Downregulated AP-1 activity is associated with inhibition of Protein-Kinase-C-dependent CD44 and ezrin localisation and upregulation of PKC theta in A431 cells

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Accepted 23 April 2002
Journal of Cell Science 115, 2713-2724 (2002) © The Company of Biologists Ltd

Summary

Progression to an invasive, metastatic tumour requires the coordinated expression and function of a number of gene products, as well as their regulation in the context of invasion. The transcription factor AP-1 regulates expression of many of those genes necessary for implementation of the invasion programme. Two such gene products, CD44 and ezrin, are both upregulated in fibroblasts transformed by v-*fos* and are commonly implicated in cell motility and invasion. Here we report that CD44 and ezrin colocalise to membrane ruffles and microvilli of A431 cells after treatment with EGF. However, A431 cells expressing dominant-negative c-Jun (TAM67), and which as a consequence fail to invade in response to EGF, also fail to correctly localise CD44 and ezrin. CD44 and ezrin are both substrates for Protein Kinase C, and we show that their EGF-dependent colocalisation requires Protein Kinase C activity. Associated with TAM67 expression and disrupted CD44 and ezrin colocalisation is the increased expression and activation of the novel PKC theta isoform. Expression of PKC theta in A431 cells results in the inhibition of cell motility and disrupted localisation of CD44 and ezrin. We propose that AP-1 regulates the integrity of Protein Kinase C signalling and identifies PKC theta as a potential suppressor of the invasion programme.

Key words: CD44, Ezrin, Protein Kinase C

Introduction

Metastatic progression is dependent on the ability of tumour cells to invade through basement membrane barriers and the extracellular matrix (ECM) to colonise distant sites in the organism. Invasion itself entails a series of processes including changes in cell-cell and cell-ECM interactions, cell morphology and the underlying cytoskeleton as well as proteolytic capability (Liotta et al., 1991). Invasion also requires new gene expression (Ozanne et al., 2000). We have shown previously that the transcription factor AP-1, composed principally of Fos and Jun heterodimers, is required for cell invasion in vitro (Hennigan et al., 1994; Lamb et al., 1997a; Malliri et al., 1998), and others have demonstrated the same requirement in vivo (Saez et al., 1995; Young et al., 1999). Having determined the profile of genes differentially expressed between Rat 1 fibroblasts and their FBR v-*fos* transformed counterparts, we have proposed that a major role of AP-1 is to control the expression of those genes necessary for invasion (Hennigan et al., 1994; Johnston et al., 2000).

Two such gene products upregulated in these libraries are CD44 and the CD44-interacting protein, Ezrin. CD44 is a transmembrane glycoprotein and the major cell surface receptor for the ECM component, hyaluronan. Extensive alternative splicing of the *CD44* gene results in a large number of CD44 isoforms that participate in functions including lymphocyte homing, cell adhesion and migration (Aruffo et al., 1990; Jalkanen et al., 1987; Thomas et al., 1992). CD44 is also known to be upregulated in a variety of tumours with metastatic or invasive properties (Gunthert et al., 1991; Sy et al., 1997). Similarly, ezrin was found to be upregulated in a number of screens designed to identify genes involved in metastasis and invasion (Jooss and Muller, 1995; Khanna et al., 2001; Nestl et al., 2001; Ozanne et al., 2000). Ezrin is a member of the Ezrin-Radixin-Moesin family of proteins (ERM) that serve as molecular crosslinkers between the actin cytoskeleton and the plasma membrane (Mangeat et al., 1999). In this capacity, ERM proteins participate in a number of cell processes including the maintenance of cell morphology, cell adhesion and motility (Bretscher et al., 2000).

CD44 expression is required for invasion by both v-*fos*-transformed and EGF-transformed fibroblasts; in these cells it localises to the tips of pseudopodial cell extensions (Lamb et al., 1997a). CD44 variant isoforms localise to the tips of 'invadapodia' in Met1 cells, where they function to localise matrixmetalloproteases for directional degradation of the ECM (Bourguignon et al., 1998; Yu and Stamenkovic, 1999; Yu and Stamenkovic, 2000). Ezrin is the only ERM protein to be upregulated by the v-*fos* oncogene, being required for the formation of the extending pseudopod (Lamb et al., 1997b).

Both CD44 and ezrin are regulated by phosphorylation. CD44 is phosphorylated exclusively on serine residues (Neame and Isacke, 1992), and the major phosphorylation site on the

CD44 cytoplasmic tail is located at serine 325, and there is an additional less well characterised Protein Kinase C (PKC) consensus site at serine 291. The significance of CD44 phosphorylation is not fully understood, although CD44 phosphorylation regulates cell migration on a hyaluronan substrate (Peck and Isacke, 1996) and may regulate association with the cytoskeleton in macrophages (Camp et al., 1991). Phosphorylation of a conserved threonine at the C-terminus of ERM proteins, together with phospholipid interaction, is required for plasma membrane localisation (Barret et al., 2000; Nakamura et al., 1999; Niggli et al., 1995). Ezrin is also tyrosine phosphorylated by the EGF receptor and the HGF receptor (Crepaldi et al., 1997; Krieg and Hunter, 1992), and while tyrosine phosphorylation is required for motility and morphogenesis in an epithelial cell line, it has no effect on cellular localisation of ezrin (Crepaldi et al., 1997). Amongst the various kinases that have been shown to be capable of phosphorylating ezrin and CD44 is PKC. Purified CD44 is phosphorylated by PKC in vitro (Kalomiris and Bourguignon, 1989). The PKCθ isoform phosphorylates moesin and ezrin in vitro (Pietromonaco et al., 1998; Simons et al., 1998), and more recently PKCα was shown to phosphorylate ezrin in vivo (Ng et al., 2001).

PKC is a family of serine-threonine kinases that are differentially regulated by lipid and calcium (Mellor and Parker, 1998). Conventional isoforms of PKC (α, βI, βII, γ) require phosphatidylserine and diacylglycerol together with calcium for activation. The novel isoforms (δ, ε, θ, μ) are calcium independent, and the atypical isoforms (λ/ι, ζ) require neither lipid or calcium for activation. A number of reports support a role for PKC in cell motility, invasion and regulation of the cytoskeleton. PKCα overexpression resulted in increased motility and adhesion of a non-metastatic mammary epithelial cell line (Sun and Rotenberg, 1999), whereas a constitutively active form of PKCα increased invasion of intestinal cells (Batlle et al., 1998). The novel PKCε isoform is involved in the regulation of cell adhesion and spreading (Berrier et al., 2000; Chun et al., 1996), whereas PKCθ regulates migration of endothelial cells (Tang et al., 1997). Atypical PKC isoforms are involved in regulating the organisation of the actin cytoskeleton (Laudanna et al., 1998; Uberall et al., 1999).

AP-1 activity is required for invasion by the squamous-cell-carcinoma-derived cell line A431 in response to EGF (Malliri et al., 1998). A431 cells overexpress the EGF receptor: treatment with EGF results in the rapid rearrangement of the actin cytoskeleton including Rac-dependent membrane ruffling and Rho-dependent cortical actin polymerisation and cell rounding, leading to increased cell motility and invasion. Downregulation of AP-1 activity in A431 cells by expression of dominant-negative c-Jun (TAM67) results in cells that are no longer motile, invasive or capable of Rac- and Rho-dependent reorganisation of the actin cytoskeleton in response to EGF (Malliri et al., 1998).

Although it is clear that a new profile of gene expression is required to implement the invasion programme, it is also necessary that the regulatory signals required for the function of each new gene product in the context of invasion are also in place. Here we investigate the regulation of CD44 and ezrin localisation in A431 cells after EGF treatment to initiate cell invasion, compared with invasion-defective A431 cells expressing TAM67 (TA cells). We find that correct EGF-dependent colocalisation of both CD44 and ezrin is defective in TA cells and provide evidence that associated upregulation of PKCθ contributes to disruption of the invasion programme.

Materials and Methods

Materials and antibodies

Human recombinant EGF was supplied by R&D Systems. TRITC- and FITC-conjugated phalloidin were purchased from Sigma. G418 was supplied by Gibco BRL. The PKC inhibitor, Ro-31-8220, was supplied by Calbiochem. A rabbit polyclonal ezrin antibody was supplied by P. Mangeat. A mouse monoclonal E1/2 CD44 antibody was provided by C. Isacke. Antibodies recognising PKC α, δ and θ were from Transduction Laboratories; PKCζ antibody was from Boehringer. The phosphoPKC (pan) antibody was purchased from Cell Signaling Technology. TPA (phorbol-12-myristate-13-acetate) was from Gibco.

