLC-β1-3), or by tyrosine kinase receptors (as for PLC-γ). The PLC-β and -γ mediated phosphatidylinositol (PtdIns) breakdown generates inositoltrisphosphate (IP_3) and diacylglycerol (Liscovitch and Cantley, 1994), which can also be generated by phospholipase C and D mediated hydrolysis of phosphatidylcholine (see Exton, 1990, for review). In turn, diacylglycerol activates a group of serine/threonine kinases, the protein kinase C family, which includes at least eleven different isoforms and represents the major class of downstream targets for lipid second messengers (see Asaoka et al., 1992; Azzi et al., 1992; Dekker and Parker, 1994; Liscovitch and Cantley, 1994, for reviews). Indeed, phospholipase A_2 generates *cis*-unsaturated fatty acids and lysophosphatidylcholine which can activate protein kinase C as well (Asaoka et al., 1992; Liscovitch and Cantley, 1994). Once protein kinase C is activated, the enzyme associates with the cell particulate fraction via RACKs (receptor for activated C-kinase) (Mochly-Rosen et al., 1991a,b), and it phosphorylates serine/threonine residues of a wide range of substrates including the myristoylated alanine-rich C kinase substrates (MARCKS), the GTPase-activated protein-43, the NF-κB transcription factor binding protein I-κB, and the amiloride-sensitive Na^+/H^+ exchanger (Azzi et al., 1992). Despite the large amount of data available on the extracellular-mediated regulation of protein kinase C, little is known about intracellular polypeptides, acting in concert with phospholipids and metal ions (Sando et al., 1992), as co-factors in modulating protein kinase C activity.

Human epidermis survives through a self-renewal process envisaging the coordinate proliferation of clonogenic, basonuclin positive, basal keratinocytes (Barrandon and Green, 1987a; Tseng and Green, 1994; Rochat et al., 1994), and their subsequent differentiation and upward migration to replace terminally differentiated cornified cells (Green, 1980; Watt, 1989;

Fuchs, 1990). In culture (Rheinwald and Green, 1975; Green et al., 1979), clonogenic keratinocytes attach to the substrate through specific integrin receptors and polarize (De Luca et al., 1990a). Cells at the periphery of the colony migrate outwards and grow under the influence of specific growth factors (Green, 1980; Barrandon and Green, 1987b; Di Marco et al., 1993a,b), while keratinocytes located in the center of the colony and committed to terminal differentiation loose their adhesive machinery (De Luca et al., 1990a; Adams and Watt, 1990; Jones and Watt, 1993), increase in size, migrate upwards and express differentiation markers such as keratins 1 and 10 (Fuchs, 1990), involucrin (Watt and Green, 1981), loricrin (Meherel et al., 1990), filaggrin (Steinert et al., 1981), cornifin (Kartasova et al., 1988) and sciellin (Kvedar et al., 1992). Keratinocyte colonies will eventually fuse, giving rise to a stratified squamous epithelium closely resembling normal human epidermis, and suitable for autologous and permanent grafting onto patients (Green et al., 1979; Green, 1980; Gallico et al., 1984; De Luca et al., 1988, 1989, 1990b; Romagnoli et al., 1990).

The protective barrier function of resting epidermis (Green, 1980) as well as the profound modification of the keratinocyte behaviour during the healing of wounds (Zambruno et al., 1995), stem from the precise and tightly regulated balance between keratinocyte migration, growth and differentiation. Although the onset of terminal differentiation has been recently linked to the loss of basal keratinocyte adhesive properties in humans (Adams and Watt, 1990), and to Ca^{2+} concentrations in mice (Dlugosz and Yuspa, 1993, 1994), the intrinsic signal(s) inducing human keratinocyte differentiation and basal cell departure from the basal layer are not yet fully understood. In the last decade strong evidence has been accumulated linking the phospholipase-mediated activation of protein kinase C to the coordinate change in gene expression occurring during keratinocyte terminal differentiation in mice (see Dlugosz and Yuspa, 1993, for complete references). Indeed, murine keratinocyte terminal differentiation is associated with changes in phospholipase C isoenzymes (Punnonen et al., 1993), and protein kinase C membrane translocation and enzymatic activation lead to stimulation of epidermal transglutaminase activity (Dlugosz and Yuspa, 1994) and cornified envelope formation (Dlugosz and Yuspa, 1993).

In an attempt to identify human keratinocyte intracellular regulators of protein kinase C, we have isolated and purified to homogeneity stratifin, an acidic homodimeric protein of unknown function (Leffers et al., 1993), and the 14-3-3 ζ isoform, both belonging to the 14-3-3 protein family (see Aitken et al., 1992, and Morrison, 1994, for reviews). We found that keratinocyte-derived stratifin harbors a pleckstrin homology (PH) domain and enhances protein kinase C activity.

MATERIALS AND METHODS

Materials and reagents

DEAE-cellulose was from Whatman (Maidston, UK). Sephadex G200, threonine-Sepharose 4B, Superdex 75 HR10/30, Mono Q HR 5/5 and ampholines (pH 4.0-6.0) were from Pharmacia (Uppsala, Sweden). Spherogel TSK2000SW was from Beckman (Berkeley, CA, USA). HPLC and FPLC were performed on a Beckman gradient liquid chromatograph equipped with a 421A system controller, 110B pumps, 210A injection valve, and 163 variable wavelength detector. The recorder was a Shimadzu C-R3A Chromatopac. Phosphatidylserine (PtdSer), diacylglycerol, histone (type III-S), leupeptin, PMSF, aprotinin, HEPES and TES buffers were from Sigma (St Louis, MO, USA). ATP was from Fluka (Buchs, Switzerland). [γ^{32}P]ATP (3,000 Ci/mmol) was from Amersham Corp. (UK). Micro-BCA protein assay was from Pierce (Rockford, Illinois, USA). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA) and HPLC reagents from BDH (Poole, Dorset, UK). Immobilon P membrane was from Millipore (Bedford, MA, USA).

Rabbit antiserum specific for 14-3-3 proteins was a gift from Dr J. Celis (Danish Centre for Human Genome Research, Aarhus, Denmark). Rabbit antisera specific for 14-3-3 isoforms were a gift from Dr Alastair Aitken (National Institute for Medical Research, London, UK)

Purification of protein kinase C and assay for protein kinase C activity

Protein kinase C was purified from adult rat brain and its activity was assayed as previously described (Kikkawa et al., 1986; Go et al., 1987).

