

The role of protein kinase C δ activation and STAT3 Ser727 phosphorylation in insulin-induced keratinocyte proliferation

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Summary

Activation of the STAT family of transcription factors is regulated by cytokines and growth factors. STAT tyrosine and serine phosphorylation are linked to the transcriptional activation and function of STAT. We have previously described a unique pathway inducing keratinocyte proliferation, which is mediated by insulin stimulation and depends on protein kinase C δ (PKC δ). In this study, we assessed STAT3 activation downstream of this pathway and characterized the role of PKC δ activation in STAT3 tyrosine and serine phosphorylation and keratinocyte proliferation. Following insulin stimulation, STAT3 interacted with PKC δ but not with any other PKC isoform expressed in skin. Activated forms of PKC δ and STAT3 were essential for insulin-induced PKC δ -STAT3 activation in keratinocyte proliferation. Abrogation of PKC δ activity inhibited insulin-induced STAT3

phosphorylation, PKC δ -STAT3 association and nuclear translocation. In addition, overexpression of STAT3 tyrosine mutant eliminated insulin-induced PKC δ activation and keratinocyte proliferation. Finally, overexpression of a STAT3 serine mutant abrogated insulin-induced STAT3 serine phosphorylation and STAT3-induced keratinocyte proliferation, whereas STAT3 tyrosine phosphorylation was induced and nuclear localization remained intact. This study indicates that PKC δ activation is a primary regulator of STAT3 serine phosphorylation and that PKC δ is essential in directing insulin-induced signaling in keratinocyte proliferation.

Key words: STAT3, PKC δ , Tyrosine phosphorylation, Serine phosphorylation, Keratinocyte, Proliferation

Introduction

STATs (signal transducers and activators of transcription) are cytoplasmic proteins that function as transcriptional activators. Seven STAT family members have now been identified including: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Akira, 1999; Akira, 2000; Horvath, 2000; Levy and Darnell, 2002). The use of isoform-specific transgenic knockout mouse models defined discreet physiological roles for each of the STAT family members. Among the seven known STAT proteins, STAT3 is unique. Only the STAT3-null phenotype in mice results in an embryonic-lethal phenotype at day 6-7 of fetal development, which cannot be compensated by any of the other STAT species expressed within the visceral endoderm (Akira, 1999). Furthermore, the importance of STAT3 for specific organ development was confirmed by experiments in which STAT3 expression was conditionally ablated in defined organs (Bromberg and Darnell, 2000; Sano et al., 2000; Takeda et al., 1997; Wen and Darnell, 1997). STAT3 has a specialized role in skin development. In the absence of STAT3 expression, both skin remodeling and hair cycle progression are severely disrupted (Sano et al., 1999; Sano et al., 2000).

The activation of STAT proteins involves tyrosine

phosphorylation, dimerization, nuclear translocation and activation of transcription by binding to DNA-response elements of target genes (Bromberg and Darnell, 2000; Levy and Darnell, 2002). The STAT proteins are distinctive among transcription factors in containing an SH2 (Src-homology 2), phosphotyrosine-binding domain. The SH2 domain interacts with sites of tyrosine phosphorylation to recruit the STATs to receptor complexes. Each of the STATs is differentially activated by various extracellular ligands including growth factors, cytokines and hormones, allowing differential intracellular processing of transcriptional signals (Bromberg and Darnell, 2000). During activation, STAT proteins can be specifically phosphorylated on both tyrosine and serine residues. Tyrosine phosphorylation of STAT3 at a single tyrosine residue (Tyr705) located at the Src-homology domain is essential for the activation of STAT3 (Bromberg et al., 1998). Similarly, a single serine site (Ser727) located in a conserved Pro-X-Ser-Pro sequence was shown to regulate STAT3-mediated transcriptional activation (Bromberg et al., 1998; Decker and Kovarik, 2000; Lim and Cao, 1999; Turkson et al., 1999). However, although STAT serine phosphorylation was shown to be induced by several factors including interferon, epidermal growth factor and IL-6, its exact role in regulation

of the STAT3 activation state has not been fully determined (Heim, 1999; Takeda et al., 1998; Zhang et al., 2000). In skin, the growth and differentiation of keratinocytes was shown to be regulated by many growth factors and cytokines including EGF family members, keratinocyte growth factor (KGF), TGF- β , insulin, insulin-like growth factor-1 (IGF-1), PDGF, HGF, IL-6, IL-1 and TNF- α (Chen et al., 1995; Marchese et al., 1990; Shen et al., 2001; Wertheimer et al., 2001; Zendegui et al., 1988). Interestingly, in skin, as in other cellular model systems, STAT3 is activated by several of these growth factors including the EGF family of proteins, HGF, PDGF and insulin, as well as by cytokines of the interleukin family such as IL-6 and IL-11 (Hashimoto, 2000; Heinrich et al., 1998; Senaldi et al., 1999; Werner and Smola, 2001).