Cell lines, culture and transfection

A431 cells, A431 cells stably expressing an empty neomycin-encoding vector (NA cells) and A431 cells expressing both a neomycin-encoding plasmid and an expression construct encoding TAM67 (TA cells) have been described previously (Malliri et al., 1998). PKCθ-expressing cell lines were established by transfecting a PKCθ expression plasmid using Fugene6 (Roche) and selecting colonies grown in the presence of G418. Cells were maintained in DMEM (Sigma) containing 10% FCS (Harlan SeraLab) and, in the case of NA, TA and A431-θ cells, 500 μg/ml G418, at 37°C, 5% CO_2. For EGF or TPA treatment, cells were plated either on tissue culture plates or on glass coverslips, allowed to attach overnight, then transferred to medium without FCS for 2 days. 10 ng/ml EGF or 100 ng/ml TPA was used to treat cells.

Wound-healing assays

A431 and A431-θ cells were grown as a confluent monolayer, serum starved for 2 days, then wounded using a disposable pipette tip. 10 ng/ml EGF was added to the medium and the extent of wound closure was determined after 36 hours.

Immunofluorescence

Cells were seeded onto glass coverslips, allowed to attach overnight, then transferred to serum-free medium for 2 days. Following various treatments, cells were fixed in 4% formaldehyde in PBS for 15 minutes and permeabilised in PBS containing 0.1% Triton X-100. Cells were blocked in 10% FCS, 0.5% BSA in PBS for 1 hour, followed by the addition of antibody diluted in blocking buffer. The antibody dilutions used were: CD44, neat; ezrin, 1:500. Cells were washed in blocking buffer then incubated in either goat-anti-mouse IgG TRITC conjugate (Sigma) (for CD44) or goat-anti-rabbit IgG FITC conjugate (Sigma) (for ezrin), both diluted in blocking buffer at 1:60, together with 500 ng/ml TRITC- or FITC-conjugated phalloidin. Cells were washed in blocking buffer followed by a final wash in PBS containing 0.1% Triton X-100. Coverslips were mounted onto glass slides using Vectashield (Vector Laboratories), followed by confocal microscopic analysis using a Biorad MRC 600 confocal illumination unit attached to a Nikon Diaphot inverted microscope. Cells were treated with PKC inhibitors for 30 minutes at 3 μM, followed by EGF treatment and fixation.

Western analysis

Cell lysates were prepared by washing cells twice in ice-cold PBS, followed by scraping into lysis buffer (20 mM Hepes pH 7.4, 5 mM EDTA, 10 mM EGTA, 5 mM Na fluoride, 1 mM KCl, 0.4% Triton

X-100, 10% glycerol, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 1 mM Na vanadate). Cleared supernatants were collected and quantified using the copper sulphate/bicinchoninic acid method (Sigma). 50 μg of protein lysate was electrophoresed on SDS-PAGE gels (no reducing agent was used for CD44 analyses). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semi-dry blotter. Filters were blocked in 5% semi-skimmed milk powder in TBST (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 hour prior to incubation with CD44 antibody (1:10 dilution), ezrin antibody (1:10,000 dilution) or PKC antibodies (at dilutions recommended by the vendor). Filters were washed in 5% semi-skimmed milk powder in TBST then incubated with a horseradish-peroxidase-conjugated sheep anti-mouse or anti-rabbit Ig (Amersham, dilution 1:5000) for 1 hour. Filters were washed in TBST followed by ECL (Amersham) to detect positive signals.

Cell fractionation

Soluble and particulate fractions were prepared as previously described (Goodnight et al., 1995). Cells were washed in ice-cold PBS, scraped into 20 mM Tris pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 20 μg/ml leupeptin, 80 μg/ml aprotinin and 0.1% 2-mercaptoethanol then sonicated for 10 seconds. The soluble supernatant was collected, and the pellet was resuspended in 1×SDS PAGE sample buffer and sonicated for 10 seconds.

Northern analysis

Total RNA was prepared using RNazol B (Biogenesis Ltd) according to the manufacturer's instructions. Electrophoresis of 20 μg of total RNA in a 1% agarose, 200 mM MOPS, 7% formaldehyde gel was followed by capillary transfer to a Hybond-N nylon filter (Amersham) and UV crosslinking using a UV Stratalinker 1800 (Stratagene). Radiolabelled PKCθ and 7S ribosomal cDNA probes were prepared by random priming using the AmershamPharmacia DNA labelling kit and α-^{32}P-dCTP (Amersham). Hybridisation was carried out at 68°C overnight in 0.25 M $NaPO_4$ pH 7.2, 7% SDS, 1 mM EDTA and 1% BSA. Filters were washed three times in 20 mM $NaPO_4$ pH 7.2, 1% SDS, 1 mM EDTA, 20 minutes, 68°C and exposed for autoradiography.

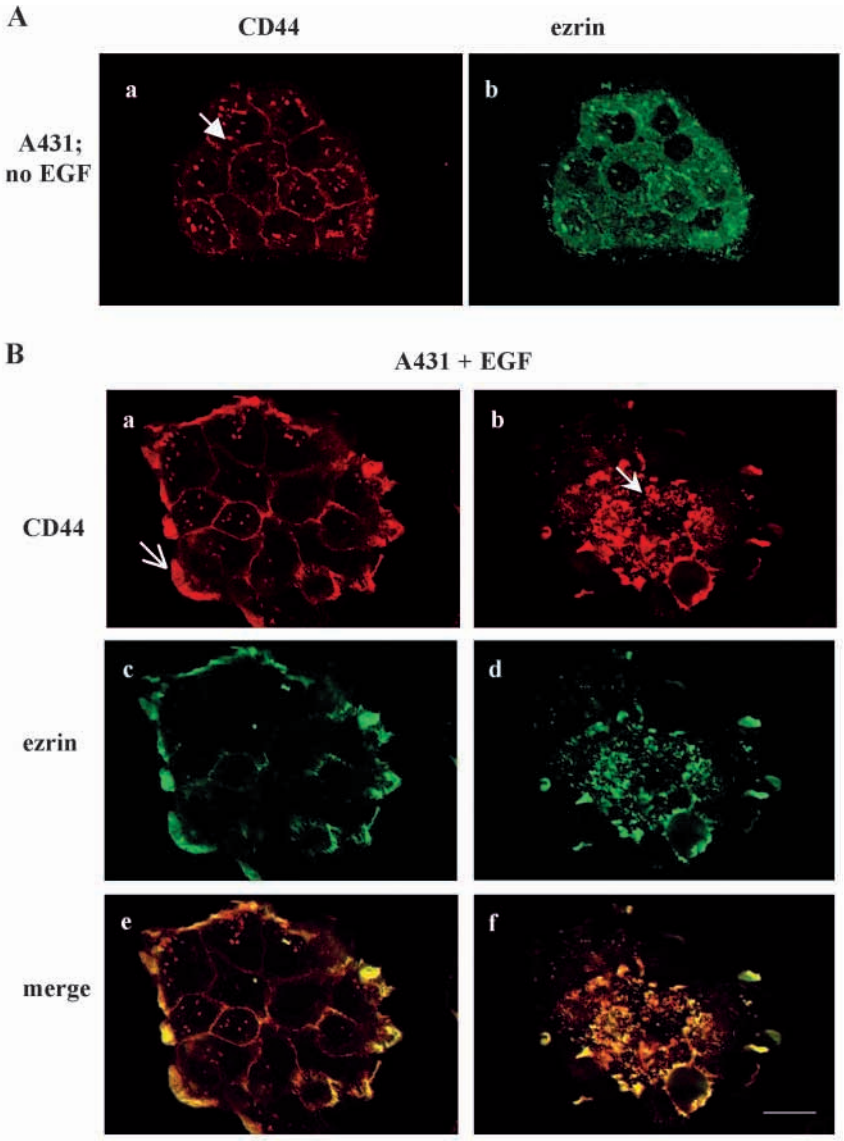

Fig. 1. CD44 and ezrin localisation in A431 cells. (A) A431 cells were serum starved for 2 days then processed for immunofluorescence using the E1/2 CD44 monoclonal antibody (a) and an anti-ezrin polyclonal antibody (b) (arrow indicates CD44-positive basal plaque; see text). (B) Serum-starved A431 cells were treated with 10 ng/ml EGF for 5 minutes before processing for immunofluorescence using the E1/2 CD44 antibody (a, b) or the ezrin antibody (c, d). Images (a, c and e) are confocal sections chosen to highlight membrane ruffling (open arrow), whereas images (b, d and f) are a higher confocal section showing microvilli (filled arrow). Panels e and f represent merged images (yellow) of a, c and b, d respectively. Bar, 10 μm.