Briefly, brain homogenates were pooled, lysed in 0.25 M sucrose, 10 mM Hepes, 10 mM mercaptoethanol, 5 mM EDTA, 0.1 mg/ml leupeptin, 2 mM PMSF, sonicated and centrifuged for 40 minutes at 100,000 ***g***. All operations were carried out at 4°C. The supernatant was collected and loaded onto a DEAE-52 column (1 cm × 10 cm) equilibrated in buffer A (10 mM Hepes, 10 mM mercaptoethanol, 1 mM EDTA). Bound proteins were eluted by application of a linear NaCl gradient (0-0.2 M) in buffer A; 20 μl of each fraction were assayed for protein kinase C activity. The peak containing protein kinase C activity was concentrated by ultrafiltration using YM10 membrane (Amicon, Beverly, MA, USA) and dialysed against buffer B (10 mM Hepes, 10 mM mercaptoethanol, 1 mM EDTA, 10% glycerol). The concentrated pool was then loaded onto a threonine-Sepharose 4B column (0.5 cm × 2 cm) equilibrated in buffer B and the proteins were eluted by a linear NaCl gradient (0-0.2 M); 20 μl of each fraction were assayed for protein kinase C activity.

Protein kinase C activity was assayed by incubating appropriate amounts of purified protein kinase C in 25 mM sodium borate, pH 7.5, 5 mM $MgCl_2$, 100 μg of histone III-S, 4 μg of PtdSer, 1 μg of diacylglycerol, 0.5 mM $CaCl_2$, 10 μM ATP, 0.5 μCi of [γ^{32}P]ATP. The mixture was incubated for 10 minutes at 30°C and the reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. For each fraction, 1 mg of bovine serum albumin was added and the mixture was washed 3 times by addition of 5 ml of 5% (w/v) trichloroacetic acid and filtered through a glass microfiber filter. Phosphate incorporation into substrate was measured by counting the adsorbed radioactivity. One unit of protein kinase C activity was defined as the amount causing the incorporation of 1 nmol of ^{32}P into histone III-S (Melloni et al., 1985).

Cell culture

3T3-J2 cells (a gift from Dr Howard Green, Harvard Medical School, Boston, MA, USA) were cultured in Dulbecco-Vogt Eagle's medium (DMEM) containing calf serum (10%), glutamine (4 mM) and penicillin-streptomycin (50 i.u./ml) and were free of mycoplasma contamination.

Human epidermal keratinocytes were obtained from skin biopsies of healthy volunteers and cultivated on a feeder-layer of lethally irradiated 3T3-J2 cells as described (Green et al., 1979). In brief, skin biopsies were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 hours. Cells were collected every 30 minutes, plated (2.5×10^4/cm^2) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2) and cultured in a 5% CO_2 humidified atmosphere in keratinocyte growth medium: DMEM and Ham's F12 media (3:1 mixture) containing fetal calf serum (FCS, 10%), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (CT,

0.1 nM), triiodothyronine (2 nM), epidermal growth factor (EGF, 10 ng/ml), glutamine (4 mM), penicillin-streptomycin (50 i.u./ml). Subconfluent primary cultures were passaged in secondary cultures as described (De Luca et al., 1988). 3T3 cells and keratinocytes were free of mycoplasma contamination.

Purification and amino acid and sequence analysis of the protein kinase C stimulator

Step 1

The protein kinase C stimulatory activity was purified from subconfluent secondary cultures. Keratinocytes were trypsinized, washed twice in DMEM, homogenized in 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) buffer, pH 7.4, containing 10% sucrose and protease inhibitors (10 mg/ml leupeptin, 0.2 U/ml aprotinin). The lysate was centrifuged for 60 minutes at 100,000 ***g*** and the supernatant was concentrated by ultrafiltration.

Step 2

The keratinocyte lysate was loaded onto a Sephadex G200 (1.5 cm × 115 cm) column equilibrated in PBS, pH 7.4; the column was eluted at 100 μl/minute and 2 ml fractions were collected; 20 μl of each fraction were assayed for protein kinase C activity. The stimulator was pooled, concentrated and dialysed against 20 mM sodium phosphate buffer, pH 7.

Step 3

The sample was loaded onto a DEAE-cellulose (DE52) column (1 cm × 2 cm) equilibrated in 20 mM sodium phosphate buffer, pH 7, and the column was washed until the absorbance at 280 nm was below 0.005. The column was then eluted by a NaCl linear gradient (0-0.6 M) at 1 ml/minute and 1 ml fractions were collected; 20 μl of each fraction were assayed for protein kinase C activity. The stimulator was pooled, concentrated by ultrafiltration and dialysed against PBS buffer, pH 7.4, overnight.

Step 4

Aliquots (250 μl) of sample were loaded onto a TSK 2000SW HPLC column (7.5 mm × 600 mm) equilibrated in PBS buffer, pH 7.4; the column was washed at 0.5 ml/minute and 0.5 ml fractions were collected; 100 μl of each fraction were assayed for protein kinase C. The size exclusion chromatography was repeated several times and fractions containing the stimulator were pooled and concentrated by ultrafiltration.

Step 5

Aliquots (250 μl) of sample were loaded onto a Superdex 75 HR10/30 FPLC column (10 mm × 300 mm) equilibrated in PBS buffer, pH 7.4; the column was washed at 1 ml/minute and 0.5 ml-fractions were collected; 100 μl of each fraction were assayed for protein kinase C activity and the stimulator was concentrated by ultrafiltration and dialysed against 20 mM Tris-HCl buffer, pH 7.5.

Step 6

Anion-exchange chromatography: 300 μl of FPLC-derived stimulator sample were loaded onto a Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl buffer, pH 7.5. Bound proteins were eluted by application of NaCl linear gradient (0-1 M) at 0.5 ml/minute for 60 minutes and 0.5 ml fractions were collected. The chromatography was repeated several times; corresponding fractions were pooled and dialysed against PBS buffer, pH 7.4; 50 μl of each fraction were assayed for protein kinase C stimulatory activity.

Protein concentrations were determined using the Bradford protein assay, the micro BCA protein assay and, when indicated, amino acid analysis.

Amino acid analysis was performed as described (Cohen and Michaud, 1993), following vapor-phase hydrolysis in 6 M HCl, 0.1% phenol at 110 °C for 24 hours. For sequence analysis, HPLC separation of peptides and amino acid sequences were performed as described (Negri et al., 1992). In order to obtain internal amino acid sequences, the protein was submitted to CNBr fragmentation using a 100-fold molar excess of CNBr over methionyl residues in 70% formic acid at room temperature for 24 hours, followed by removal of excess reagent under a stream of N_2.