The protein kinase C (PKC) family of serine-threonine kinases plays an important regulatory role in a variety of biological phenomena (Dekker and Parker, 1994; Toker, 1998). The family is composed of at least 11 individual isoforms. In skin, protein kinase C (PKC) signaling was shown to be a major intracellular mediator of proliferation and differentiation pathways (Denning et al., 1996; Dotto, 1998; Matsui et al., 1992; Ohba et al., 1998). Utilizing both pharmacological activators of PKC as well as genetic approaches, we and others identified distinct roles for specific PKC isoforms in the induction of keratinocyte proliferation and differentiation in vivo and in vitro (Alt et al., 2001; Dlugosz et al., 1992; Dotto, 1998; Verma, 1988). Specifically, we previously showed that growth factors such as insulin-like growth factor-1 (IGF-1) and insulin, although similar in their structure, diverge in their downstream signaling to mediate skin keratinocyte proliferation. Furthermore, insulin-induced proliferation is uniquely mediated by activation of PKC δ (Jain et al., 1999; Novotny-Diermayr et al., 2002; Shen et al., 2001). In this study we demonstrate STAT3 activation and serine phosphorylation downstream of insulin-induced PKC δ activation and keratinocyte proliferation. However, although PKC δ activation is indispensable for insulin action in keratinocytes proliferation, eliminating STAT3 serine phosphorylation does not abrogate insulin-mediated PKC δ activation and keratinocyte proliferation.

Results

The role of STAT3 in signaling of keratinocyte proliferation

In previous studies, we identified a unique pathway involved in mediating insulin action in keratinocytes. This pathway uses the activation of PKC δ as a crucial signaling factor downstream of insulin to induce keratinocyte proliferation. One of the factors involved downstream of PKC δ signaling and which is affected by various cytokines and growth factors, is STAT3 (Boulton et al., 1995; Jain et al., 1999). To determine whether insulin signaling in keratinocytes may mediate its effects via STAT3 activation, we first examined the ability of insulin to regulate the phosphorylation state of STAT3 in these cells. As early as 5 minutes following insulin stimulation, tyrosine phosphorylation of STAT3 was induced; phosphorylation peaked by 15 minutes following stimulation (Fig. 1A). The phosphorylation of STAT3 was accompanied by its nuclear translocation, as observed by immunofluorescence (Fig. 1B).

Specificity of STAT3 activation to the insulin-signaling pathway was further corroborated by following STAT3

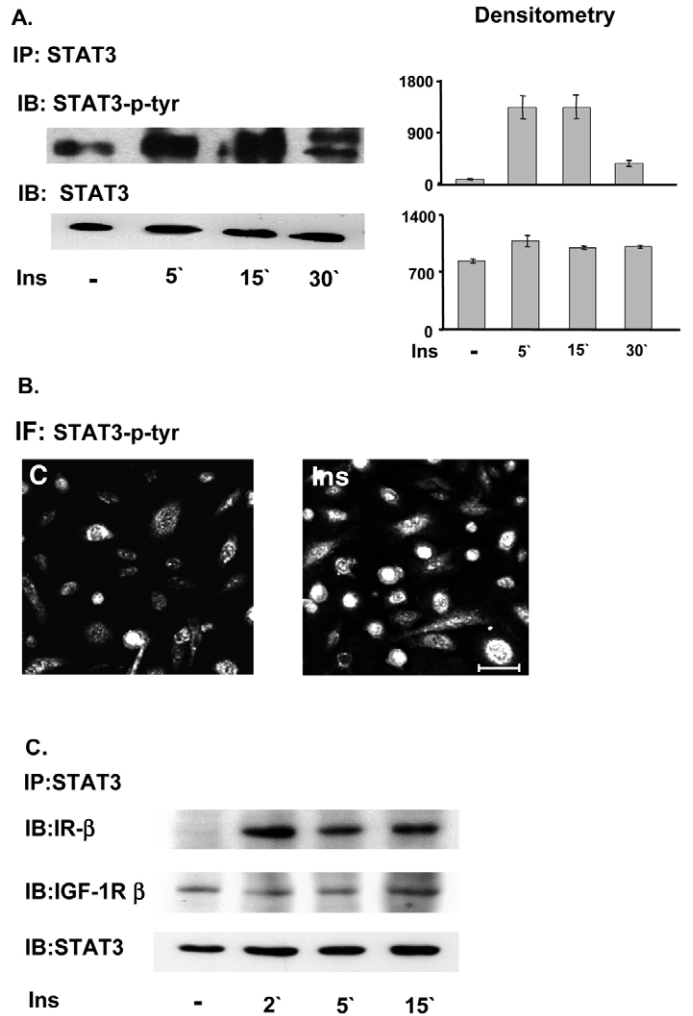
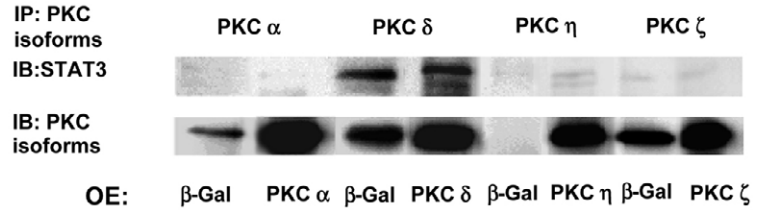