Results

EGF induced colocalisation of CD44 and ezrin in A431 cells

Because fibroblasts transformed with the FBR v-*fos* oncogene require both CD44 and ezrin expression and localisation to the extending tips of pseudopodia for motility and invasion (Lamb et al., 1997a; Lamb et al., 1997b), we determined whether the same assembly of proteins occurs in specific locomotory structures of A431 cells treated with EGF. Cells were serum starved for 2 days then treated with EGF for five minutes to induce the formation of membrane ruffles, microvilli and the polymerisation of cortical actin. In untreated cells, CD44 is localised to sites of cell-cell contact (Fig. 1Aa), a distribution that has been reported previously in other epithelial cell models (Neame and Isacke, 1993). However, unlike MDCK cells, CD44 also concentrates in actin-rich plaques on the basal surface of the cells. These structures may represent podosomes, which are adhesion structures previously described in monocyte-derived cell types and fibroblasts transformed by tyrosine kinases (Marchisio et al., 1987). There is very little detectable CD44 on either the apical surface of the cell or at the plasma membrane that is not in contact with an adjacent cell. Ezrin does not colocalise with CD44 in serum-starved A431 cells, where it resides in the cytosol (Fig. 1Ab).

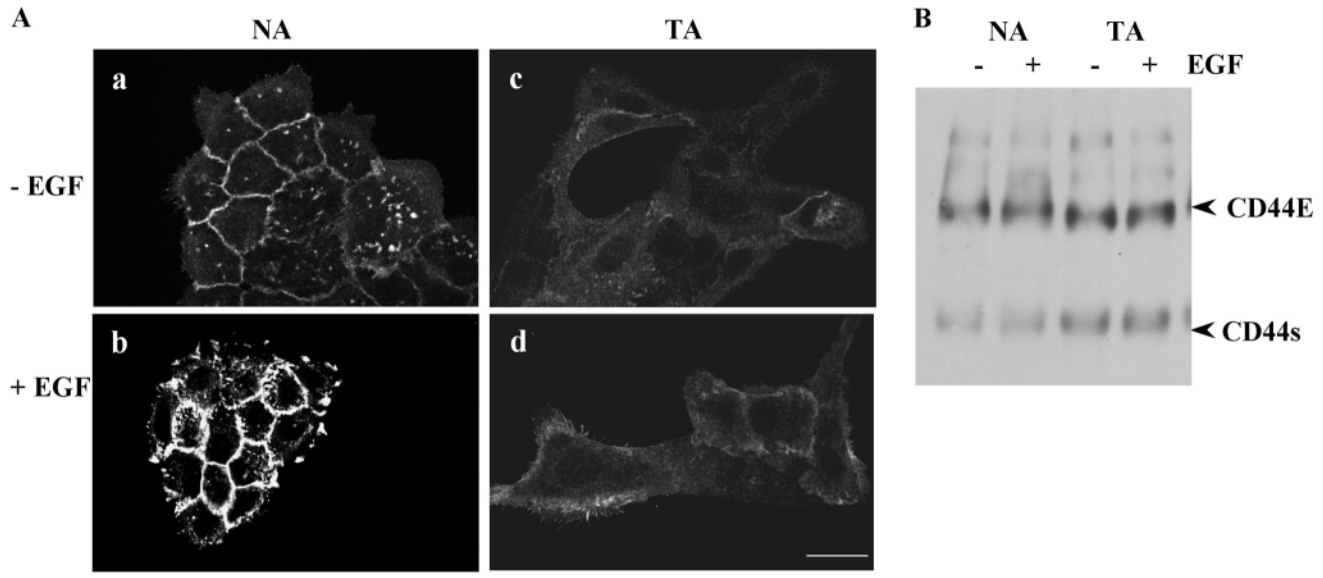

Fig. 2. CD44 localisation and expression in A431 cells expressing dominant-negative Jun. (A) NA and TA cells were serum starved for 2 days (a, c) or treated with EGF for 5 minutes (b, d), then processed for immunofluorescence using the E1/2 CD44 monoclonal antibody. Bar, 10 μm. (B) Whole cell extracts from serum-starved and EGF-treated NA and TA cells were prepared and analysed by western blotting using the E1/2 CD44 monoclonal antibody. CD44s is the standard 85 kDa isoform; CD44E indicates the 145 kDa, epithelial isoform.

After a 5 minute exposure to EGF, a proportion of CD44 swiftly localises to membrane ruffles and also concentrates in newly formed apical microvilli (Fig. 1B,a,b). We found identical, localisation of the standard 85 kDa form of CD44 (CD44s)-GFP fusion protein in A431 cells before and after EGF treatment (data not shown). Similarly, EGF results in an identical relocalisation of ezrin to membrane ruffles and microvilli (Fig. 1Bc,d) (Bretscher, 1989), precisely overlapping CD44 localisation (Fig. 1Be,f). Thus, signals from the EGF receptor result in the assembly of functionally interacting proteins at locomotory structures in a similar manner to that observed in FBR cells, where the activity of each is required for cell invasion.

Expression of dominant-negative c-Jun (TAM67) results in CD44 and ezrin mislocalisation

Previously we have shown that A431 cells require AP-1 activity for EGF-induced invasion, motility and associated reorganisation of the actin cytoskeleton (Malliri et al., 1998). We therefore examined whether the localisation of two gene products required for invasion, CD44 and ezrin, was affected

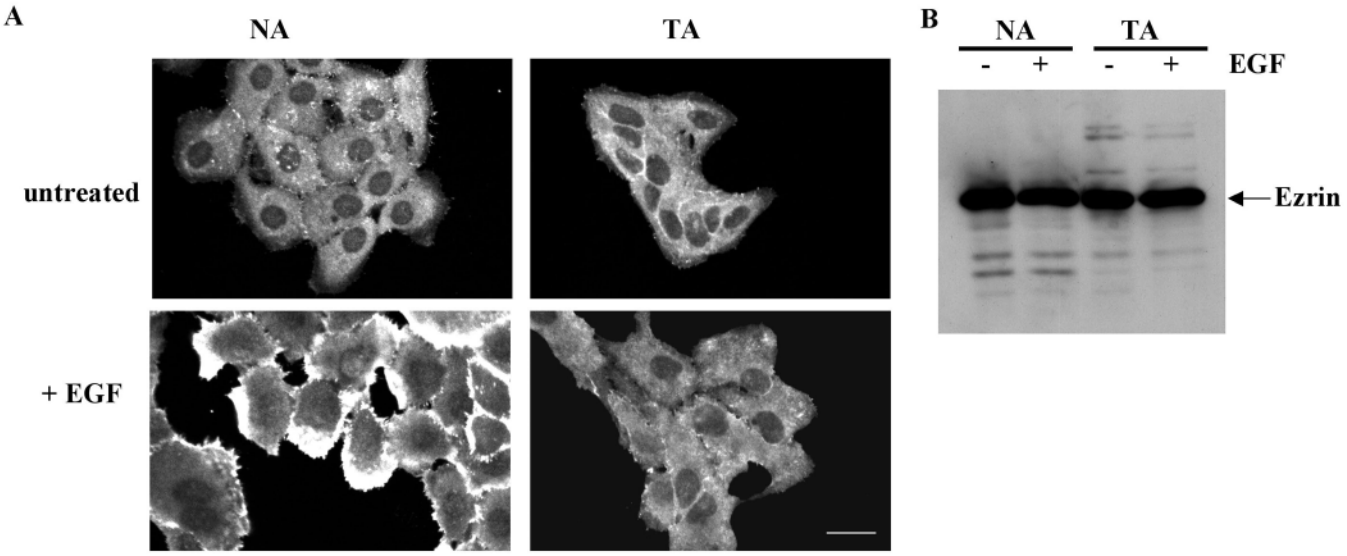

Fig. 3. Ezrin localisation and expression in A431 cells expressing TAM67. (A) NA and TA cells were serum starved for 2 days followed by treatment with 10 ng/ml EGF for 5 minutes. Cells were fixed then immunostained for ezrin distribution and counter-stained with phalloidin to visualise actin organization. Bar, 10 μm. (B) Whole cell extracts from serum-starved and EGF-treated NA and TA cells were prepared and analysed by western blotting using an ezrin polyclonal antibody.

in A431 cells expressing TAM67 (TA cells) (Malliri et al., 1998). CD44 localisation is completely disorganised in TA cells (Fig. 2A, compare c with a). CD44 is mostly absent from sites of cell-cell contact and the intensity of staining is greatly reduced compared with NA cells (A431 cells expressing an empty neomycin expression vector). Upon EGF treatment of TA cells, CD44 localisation remains generally disorganised and has a lower signal intensity (Fig. 2A, compare d with b).