SDS-PAGE, isoelectrofocusing, PVDF blot and immunoblot

Samples were precipitated with 10% (v/v) trichloroacetic acid, resuspended in 20 μl of 0.06 M Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, in the presence or in the absence of 2% 2-mercaptoethanol, heated for 3 minutes at 100°C and electrophoresed on 12.5% polyacrylamide SDS-gels in Laemmli buffer. Gels were silver stained. For isoelectrofocusing, samples were applied to a preformed pH gradient (4.0-6.0) in urea and focused to equilibrium. Gels were soaked in 11.5% trichloroacetic acid, 0.136 M 5-sulfosalicylic acid, and silver stained. The pH gradient was determined by cutting the gel into 1 cm strips and measuring the pH of each strip in 1 ml of milliQ distilled water. For polyvinylidene difluoride (PVDF) blots, samples (200 pmol) were electrophoresed onto 12.5% polyacrylamide SDS-gels and blotted onto PVDF membranes (Immobilon-P, Millipore) as described (Matsudaira, 1987). Blots were stained with Coomassie Blue and bands were directly processed for amino acid analysis and sequence. For immunoblots, samples were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose filters. Filters were soaked in 3% non fat dry milk/TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) at 4°C overnight. Rabbit antisera recognizing all 14-3-3 proteins or specific isoforms were added (1:200-1:1,000) and filters were incubated for 2 hours at room temperature. Filters were washed three times with TTBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20), incubated for 1 hour with sheep anti-rabbit alkaline phosphatase IgG (1:2,000), and extensively washed with TTBS. The phosphatase substrate 4-nitrophenylphosphate was then added to visualize specific bands.

RESULTS

Protein kinase C was prepared from adult rat brain as described in Materials and Methods. Its activity was assayed using the standard C-kinase activation assay involving incorporation of ^{32}P radioactivity from [^{32}P]ATP into histone III-S (see Materials and Methods). Preliminary experiments have shown that extracts prepared from sub-confluent human keratinocytes, from different cell strains in primary or secondary culture, stimulate protein kinase C-dependent histone phosphorylation (not shown). To elicit its activity, the keratinocyte extract required the presence of phosphatidylserine (PtdSer), diacylglycerol and 0.5 mM $CaCl_2$.

Purification of protein kinase C stimulator

The keratinocyte-derived protein kinase C stimulator was purified by sequential chromatography and ion-exchange analysis, as described in Materials and Methods. Fig. 1 shows the position of two peaks of protein kinase C stimulatory activity eluted from four Sephadex G200 columns loaded with extracts prepared from four different normal human keratinocyte strains in sub-confluent secondary culture. The first peak eluted in the void volume, while the second peak eluted as a protein of approximately 70 kDa in molecular mass (Fig. 1, hatched areas). The second peak was further analyzed by sequential DEAE-cellulose, HPLC and FPLC columns. As

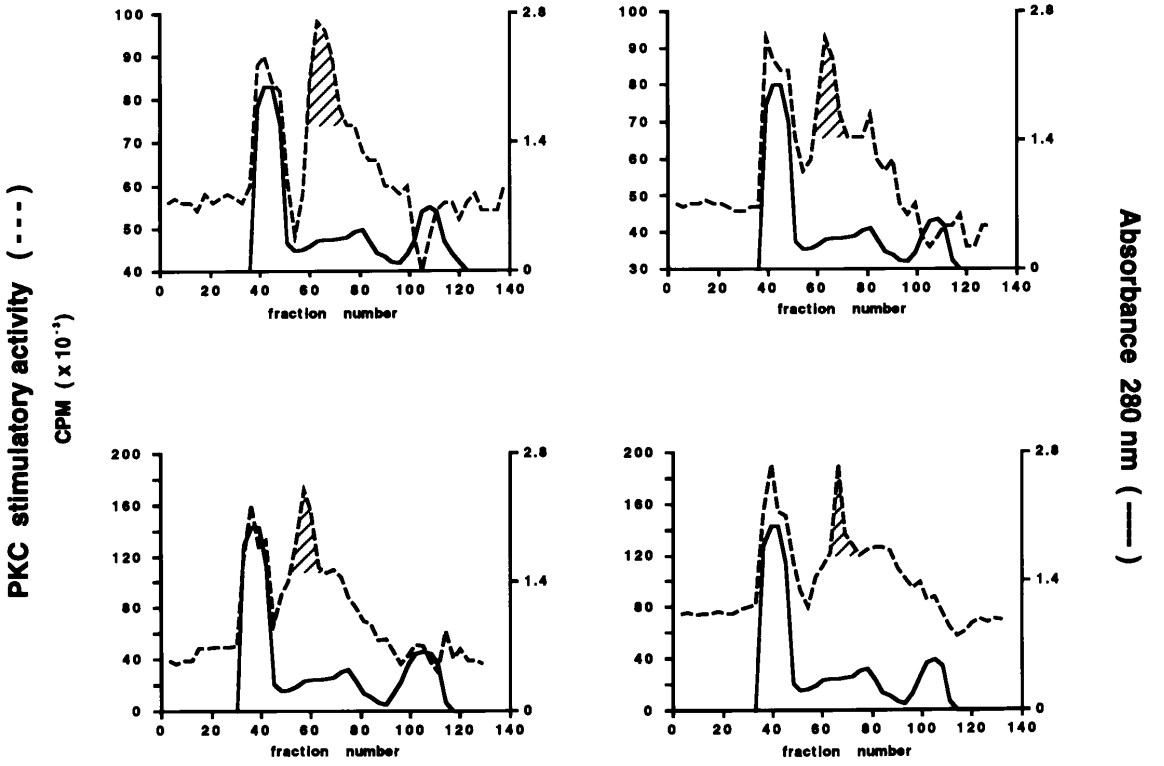

Fig. 1. Sephadex G200 chromatography. Keratinocyte lysates, prepared from four different normal human keratinocyte strains, were loaded onto Sephadex G200 columns and eluted as described; 20 µl of each fraction were assayed for protein kinase C activity. The stimulatory activity detected in the 70 kDa region (hatched area) was further characterized.

shown in Fig. 2 (hatched area), a broad peak of protein kinase C stimulatory activity eluted from the DEAE-cellulose column at approximately 0.2 M NaCl. When analyzed by size-exclusion chromatography (TSK 2000SW HPLC), the dialysed DEAE-derived protein kinase C stimulator eluted as two discrete peaks with estimated molecular masses of 70 kDa and 30 kDa respectively (Fig. 3A, hatched areas). The 70 kDa peak was always more abundant, and in some experiments the 30 kDa peak was barely detectable. Analysis by SDS-PAGE on a 12.5% polyacrylamide slab gel, followed by silver staining (Fig. 3B), showed that both the 70 kDa (lanes 1 and 2) and the 30 kDa (lane 5) peaks had identical electrophoretic mobilities and an apparent molecular mass of 30 kDa. The electrophoretic mobility was not affected by the presence of 0.25 M 2-mercaptoethanol, suggesting that the 70 kDa peak represents a non disulfide-linked dimer of the 30 kDa protein. When loaded on an FPLC (Superdex 75 HR10/30) column (Fig. 4A), the concentrated peak of HPLC-derived protein kinase C stimulator eluted as a single peak (coincident with the major peak of 280 nm absorbance) of 70 kDa in molecular mass. Analysis of the FPLC peak by SDS-PAGE, performed both under non-reducing (Fig. 4B, lanes 1-4), and reducing (not shown) conditions, showed the presence of a 30 kDa protein migrating as a distinct doublet, further suggesting the formation of a 30 kDa dimer generating the 70 kDa protein observed in both the HPLC and FPLC columns. The absence of disulfide bonds was further confirmed by lack of molecular mass shift when the protein kinase C stimulator was eluted from size-exclusion chromatography columns under reducing conditions (not shown). Isoelectric focusing showed that the pI of the FPLC-eluted protein kinase C stimulator is approximately 4.5 (Fig. 4A, inset). The FPLC-eluted protein kinase C stimulator was also analysed on native gels, where it migrated as a single band

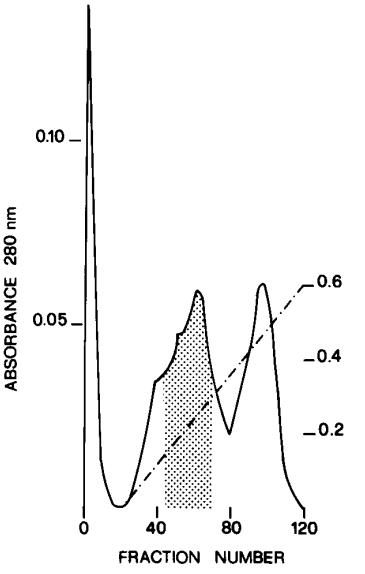

Fig. 2. DEAE-52 ion-exchange chromatography. Stimulatory activity from Sephadex G200 was applied onto a DEAE-52 column after desalting. Bound proteins were eluted by a NaCl linear gradient (0-0.6 M) and a broad peak of stimulatory activity was detected at approximately 0.2 M NaCl (hatched area). The concentration of protein giving a 2-fold increase in protein kinase C activity was 0.18 mg/ml.

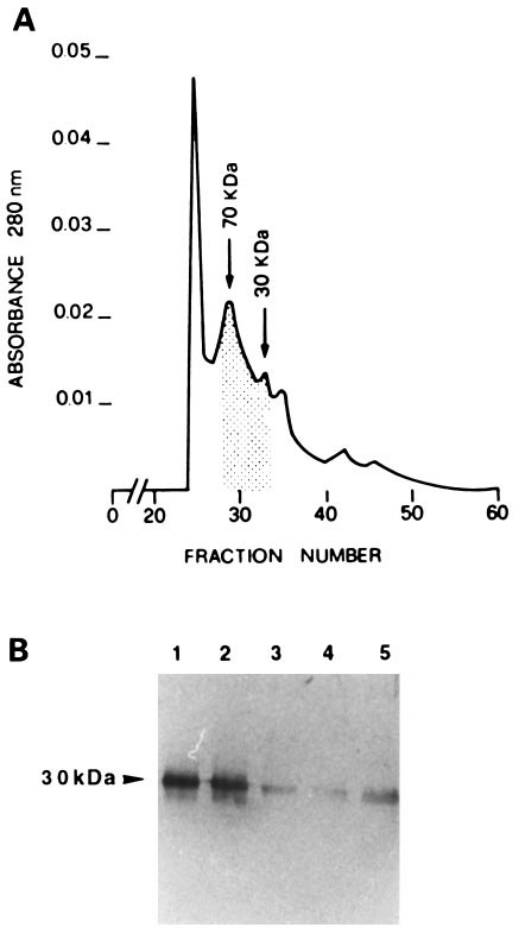

Fig. 3. HPLC size-exclusion chromatography and SDS-PAGE. DEAE-52-derived protein kinase C stimulator was loaded onto Spherogel TSK 2000SW columns and eluted as described. Two discrete peaks of protein kinase C stimulatory activity, with estimated molecular mass of 70 and 30 kDa, respectively (A), were eluted; 100 μl of each fraction were analysed by SDS-PAGE on a 12.5% gel and stained with silver (B). The concentration of protein giving a 2-fold increase in protein kinase C activity was 0.09 mg/ml.

(not shown). The protein was extracted from the native gel and maintained its protein kinase C stimulatory activity. The 30 kDa doublet was the only protein visualized in silver stained SDS-PAGE gels of FPLC-eluted protein kinase C stimulator, even when the gels were overloaded. However, in some preparations of FPLC a small amount of co-purified protein (≤3% of total protein), migrating as a 58-60 kDa band, was detected (see Fig. 4C, at arrow). By sequence analysis, this co-purified protein was identified as calreticulin (see Michalak et al., 1992 for a recent review), a high affinity Ca^{2+} binding protein unrelated to protein kinase C, confined to the cell endoplasmic reticulum compartment where it functions as a Ca^{2+} storage protein.

Activity of the protein kinase C stimulator

The purified protein lost its protein kinase C stimulatory activity after heating for 3 minutes at 100°C. The possibility that the protein kinase C stimulator was itself a substrate for protein kinase C was ruled out by the lack of ^{32}P incorporation in the absence of histone III-S. The possibility of an intrinsic kinase activity of the protein was unlikely, since no histone phosphorylation was observed in the absence of added protein kinase C. The C-kinase stimulatory activity of the protein was Ca^{2+} dependent and required the presence of both PtdSer and diacylglycerol. The specific activity of the stimulator at each stage of its purification, measured as cpm of ^{32}P incorporated into histone III-S per μg of purified protein, is shown in Table 1. Based on Bradford protein determination, the stimulator was purified 10,000-fold, but amino acid analysis (see below) showed a 32,000-fold purification with a recovery of activity of approximately 6%. Fig. 5 shows the protein kinase C activity measured in the presence of increasing concentrations of the FPLC-eluted stimulator. The 0% stimulation defines the full protein kinase C-dependent histone phosphorylation attained in the presence of PtdSer, diacylglycerol and 0.5 mM $CaCl_2$. The stimulatory effect was dose-dependent and the concentration of stimulator necessary for the half maximal activation ($V_{max}/2$) was 83 nM based on amino acid analysis and considering its dimeric active form (see below). Eleven different preparations of FPLC-eluted protein kinase C stimulator, purified from eight different keratinocyte strains, were assayed. The $V_{max}/2$ was comparable in all experiments, but the maximal protein kinase C stimulation was variable, ranging from 80 to 380% above the control. The stochiometric analysis (maximal activation at 55 picomoles of FPLC-derived protein/73 picomoles of protein kinase C) suggests that, on a molar basis, protein kinase C stimulation is achieved by direct 1:1 interaction of protein kinase C and the FPLC-derived stimulator.