The immunolocalisation data suggested either that CD44 was now uniformly distributed over the cell surface of TA cells or that CD44 expression levels were greatly reduced in these cells. Western analysis showed that the expression levels and isoform profile of CD44 is unaffected in TA cells (Fig. 2B). A431 and TA cells both express the standard 85 kDa form (CD44s), the 145 kDa epithelial isoform (CD44E) and a >200 kDa form that most probably corresponds to CD44v3 8-10, which is expressed in A431 cells (Grimme et al., 1999). Thus CD44 localisation, rather than isoform expression levels, is affected by downregulated AP-1 activity. Although it is known that CD44 is an AP-1 target gene, our results are consistent with other reports showing that dominant-negative versions of c-Jun (Johnston et al., 2000; Young et al., 1999) and JunD (Ui et al., 2000) do not affect expression of all AP-1 target genes.

Likewise, ezrin fails to relocalise to the plasma membrane or microvilli in response to EGF in TA cells, remaining in the cytosol of both resting and EGF-treated TA cells (Fig. 3A). Ezrin protein levels are also equal in NA and TA cells (Fig. 3B). Invasion-defective TA cells, therefore, are incapable of assembling two proteins necessary for cell motility and invasion into locomotory structures. AP-1 activity is required for expression of the gene(s) necessary to signal correct ezrin and CD44 localisation from the EGF receptor.

Protein Kinase C activity is required for ezrin and CD44 localisation

PKC is capable of phosphorylating both CD44 and ezrin, and the localisation of ERM proteins to the membrane is regulated in part by phosphorylation of a conserved C-terminal threonine (Nakamura et al., 1999). To address whether PKC activity is required for CD44 and ezrin localisation in A431/NA cells, we inhibited PKC activity using the broad spectrum PKC inhibitor,

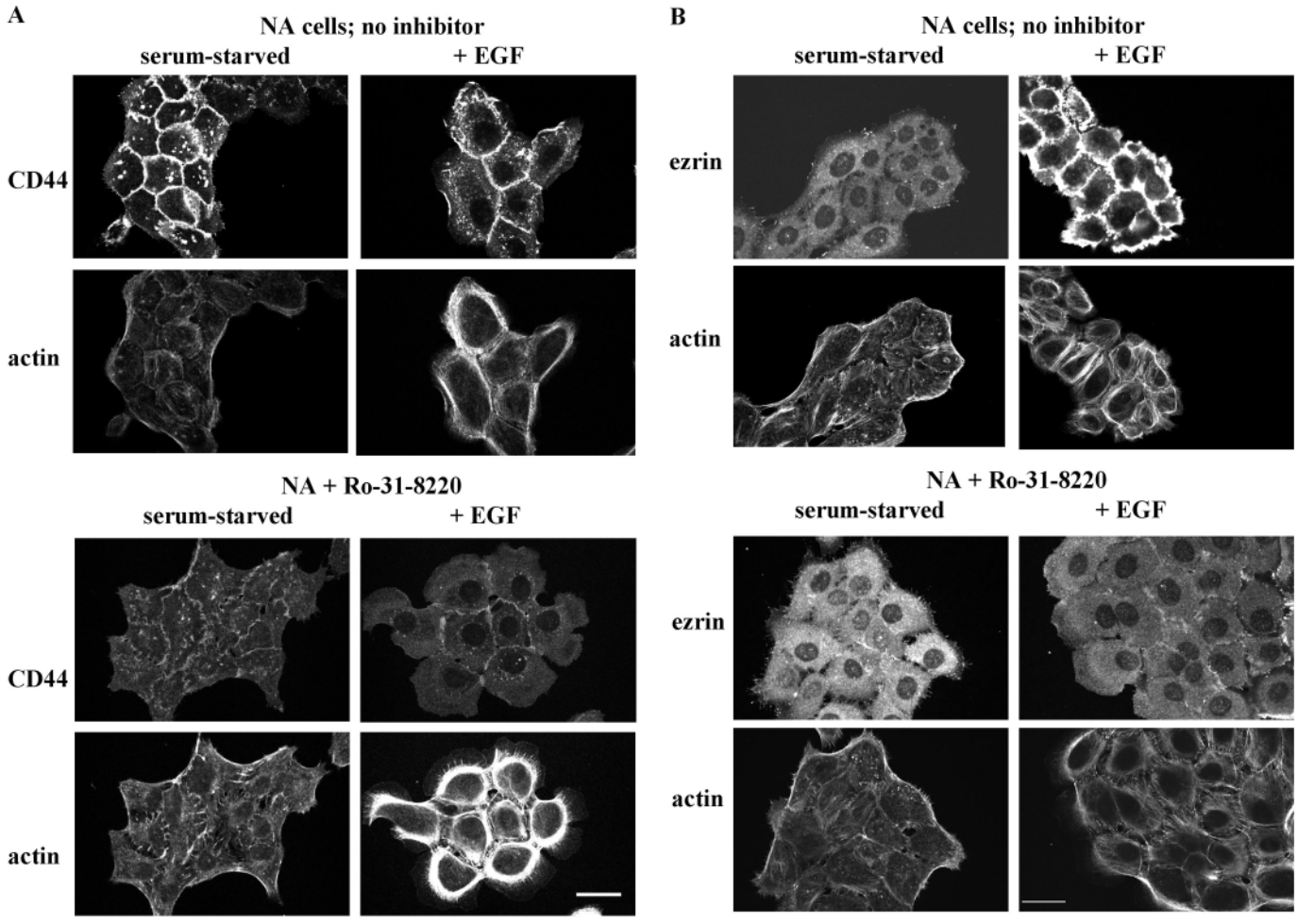

Fig. 4. Effect of Protein Kinase C inhibition on CD44 and ezrin localisation in A431 cells. NA cells were serum starved for 2 days then pretreated with or without 3 μM Ro-31-8220 for 30 minutes, followed by treatment with 10 ng/ml EGF for 5 minutes. Cells were fixed then immunostained for either CD44 (A) using the E1/2 monoclonal antibody and counterstaining with FITC-conjugated phalloidin or ezrin (B) using a polyclonal ezrin antibody and counterstaining with TRITC-conjugated phalloidin. Bar, 10 μm.

Ro-31-8220 (identical results were obtained using another PKC inhibitor, GF109203X) (data not shown).

Serum-starved cells were pretreated with 3 μM Ro-31-8220 for 30 minutes before EGF treatment and fixation. In both EGF-treated and untreated cells, CD44 localisation is affected (Fig. 4A). After inhibitor pre-treatment, serum-starved cells have a disrupted actin cytoskeleton, and the cells appear to be detaching from each other. CD44 staining is reduced in cell-cell contacts and appears as a uniform signal over the cell surface (Fig. 4A). However, when the same inhibitor-treated cells were stained for E-cadherin, a marker for the integrity of cell-cell contacts, the same disrupted staining pattern resulted (data not shown). This suggests that the effect of the PKC inhibitor was an indirect effect caused by disruption of cell-cell contacts, and we conclude that the PKC inhibitor has not directly affected CD44 localisation in untreated cells.

Upon EGF treatment, however, Ro-31-8220-treated A431 cells still reorganise the actin cytoskeleton, producing large membrane lamellipodia and cortical actin polymerisation, yet CD44 remains localised to cell-cell contacts and does not mobilise to the newly formed membrane structures (Fig. 4A). EGF-induced CD44 localisation is therefore directly dependent on PKC activity and is not an indirect consequence of effects on the actin cytoskeleton. Ezrin distribution in A431 cells is again affected by the presence of the PKC inhibitor (Fig. 4B). In Ro-31-8220-treated serum-starved cells, ezrin remains cytosolic; however, upon EGF treatment, ezrin fails to efficiently relocalise to the plasma membrane even though lamellipodia formation and cortical actin polymerisation have occurred (Fig. 4B). We conclude that CD44 and ezrin localisation requires PKC activity and suggest that the failure of CD44 and ezrin to localise in TA cells may be caused by aberrant PKC signalling in the cells.