Amino acid and sequence analysis

Amino acid and sequence analysis of the protein kinase C stimulator were performed from the FPLC peak and from the 30 kDa band cut from a polyvinylidene difluoride blot of an SDS-polyacrylamide gel. Identical results were obtained. The intact protein was submitted to Edman degradation. No results were obtained, suggesting that the N-terminal residue was blocked. Internal amino acid sequences were therefore generated by chemical fragmentation using cyanogen bromide. The resulting peptides were separated by reverse phase-HPLC and sequenced. The amino acid sequence allowed the unambigous identification of the FPLC-derived protein kinase C stimulator as a mixture of the highly homologous σ and ζ isoforms of protein 14-3-3 (Fig. 6, boxes). Both isoforms belong to a group of highly conserved, acidic, dimeric proteins, called 14-3-3 proteins (Aitken et al., 1992; Leffers et al., 1993; see Discussion). The ζ isoform is ubiquitous. The σ isoform is specific for stratified epithelia (Leffers et al., 1993), and hereafter it will be called with its original name, stratifin (Leffers et al., 1993). No other sequences were identified, suggesting the absence of other 14-3-3 isoforms or of other unrelated proteins. These conclusions are further confirmed by the observation that: (i) 14-3-3 proteins are homodimers of identical 30 kDa subunits (Aitken et al., 1992); (ii) their N-terminal residue is acetylated; (iii) the amino acid composition deduced from their sequence agrees with the one determined for the FPLC-derived protein (not shown).

The PH domain is a region of approximately 120 amino acids present in many proteins involved in intracellular signaling pathways, including serine/threonine and tyrosine kinases, GTP binding proteins and cytoskeletal proteins (Gibson et al., 1994). The crystal structure of the human

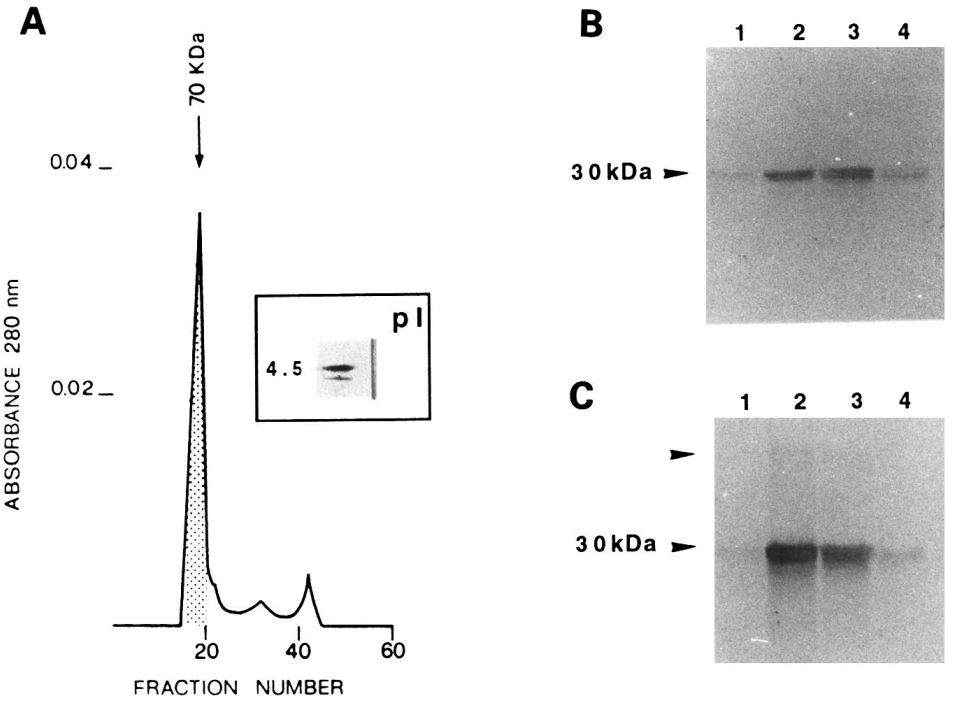

Fig. 4. FPLC size-exclusion chromatography, SDS/PAGE and isolectrofocusing. TSK 2000SW-derived peaks were pooled and loaded onto Superdex 75 and eluted as described. A single peak (A, hatched area), coincident with the 280 nm absorbance was eluted. Analysis by SDS-PAGE on a silver stained 12.5% polyacrylamide gel, showed a 30 kDa protein migrating as a distinct doublet (B). Isoelectrofocusing revealed that pI is approximately 4.5 (A, inset). In some preparations a small amount of co-purified protein, migrating as a 58-60 kDa protein, was detected (C, arrowhead) when gels were overloaded. The concentration of protein giving a 2-fold increase in protein kinase C activity was 0.01 mg/ml.

dynamin PH domain has recently been defined (Ferguson et al., 1994). Besides the highly conserved tryptophan residue, located in the 6B domain at the α-helix C-terminal (Fig. 6A, at arrow), PH domains are quite divergent in terms of amino acid sequences (Gibson et al., 1994), and this divergency hinders their detection by using conventional computerized programs. However, by using the Profile Analysis Essai of the GCG package (version 8, Genetic Computer Group, Madison, WI, USA) combined with the analysis program SearchWise kindly provided Dr E. Birney (Gibson et al., 1994), after final manual arrangement, we were able to detect, in stratifin and in the 14-3-3 ζ isoform, the six motifs forming PH domains (Fig. 6A, between closed circles). The sequence of the stratifin PH domain has been aligned with PH domains of eight different proteins. The consensus sequences (CONS) of all six PH domains (PH d) are shown (Fig. 6B).

Mono Q anion-exchange cromatography and immunoblot

In an attempt to separate the two 14-3-3 isoforms, aliquots of the FPLC-derived 14-3-3 proteins were analysed by Mono Q anion exchange chromatography. As shown in Fig. 7, two discrete peaks (peak 1 and peak 2) were eluted (at approximately 0.5 M NaCl) from the Mono Q column, peak 1 being more abundant than peak 2. Most of the protein kinase C stimulatory activity was recovered from peak 1. Analysis by SDS-PAGE on a 12.5% polyacrylamide slab gel, followed by silver staining (Fig. 7, inset A), showed that both peaks had identical

Table 1. Purification steps and evaluation of specific stimulatory activity

Step	Total protein (mg)	Specific activity (cpm/μg)	Purification (-fold)
Extract	640	8.85	0
Sephadex G200	120	22.14	5.33
DEAE 52	7.8	576.73	82
Spherogel TSK2000SW	0.360	1995.26	1777.7
Superdex 75	0.064	5433.66	10000
	0.02*		32000*

Values were based on Bradford protein assay.
*Indicates values from FPLC based on amino acid analysis.