Phorbol ester activation of conventional and novel Protein Kinase C is insufficient for relocalisation of ezrin and CD44

We next established whether activation of PKC alone is sufficient to relocalise both proteins and whether direct activation of PKC in TA cells would result in ezrin or CD44 relocalisation. This was achieved by determining the distribution of CD44 and F-actin (Fig. 5A,B) and ezrin (Fig. 5C,D) in NA and TA cells before and after treatment with phorbol ester (TPA), a potent activator of conventional and novel PKC isoforms. Treatment of NA cells results in actin concentrating in sites of cell-cell contact and an increase in actin cables, with little or no membrane ruffling observed (Fig. 5A). This is consistent with a previous report describing the effect of phorbol ester on the actin cytoskeleton in A431 cells (Vaaraniemi et al., 1999). Interestingly, TPA treatment of TA cells results in the same reorganisation of the actin cytoskeleton (Fig. 5B). Although TPA- and EGF-induced reorganisation of the actin cytoskeleton is different (compare Fig. 4A,B upper

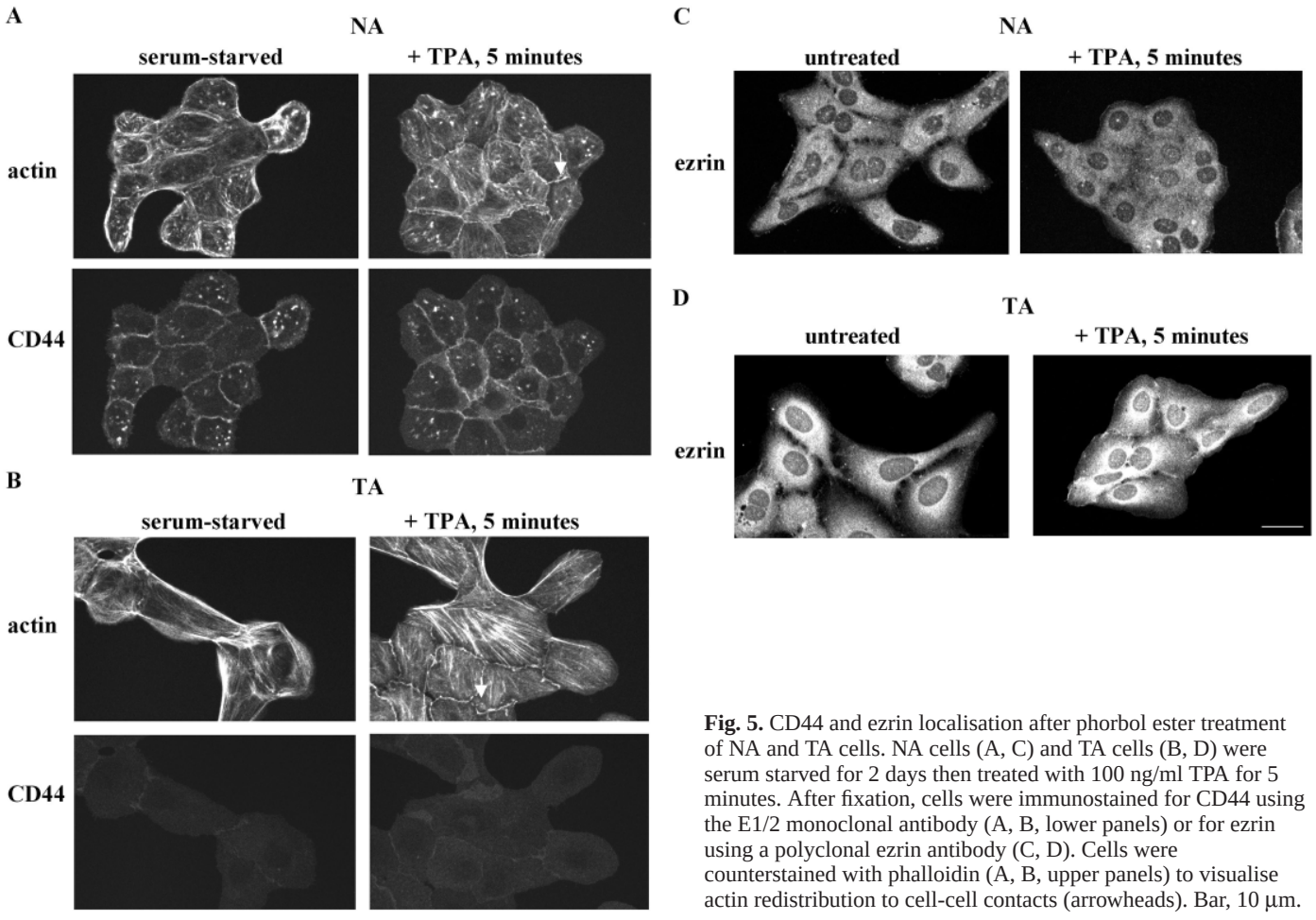

Fig. 5. CD44 and ezrin localisation after phorbol ester treatment of NA and TA cells. NA cells (A, C) and TA cells (B, D) were serum starved for 2 days then treated with 100 ng/ml TPA for 5 minutes. After fixation, cells were immunostained for CD44 using the E1/2 monoclonal antibody (A, B, lower panels) or for ezrin using a polyclonal ezrin antibody (C, D). Cells were counterstained with phalloidin (A, B, upper panels) to visualise actin redistribution to cell-cell contacts (arrowheads). Bar, 10 μm.

panels with Fig. 5A), it is clear that PKC can be activated in TA cells and can transduce signals to effector molecules required for reorganisation of the actin cytoskeleton.

TPA treatment however has no effect on CD44 distribution in either NA or TA cells. CD44 remains tightly associated with cell junctions and basal surface plaques in NA cells (Fig. 5A), and no change in organisation or intensity of CD44 staining was seen in TA cells treated with TPA (Fig. 5B). Similarly, ezrin distribution is not affected by TPA treatment of either NA or TA cells (Fig. 5C,D). Ezrin remains cytosolic in the presence of TPA, even though PKC activation has resulted in actin cytoskeletal rearrangements. Therefore, PKC is necessary for CD44 and ezrin localisation to the plasma membrane of A431 cells; however, additional signal(s) from the EGF receptor are required, and these signals are not delivered in TA cells. These conclusions, however, do not extend to atypical PKC isoforms that are not activated by phorbol ester.

Aberrant Protein Kinase C isoform expression in AP-1-deficient TA cells

In light of the effects seen using PKC inhibitors on CD44 and ezrin localisation, we next investigated whether expression of TAM67 resulted in changes to PKC isoform expression. Liu et al. have previously shown that a single representative isoform from each PKC subgroup is expressed in A431 cells (conventional, PKCα; novel, PKCδ; atypical, PKCζ) (Liu et al., 1994). While PKCα and PKCδ expression levels are unchanged in TA cells, PKCζ expression is reduced three-fold in TA cells (Fig. 6A). More significantly, however, we found that an extra novel PKC isoform, PKCθ, is expressed in TA cells (Fig. 6A). The 3.4 kb PKCθ transcript is also upregulated in TA cells and absent from NA cells, indicating transcriptional upregulation of PKCθ (Fig. 6B). We have confirmed PKCθ upregulation by western analysis of a number of independent TA cell lines, finding that all cell lines expressing TAM67 also express PKCθ (data not shown). Together, these data establish that PKC expression levels and isoform profile are altered in TA cells by the addition of an extra novel isoform and suggest that signalling pathways through PKC may too be affected.