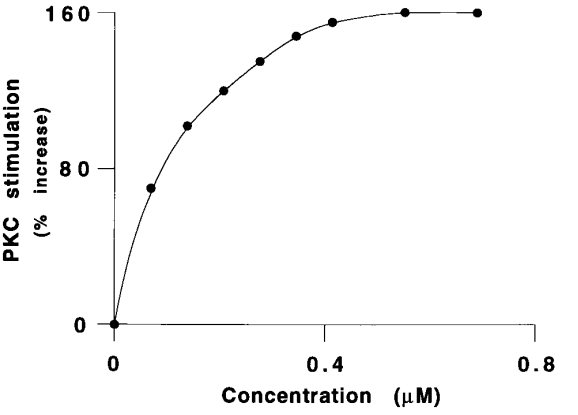

Fig. 5. Stimulation of protein kinase C activity. Protein kinase C activity was measured in the presence of increasing concentrations (determined by amino acid analysis) of FPLC-eluted stimulator. The 0% stimulation defines the full protein kinase C-dependent histone phosphorylation in the presence of PtdSer, diacylglycerol and 0.5 mM $CaCl_2$. Each point is the average of triplicates. These data are representative of several experiments: eleven different preparations of FPLC-eluted protein kinase C stimulator, purified from eight different keratinocyte strains, were assayed. The $V_{max}/2$ was comparable in all experiments, but the maximal protein kinase C stimulation (160% in the experiment showed here) was variable, ranging from 80 to 380% above the control.

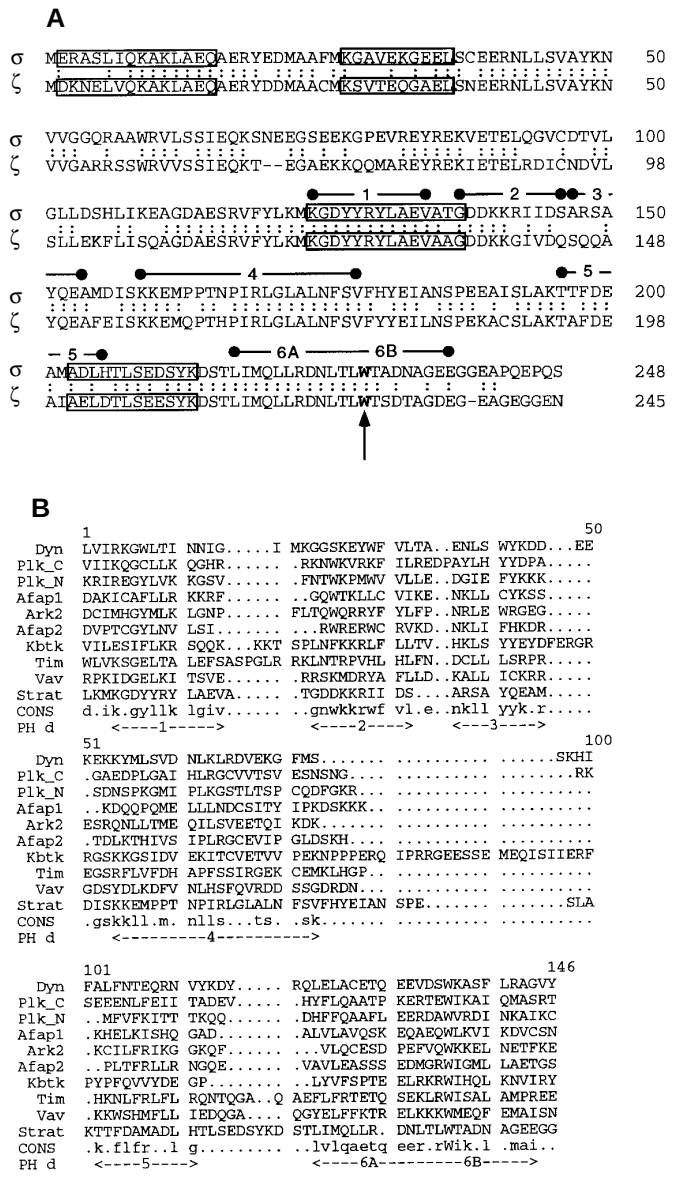

Fig. 6. Sequence analysis. (A) Alignment of the amino acid sequences of the σ isoform (stratifin) and ζ isoform of human 14-3-3 proteins. The amino acid sequences of peptides obtained by CNBr fragmentation of the FPLC-derived stimulator are boxed. Motifs forming the PH domain are indicated between filled circles. The arrow indicates the highly conserved tryptophan residue located in the 6B domain at the C terminus. (B) The sequence of the stratifin (Strat) PH domain has been aligned with PH domains of eight different proteins: dynamin-1 (Dyn), pleckstrin C- and N-termini (Plk_C, Plk_N), actin filament-associated protein (Afap1), beta-adrenergic receptor kinase 2 (Ark2), neural actin filament protein (Afap2), tyrosine-protein kinase BTK (Kbtk), guanine nucleotide regulatory protein (Tim), Vav oncogene (Vav). The consensus sequences (CONS) of all six PH domains (PH d) are shown.

electrophoretic mobilities and an apparent molecular mass of 30 kDa. Western blot analysis using a rabbit antiserum raised against 14-3-3 proteins (a gift from Dr J. Celis) identified both peaks as 14-3-3 members (Fig. 7, inset B, lanes 1 and 2), indicating that the separation of the two isoforms had been achieved. Western blot analysis was then performed using a rabbit antiserum specific for stratifin (a gift from Dr A. Aitken) and raised against the N terminus sequence MERASLIQKAC of the protein. As shown in Fig. 7 (inset C, lane 1), stratifin was identified only in peak 1, where most of the protein kinase C stimulatory activity was recovered.

Experiments generated with recombinant human stratifin have confirmed these data and have shown that, at variance with the ζ isoform, stratifin enhances the enzymatic activity of distinct keratinocyte-derived protein kinase C isoforms (E. Dellambra et al., unpublished).

DISCUSSION

In this paper we report the isolation and identification of stratifin as a keratinocyte-derived stimulator of protein kinase C-mediated phosphorylation. Stratifin was originally sequenced from a human keratinocyte protein database (Leffers et al., 1993), but its function was unknown.