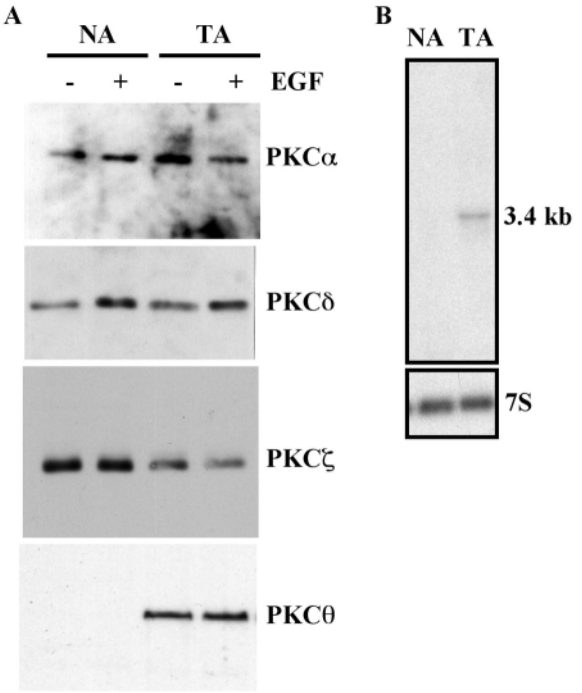

Fig. 6. Protein Kinase C isoform expression in NA and TA cells. (A) NA and TA cells were serum starved for 2 days then left untreated or treated with 10 ng/ml EGF for 5 minutes. Whole cell extracts were prepared and analysed by western blotting using antibodies for PKCα, PKCδ, PKCζ and PKCθ. (B) Total RNA was prepared from NA and TA cells then 20 μg samples were electrophoresed, blotted then probed with a radiolabelled PCR fragment of human PKCθ. Equivalent loading of RNA samples was demonstrated using a 7S ribosomal cDNA probe.

PKCθ translocates to the cellular particulate fraction upon EGF treatment

In order to determine whether PKCθ is activated in response to EGF and whether the increased expression of an extra isoform might interfere with the activation of endogenous PKC isoforms, we examined translocation of PKC isoforms from the cytosol to the plasma membrane after treatment with EGF or TPA. Soluble and insoluble protein fractions were prepared from serum-starved, EGF-treated and TPA-treated NA cells and the same for TA cells, followed by western analysis to determine the relative distribution of each PKC isoform (Fig. 7A).

Phorbol ester treatment of A431 cells results in the expected shift of PKCα from the cytosol to the membrane fraction (Fig. 7A). TA cells treated with TPA are also capable of translocating PKCα, indicating that signalling events independent of the EGF receptor function equivalently in A431 and TA cells. There is no detectable PKCα in the membrane fraction after EGF treatment of NA or TA cells. It may be that only a very small, undetectable fraction of PKCα translocates after EGF treatment or that EGF does not activate PKCα. Nevertheless, there is no difference in the cellular partitioning of PKCα between NA and TA cells.

In unstimulated NA cells, PKCδ can be detected in both the cytosolic and membrane fractions. Treatment with TPA results in a small increase in PKCδ in the particulate fraction; however EGF treatment does not result in any significant change in the amount of PKCδ in either fraction (Fig. 7A). The distribution of PKCδ is equivalent in NA and TA cells. These data suggest either that PKCδ is not being activated in response to EGF or that the sensitivity of this assay is such that a small change in cellular localisation will not be detected. Nevertheless, there is no difference in PKCδ fractionation in response to EGF by either cell type. Atypical PKCζ distribution remains largely cytosolic, with only a small amount detected in the membrane fraction (Fig. 7A). This partitioning pattern is conserved in the TA cells and does not change after TPA or EGF treatment. This is consistent with the inability of PKCζ to bind phorbol ester and has been seen also in fibroblasts overexpressing PKCζ (Goodnight et al., 1995).

We next examined the distribution of PKCθ in TA cells to determine whether it was activated by EGF. In unstimulated cells, PKCθ is detected in both the cytosolic and membrane fractions; however after either TPA or EGF treatment, there is a large shift of PKCθ to the membrane fraction (Fig. 7A), indicating PKCθ activation in TA cells treated with EGF. In

summary, the fractionation of PKC after growth factor treatment shows that there is no gross effect on endogenous PKC membrane translocation owing to the expression of PKCθ. PKCθ, however, is clearly sent to the membrane of EGF-treated TA cells, indicating activation of the kinase in response to growth factors.

EGF-induced phosphorylation of endogenous PKCα and δ are equivalent in NA and TA cells

A parallel approach to confirm the activation state of endogenous PKC in NA and TA cells utilised phosphospecific PKC antibodies. A phosphoPKC (pan) antibody capable of detecting a subset of phosphorylated PKC isoforms was used in western analysis of EGF- and TPA-treated NA and TA cell lysates (Fig. 7B). The antibody efficiently recognised phosphorylated PKCα in response to both EGF and TPA in both cell types. This is inconsistent with the fractionation data where EGF fails to relocalise PKCα to the membrane; however it may be that the sensitivities of each assay differ such that only a small undetectable amount of PKCα fractionates to the membrane. Neither TPA or EGF resulted in an increased phosphorylation of PKCδ in either NA or TA cells, suggesting that PKCδ is not efficiently activated in A431 cells at the timepoint tested. Nevertheless, it is evident that the activation profile of PKCα and PKCδ by EGF is equivalent in both NA and TA cells, and therefore the expression of PKCθ in TA cells does not appear to affect activation of endogenous conventional or novel PKC isoforms.

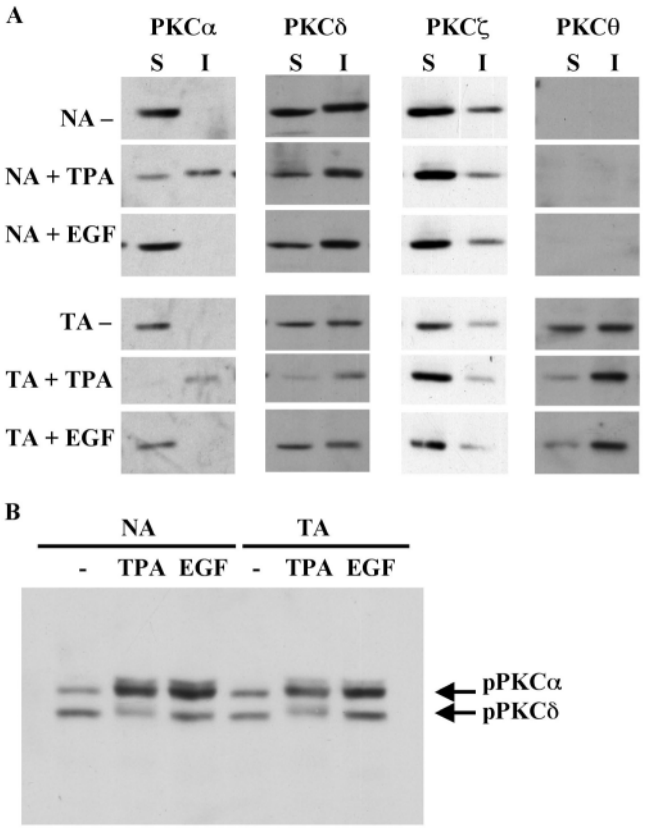

Fig. 7. Protein Kinase C activation in NA and TA cells. (A) NA and TA cells were serum starved for 2 days then either left untreated or treated with 10 ng/ml EGF or 100 ng/ml TPA for 5 minutes. Cells were scraped into lysis buffer and soluble (S) and insoluble (I) fractions were prepared as described in the Materials and Methods. Equal amounts of each fraction were electrophoresed on 10% SDS-PAGE gels and analysed by western blotting using antibodies recognising PKCα, PKCΔ, PKCζ and PKCθ. (B) PKC phosphorylation in NA and TA cells. Whole cell extracts were prepared from NA and TA cells that had been serum starved for 2 days then left either untreated or treated with 10 ng/ml EGF or 100 ng/ml TPA for 5 minutes. Western analysis followed using polyclonal antibodies recognising phosphoPKC (pan) antibody, which, in A431 cells, recognises phosphoPKCα and phosphoPKCδ only. This was confirmed by probing adjacent lanes with PKCα and PKCδ antibodies and showing co-migrating bands (data not shown).