Stratifin belongs to a large group of highly conserved and highly homologous, acidic, dimeric proteins, the 14-3-3 family, expressed in mammalian tissues, *Xenopus*, *Drosophila melanogaster*, plants and yeast (see Aitken et al., 1992, for review). Several 14-3-3 isoforms (α-η) are expressed in mammalian brain, where they activate tyrosine and tryptophan hydroxylases (see Aitken et al., 1992 for review). 14-3-3 proteins regulate *N*-acetyl-transferase in the pineal gland (ε isoform), and arachidonic acid metabolism in platelets (ζ isoform), and stimulate, in concert with protein kinase C, Ca^{2+}-dependent exocytosis in permeabilized adrenal medulla cells (see Aitken et al., 1992 for review). Some 14-3-3 proteins appear to be ubiquitous (as for the ζ isoform), while others are tissue specific (as for stratifin in the stratified epithelia and as for the τ isoform in T-cells). One of the most conserved regions of 14-3-3 proteins, including stratifin, contains a stretch of residues highly homologous to the C terminus of a family of Ca^{2+}, lipid and membrane-binding proteins named lipocortins/annexins (Aitken et al., 1990). This sequence is responsible for the binding of annexins to protein kinase C and is also involved in the binding of protein kinase C to RACKs at the cell membrane (Mochly-Rosen et al., 1991a,b). The presence of this particular amino acid sequence suggests a role for 14-3-3 proteins in protein kinase C membrane translocation and activation. Interestingly, it has been shown that the binding of a synthetic peptide (with the above amino acid sequence) to protein kinase C is increased in the presence of protein kinase C activators (Ron et al., 1994); Moreover, microinjection of this peptide in *Xenopus* oocytes inhibited insulin-induced protein kinase C translocation and protein kinase C-mediated oocyte maturation (Ron et al., 1994).

Nevertheless, contrasting data exist in literature concerning the role of 14-3-3 proteins as regulators of protein kinase C. Indeed, 14-3-3 proteins, with the notable exception of stratifin, have been reported to inhibit protein kinase C-mediated phosphorylation of both histone III-S and synthetic MARCKS peptides (Toker et al., 1992; Robinson et al., 1994). It has also been shown that the Exo-1 isoform, which stimulates exocytosis (Morgan and Burgoyne, 1992a), does not regulate protein kinase C activity at all (Morgan and Burgoyne, 1992b). In contrast, Isobe and colleagues (1992) and Chen and Wagner (1994), have demonstrated that 14-3-3 proteins actually stimulate protein kinase C dependent histone phosphorylation,

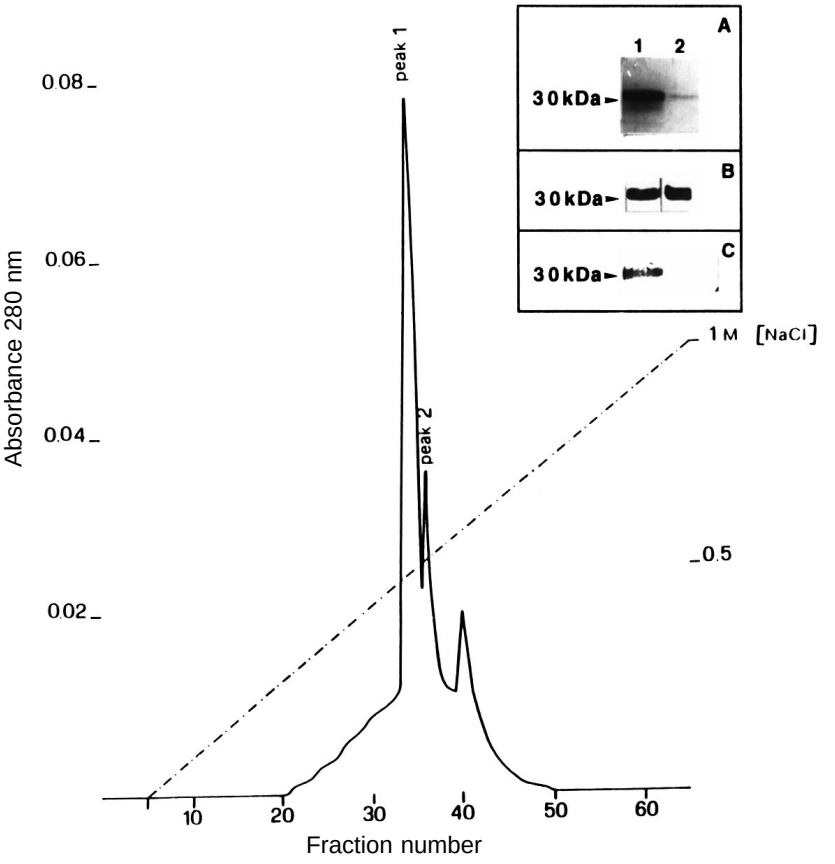

Fig. 7. Mono Q anion-exchange chromatography and immunoblot. FPLC-derived stimulator was applied to a Mono Q column. Bound proteins were eluted by a NaCl linear gradient (0-1 M) and two discrete peaks (peak 1 and peak 2) were eluted at approximately 0.5 M NaCl; 200 μl of each peak were analysed by SDS-PAGE on a 12.5% gel and silver-stained (inset A, lanes 1 and 2). The protein kinase C stimulatory activity was recovered mainly from peak 1. Peak 1 and peak 2, obtained from several Mono Q columns, were pooled and concentrated. Equal amounts of peak 1 and peak 2 were then analysed by western blotting (insets B and C, lanes 1 and 2) using a pan-14-3-3 rabbit antiserum (inset B) or a rabbit antiserum specific for stratifin and raised against the N terminus sequence of the protein (inset C).

in the presence of calcium, diacylglycerol and PtdSer. These discrepancies have been related to the type of substrate (Chen and Wagner, 1994), but data from Robinson et al. (1994) seem to rule out this possibility.

In our experiments, stratifin stimulates protein kinase C-mediated histone phosphorylation up to 3-4-fold in assays where protein kinase C is already maximally stimulated. Keratinocyte-derived 14-3-3 has a $V_{max}/2$ of 83 nM, which is at least 5-fold lower than the IC_{50} of the inhibitory effects reported for other isoforms, both purified or recombinant. The effect of lower concentrations of other 14-3-3 members on protein kinase C activity has not been reported. Thus, a careful evaluation of protein concentrations used in these experiments should be made, to rule out the possibility of a biphasic effect of all 14-3-3 members. The mechanism by which stratifin stimulates protein kinase C activity is currently unknown. However, the binding of 14-3-3 proteins to the regulatory subunit of protein kinase C (Robinson et al., 1994) and the lack of stimulatory effect of stratifin in the absence of diacylglycerol and PtdSer, suggest a positive regulation of an activated protein kinase C rather than a direct protein kinase C activation. Stratifin might thus either act as a co-factor in stabilizing the conformation of protein kinase C, or act on the substrate itself, or link protein kinase C to selected substrates and increase its phosphorylation activity towards specific targets. A number of observation discussed hereafter might support the latter hypothesis.