Expression of PKCθ in A431 cells mimics aspects of the TA phenotype

To determine whether any of the effects described in TA cells were attributable to PKCθ, we established stable A431 cell

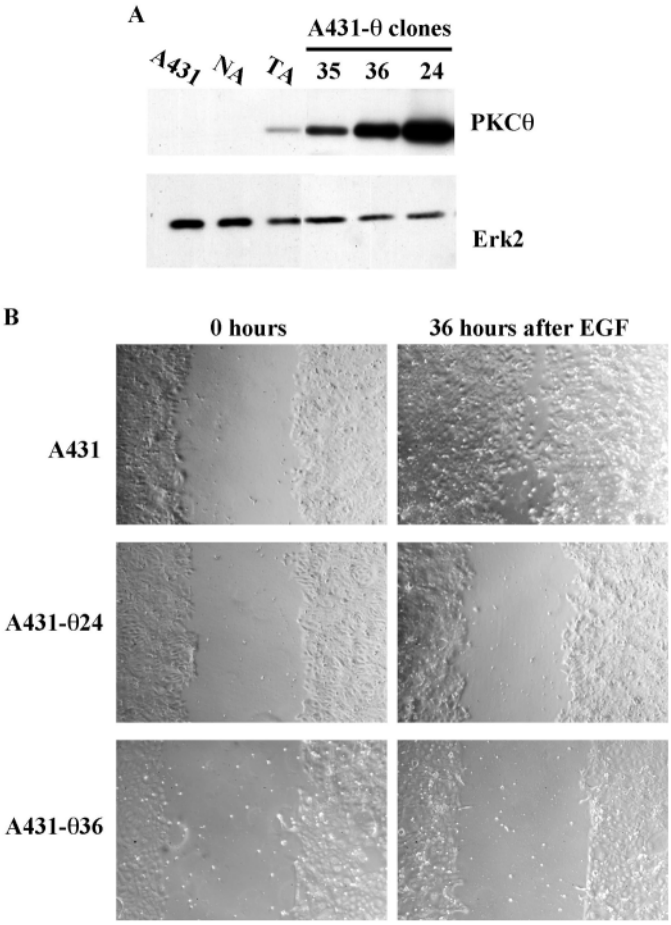

Fig. 8. PKCθ expression in A431 cells mimics aspects of TAM67 expression. (A) Protein lysates from A431, NA, TA and three A431-θ lines were analysed by western blotting to determine relative expression levels of PKCθ. The filter was reprobed with an Erk2 antibody to demonstrate equivalent protein levels in each lane. (B) Confluent monolayers of A431 and A431-θ cells (clones 24 and 36) were serum starved for 2 days, then wounded using a plastic tip. Cells were treated with 10 ng/ml EGF, then photographed after 36 hours.

lines expressing PKCθ (A431-θ) (Fig. 8A). Because TA cells are non-motile and non-invasive, we determined firstly whether motility had been similarly affected by PKCθ expression. EGF induces the closure of a wound made in a monolayer of serum-starved A431 cells after 36 hours; however, A431-θ cell lines are unable to migrate into the wound (Fig. 8B), indicating a defect in cell motility. Expression of PKCθ is therefore sufficient to disrupt EGF-receptor-directed signalling to implement the invasion programme.

We next examined the effect of PKCθ expression on EGF-induced ezrin (Fig. 9) and CD44 (Fig. 10) localisation, together with actin cytoskeletal rearrangements, by immunofluorescence. While the distribution of actin, ezrin and CD44 appears unaffected in serum-starved A431-θ cells (Fig. 9A, Fig. 10A), confocal sections perpendicular to the substratum reveal that A431-θ cells are significantly flatter than A431 cells (Fig. 9B, Fig. 10B). Upon EGF treatment, A431-θ cells do round up and increase their height in response to EGF; however this is still reduced compared with EGF-treated A431 cells. EGF-treated TA cells similarly do not round or increase in height compared with A431 cells. Cell rounding in EGF-treated A431 cells is a consequence of F-actin cortical polymerisation and cell contraction. PKCθ expression in A431-θ cells similarly has effects on actin cytoskeletal reorganisation. F-actin cortical polymerisation and cell contraction are reduced in A431-θ cells. More significantly, very little membrane ruffling is produced in response to EGF; however A431-θ cells still form apical microvilli (Figs 9 and 10). This is in contrast to TA cells where neither structure is formed. PKC-θ therefore has partially mimicked TAM67 expression by affecting a subset of cytoskeletal events but still results in non-motile cells.

Ezrin and CD44 localisation is partially affected in EGF-treated A431θ cells. There is reduced membrane localisation by both proteins in response to EGF (Fig. 9A, Fig. 10A); however, CD44 and ezrin can localise to apical microvilli (Fig. 9B, Fig. 10B). In both cases, the absence of membrane ruffles accounts for the lack of either protein at the membrane. Again this partially mimics the effect of TAM67 expression, where extension of membrane ruffles does not occur, and consequently the mislocalisation of CD44 and ezrin in TA cells. PKCθ expression therefore has disrupted ezrin and CD44 localisation to membrane ruffles, and this may be an indirect effect resulting from effects on the actin cytoskeleton. When membrane structures, such as microvilli, are formed, ezrin and CD44 are able to localise. We conclude that PKCθ expression in A431 cells contributes to the disruption of cell invasion by TAM67, most probably through effects on the actin cytoskeleton, which in turn, affects membrane ruffling and therefore localisation of CD44 and ezrin to these structures and consequently cell motility.

Discussion

The process of cell invasion requires the co-ordinate expression and interaction of an invasion-specific proteome, which, working in a coordinated manner, facilitates cell invasion. This genetic programme of invasion requires not only changes in gene expression but also the implementation of new signalling pathways to regulate new gene products in the context of cell invasion. This has been demonstrated by the regulated colocalisation of CD44 and ezrin and their deregulated localisation in cells where AP-1 activity is inhibited. Thus, AP-1 controls cell invasion on at least two levels. Firstly, AP-1 regulates the expression of genes directly necessary for cell invasion, such as matrix metalloproteases, and adhesion proteins. AP-1 regulates the invasion process at a second level by controlling the expression of intracellular signalling proteins necessary for the correctly regulated function of new gene products in the invasion programme.

Constitutive expression of dominant-negative Jun (TAM67)

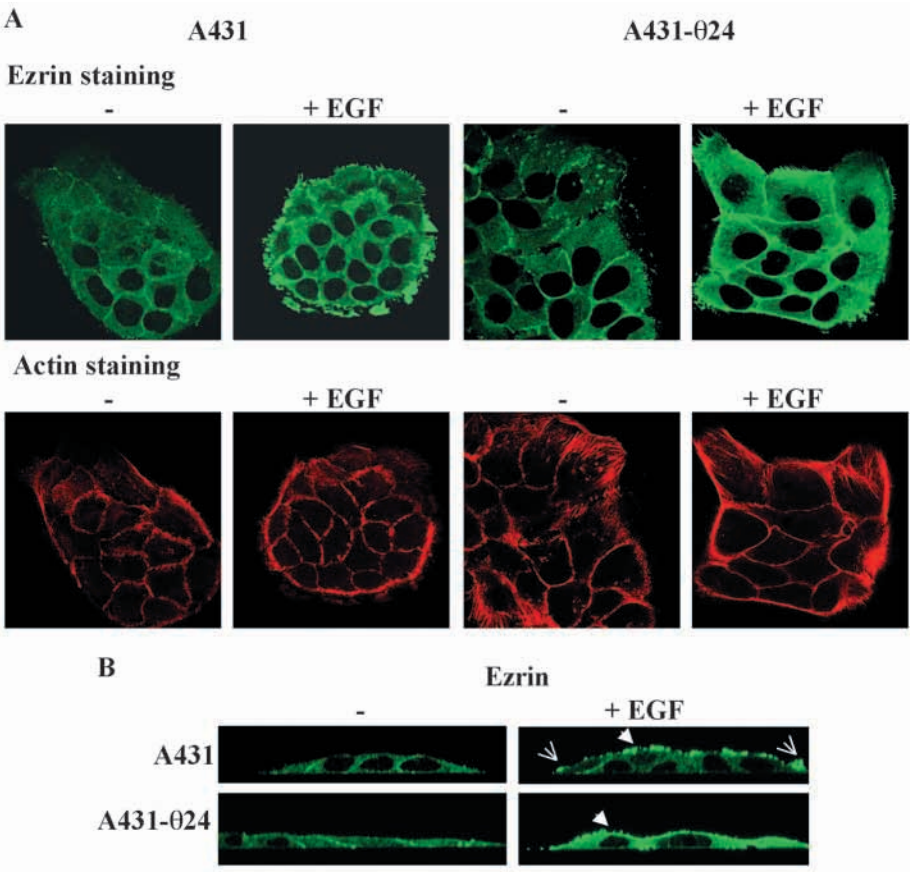

Fig. 9. PKCθ expression affects correct ezrin localisation in response to EGF. (A) A431 and A431-θ cells (clone 24) were plated onto coverslips, serum starved for 2 days, then left untreated or treated with 10 ng/ml EGF for 5 minutes. Cells were stained for actin (red) or ezrin (green) and analysed by confocal microscopy. (B) Confocal sections perpendicular to the substratum were taken to visualise ezrin localisation to microvilli (closed arrow) and membrane ruffles (open arrow).

results in changes in gene expression, some of which will be through directly affecting AP-1 activity, whereas others may be the result of interfering with the expression and activity of other transcription factors (e.g. Li et al., 2000). CD44 is a known AP-1 target gene, and downregulation of its expression might have been expected in TA cells. However, we have found that expression of TAM67 is incapable of downregulating 14% of genes that are upregulated in v-*fos* expressing fibroblasts (Johnston et al., 2000). Our findings are also consistent with others where expression of dominant-negative versions of c-jun (Young et al., 1999) or JunD (Ui et al., 2000) does not affect expression of all AP-1 targets. It is worth noting nevertheless that when used in an inducible system TAM67 was able to revert expression of two AP-1-dependent genes (Li et al., 2000). The future use of an inducible TAM67 in A431 cells could extend our findings, revealing the timing of PKCθ expression and indeed whether PKCθ upregulation is a direct consequence of downregulated AP-1.

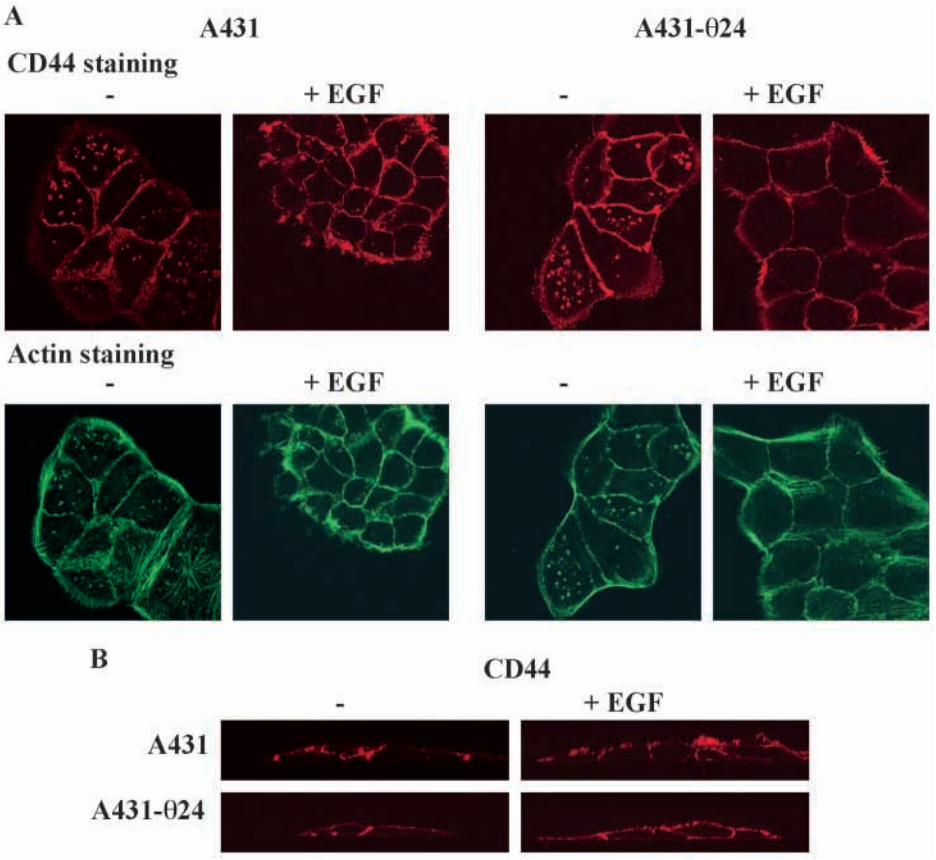

Fig. 10. EGF-induced CD44 localisation is disrupted in A431-θ cells. (A) A431 and A431-θ cells (clone 24) were plated onto coverslips, serum starved for 2 days, then left untreated or treated with 10 ng/ml EGF for 5 minutes. Cells were stained for actin (green) or CD44 (red) and analysed by confocal microscopy. (B) Confocal sections perpendicular to the substratum were taken to visualise CD44 distribution to apical structures.

Inhibiting AP-1 activity by TAM67 in A431 cells results in the failure of the EGF receptor to colocalise CD44 and ezrin to the plasma membrane. Here we show that PKC activity is required for CD44 and ezrin translocation. Further, TAM67 expression is associated with abnormal PKC expression and function. We have previously shown that downregulation of AP-1 activity in A431 cells similarly results in the uncoupling of signalling events from the EGF receptor to the reorganisation of the actin cytoskeleton (Malliri et al., 1998). These events were shown to be dependent on Rac and Rho GTPase activities, and these signalling pathways failed to correctly signal to the cytoskeleton in TA cells. We have subsequently shown that Rac and Rho GTPases are required for CD44 and ezrin localisation, suggesting that an intact cytoskeleton is required for their localisation (G.S., unpublished). Nevertheless, the requirement for PKC activity for CD44 and ezrin localisation is independent of actin cytoskeletal rearrangements, as CD44 and ezrin both fail to localise to the plasma membrane in the presence of PKC inhibitors, even though actin polymerisation and lamellipodial extensions are produced (Fig. 4A,B). Thus two signalling pathways downstream of the EGF receptor are perturbed in TAM67-expressing cells, each presumably contributing to the non-invasive state of the cells.

There are several mechanism(s) through which the introduction of an extra PKC isoform in TA cells could affect endogenous PKC function. Firstly, PKCθ might block the function of endogenous isoforms either by competing for upstream activators or by binding to PKC-binding partners and substrates. PKCθ is capable of phosphorylating the conserved C-terminal threonine of the ERM family member, moesin and ezrin (Simons et al., 1998). Although it seems contradictory that ezrin should not localise in TA cells if PKCθ is now expressed, it is known that other events, such as phosphatidyl-inositol 4,5-bisphosphate [PtdIns(4,5)P_2]-binding is required for ezrin localisation (Barret et al., 2000), and it may be that additional signals, including those to localise the kinase, are lacking in TA cells. Nevertheless, it cannot be predicted how PKCθ will behave in epithelial cells in response to EGF receptor signalling compared to its function in T cells.

Secondly, PKCθ could phosphorylate a new set of substrates in the cell, independently of endogenous PKC function, which somehow interferes with the correct localisation of CD44 and ezrin. Thirdly, PKCθ may interfere with signalling from the EGF receptor by interacting with signalling molecules that are not normally associated with endogenous PKC in A431 cells. To this end, PKCθ is known to associate with a number of signalling molecules, including Vav1, a GDP-exchange factor for Rac (Moller et al., 2001), and the Src family tyrosine kinase, Lck (Liu et al., 2000).

The data presented suggest that both ezrin and CD44 receive some common regulatory signal(s) to determine their cellular localisation and subsequent function; however it may be that

signalling to one protein may suffice to localise the other. The phosphorylation of CD44 appears to be complex, and a number of kinases have been described as capable of phosphorylating CD44. ROKα is another candidate kinase for both CD44 and ezrin phosphorylation (Bourguignon et al., 1999; Matsui et al., 1998); however, we have found no differences in ROKα expression levels in TA and NA cells (G.S., unpublished). Ezrin phosphorylation at threonine 567 by PKCα has been demonstrated in a breast cancer cell line (Ng et al., 2001). Although PKCα phosphorylation is evident in A431 cells we do not see an accompanying shift to the insoluble fraction even though phorbol ester induces efficient translocation. It seems likely that EGF is an inefficient activator of PKCα and that only a small amount is translocated.

The inhibition of cell motility, membrane ruffling and associated localisation of CD44 and ezrin to these structures by PKCθ expression supports its contribution to the effects attributed to TAM67. Indeed, the inhibition of membrane ruffling suggests that PKCθ may interfere with signalling through Rac GTPase. Further investigation of the mechanism of inhibition by PKCθ will reveal not only the endogenous signalling pathways required for invasion that PKCθ has affected but will also shed light on the potential of PKCθ as a novel suppressor of cell invasion.

We are especially grateful to Paul Mangeat for providing the ezrin antibody and comments, to Clare Isacke for invaluable reagents and discussions and to Amnon Altman for providing the PKCθ expression plasmid. We thank members of R10 for helpful contributions, Peter McHardy for help with confocal microscopy, Walter Kolch for valuable discussions and Linda Scott, Margaret Frame and John Wyke for comments on the manuscript. This work was supported by the Cancer Research UK and The West of Scotland Women's Bowling Association.

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