We show that stratifin (and 14-3-3 ζ) harbors a pleckstrin homology (PH) domain. The PH domain was first identified as a new module in pleckstrin, the major protein kinase C substrate in platelets (Hastam et al., 1993; Mayer et al., 1993). During the last year, PH domains have been found in several signaling and cytoskeletal proteins (see Gibson et al., 1994, for a review), including ras^{GAP}, the β-adrenergic receptor kinase, the μ isoform of protein kinase C, the β, γ and δ isoforms of PLC, SOS, Bruton tyrosine kinase and members of the src family of tyrosine kinases. PH domain containing proteins can be translocated to the cell membrane. Indeed, in addition to the PH domain C terminus ability to bind the βγ complex of heterotrimeric G proteins (Touhara et al., 1994; Tsukada et al., 1994), the N terminus of the PH domain has been recently shown to bind phosphatidylinositol-4,5-bisphospate (Harlan et al., 1994). The different and probably independent roles of the N and C termini of PH domains are further suggested by the PH domain distribution in PLCγ, where the C-terminal half of the domain is separated from the N terminus by a very long intervening amino acid sequence containing SH2 and SH3 domains (Hastam et al., 1993; Musacchio et al., 1993). Indirect evidence suggests that the PH domain may also recognize serine or threonine phosphorylated peptides (see Gibson et al., 1994, for a comprehensive review), and very recently it has been shown that the PH domain of Bruton tyrosine kinase interacts with protein kinase C (Yao et al., 1994). Altogether, these data suggest a conceptual analogy with proteins containing SH2 and SH3 domains (reviewed by Pawson and Schlessinger, 1993) and argue in favour of a role of PH domains in directing protein-protein interactions. Moreover, recent data have shown that 14-3-3 proteins bind to Raf-1 (Fantl et al., 1994; Freed et al., 1994; Irie et al., 1994; Fu et al., 1994), probably through the binding of the 14-3-3 annexin-like

domain to a zinc-finger-like region contained within the C-terminus of Raf-1. This leads to membrane translocation of Raf-1 and strong activation of its kinase activity (Fantl et al., 1994; Freed et al., 1994; Irie et al., 1994; Fu et al., 1994). However, the interaction of 14-3-3 proteins with Raf-1 is not sufficient per se to stimulate the kinase activity of Raf-1 (Freed et al., 1994; Shimizu et al., 1994), suggesting that 14-3-3 proteins may be required in a common pathway of Raf activation envisaging other mediators endowed with serine/threonine kinase activity.

Therefore, it is tantalizing to speculate that stratifin, as well as other 14-3-3 members, can act as activator or modulator of signaling proteins by linking together, at the cell membrane and through the PH domain, the Ras-Raf complex and protein kinase C (as well as other signaling molecules). Since the interaction of 14-3-3 proteins with Raf-1 is not sufficient to stimulate Raf kinase (Freed et al., 1994; Shimizu et al., 1994), 14-3-3-dependent Raf-1 activation could be accounted for by protein kinase C-dependent serine/threonine phosphorylation of Raf driven by the mutual interaction with 14-3-3 proteins (see also Daum et al., 1994; Kharbanda et al., 1994). Indeed, it has been reported that: (i) protein kinase C can interact in vivo with Ras (Diaz-Meco et al., 1994); (ii) protein kinase C-mediated serine phosphorylation activates Raf-1 (Carrol and May, 1994); (iii) phospholipase A_2 mediated protein kinase C activation stimulates mitogen-activated protein kinase (MAP kinase) (Qiu and Leslie, 1994); (iv) protein kinase C activates MEK kinase through phosphorylation of its N-terminal non-catalytic domain (Blumer et al., 1994).

Thus, the activation of protein kinase C is an integral part of the signal-induced degradation cascade of various membrane phospholipids, as well as of the kinase cascade elicited by the stimulation of tyrosine kinase receptors; stratifin, as well as other 14-3-3 proteins, can be an important part of these mechanisms by linking signaling proteins at the cell membrane and by modulating their function.

Stratifin is specifically expressed in keratinocytes forming stratified squamous epithelia (Leffers et al., 1993). The tissue-specific expression of stratifin is of particular interest in relation to its potential role in regulating keratinocyte terminal differentiation. Indeed, protein kinase C has been related to the regulation of the tight balance between keratinocyte growth and terminal differentiation, responsible for the maintenance of the proper epidermal morphology, both in humans and in other mammalian species (Matsui et al., 1992; Gherzi et al., 1992; Dlugosz and Yuspa, 1993, 1994; Punnonen et al., 1993; Fisher et al., 1993). Terminal differentiation of human epidermal keratinocytes has been associated with an up-regulation of protein kinase C activity (Matsui et al., 1992), and phorbol ester stimulation of protein kinase C inhibits human epithelial cell growth (Jetten et al., 1989). Protein kinase C is a multigene family of related isoenzymes composed of conventional, Ca^{2+} dependent (isoforms α, βI, βII, γ), novel, Ca^{2+} independent (isoforms δ, ε, η, ϕ), atypical, diacylglycerol independent (isoforms ζ,$\upsilon\lambda$) and potentially *trans*-membrane (isoform μ) isoenzymes (see Asaoka et al., 1992; Azzi et al., 1992; Dekker and Parker, 1994; Liscovitch and Cantley, 1994, for reviews). Gherzi et al. (1992), reported that in vitro reconstituted human epidermis expresses the α isoform of Ca^{2+} dependent and the δ and η isoforms of Ca^{2+} independent protein kinase C. Normal human keratinocyte differentiation was associated with a decrease in the mRNA level of the α and δ isoform and to an increase in the mRNA level of the η isoform (Gherzi et al., 1992). By reverse transcription/polymerase chain reaction on primary cultures of human keratinocytes, it has been shown that keratinocytes express also the Ca^{2+} independent ε isoform as well as the atypical, non-diacyglycerol responsive ζ protein kinase C isoform (Fisher et al., 1993; Reynolds et al., 1994).

We are currently studying the expression of protein kinase C isoenzymes in pure cultures of cloned human keratinocytes. We are also investigating whether the topography of protein kinase C isoforms within the epidermal cell layers matches the stratifin distribution, and whether stratifin is able to regulate the entire protein kinase C machinery of normal human keratinocytes or to modulate the enzymatic activity of selected protein kinase C isoforms (E. Dellambra et al., unpublished), in order to correlate this with the onset of keratinocyte terminal differentiation.

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