Fig. 1. Insulin induces STAT3 activation and nuclear translocation in mouse primary keratinocytes. (A) Keratinocytes following 5 days in culture were stimulated with 10^{-7} M insulin for the times indicated. STAT3 immunoprecipitates were probed with anti-p-tyr-STAT3 (top panel). Equal loading of gels was confirmed by reblotting with STAT3 antibody (bottom panel). Relative optical density of four representative blots is presented in arbitrary units (mean \pm s.d.). (B) Keratinocytes were plated on glass slides. Cultures (5 days old) were stimulated with insulin for 5 minutes, fixed in ethanol and analyzed by immunofluorescence, using anti-p-tyr-STAT3, followed by FITC-conjugated secondary antibody. Cells were viewed by confocal microscopy. (C) Keratinocytes were stimulated with 10^{-7} M insulin for the times indicated. STAT3 immunoprecipitates were probed with anti-IR β antibody (top panel) and with anti-IGF 1R β antibody (middle panel). Equal loading of gels was confirmed by reblotting with STAT3 antibody (bottom panel). The experiment was repeated twice. Bar, 20 μ m.

association with the insulin receptor. As early as 2 minutes following insulin stimulation, activated STAT3 was physically associated with the insulin receptor (Fig. 1C). Moreover, insulin did not induce association of STAT3 with the insulin-like growth factor-1 (IGF-1) receptor, suggesting that the specificity of insulin-induced STAT3 activation to insulin receptor mediated signaling (Fig. 1C).

Fig. 2. Protein kinase C δ (PKC δ) specifically associates with STAT3. Keratinocytes (5 day cultures) were infected with isoform-specific PKC α , δ , η and ζ recombinant adenoviruses, or with adenovirus encoding β -galactosidase (β -Gal) as a control, for 1 hour. OE, overexpression.

Following infection, cells were incubated for 24 hours, then extracted and immunoprecipitated (IP) with antibodies against PKC α , PKC δ , PKC η and PKC ζ . Immunoprecipitates were subjected to western blot analysis using isoform-specific anti-PKCs or anti-STAT3 antibodies. Results presented are representative of at least three experiments.



Effects of insulin on the PKC δ -STAT3 complex

Our previous studies identified PKC δ as a primary mediator of insulin signaling in keratinocyte proliferation. Since PKC δ activation was previously linked to STAT3 signaling, we next investigated whether the PKC δ isoform could physically bind to STAT3. Using recombinant PKC adenoviruses, keratinocytes were transduced with recombinant PKC adenoviruses where β -gal-infected keratinocytes were used as

controls for the viral infection (Miyake et al., 1996; Ohba et al., 1998). Lysates were subjected to immunoprecipitation with antibodies to PKC isoforms expressed in skin including: PKCs α , δ , ζ and η , and immunoprecipitates were then subjected to western blot analysis using a STAT3-specific antibody. The overexpression of various PKC isoforms effectively induced protein expression five- to tenfold above basal levels associated with constitutive activation of the PKCs isoforms (Fig. 2) (Alt et al., 2004). Under unstimulated basal conditions, STAT3 was associated with PKC δ but not with PKC isoforms α , ζ or η , expressed in skin (Fig. 2).

Furthermore, as can be seen in Fig. 3A, following stimulation by insulin, a dramatic increase of STAT3 serine phosphorylation was observed within the complex. Tyrosine phosphorylation was also increased, indicating STAT3 activation. However, no induction in STAT3 tyrosine or serine phosphorylation was observed in PKC immunoprecipitates of insulin-stimulated keratinocytes and keratinocytes overexpressing PKC isoforms α , ζ or η (Fig. 3B and results not shown). Although PKC-overexpressing keratinocytes expressed similar levels of each of the PKC isoforms, constitutive association and phosphorylation of STAT3 was evident only with PKC δ (Fig. 2).

A direct link between PKC δ activity and complex formation and activation was next confirmed by abrogation of PKC δ activity. Overexpression of a kinase inactive PKC δ form (dominant-negative PKC) reduced STAT3-PKC δ complex formation (Fig. 4A,B) and decreased both tyrosine

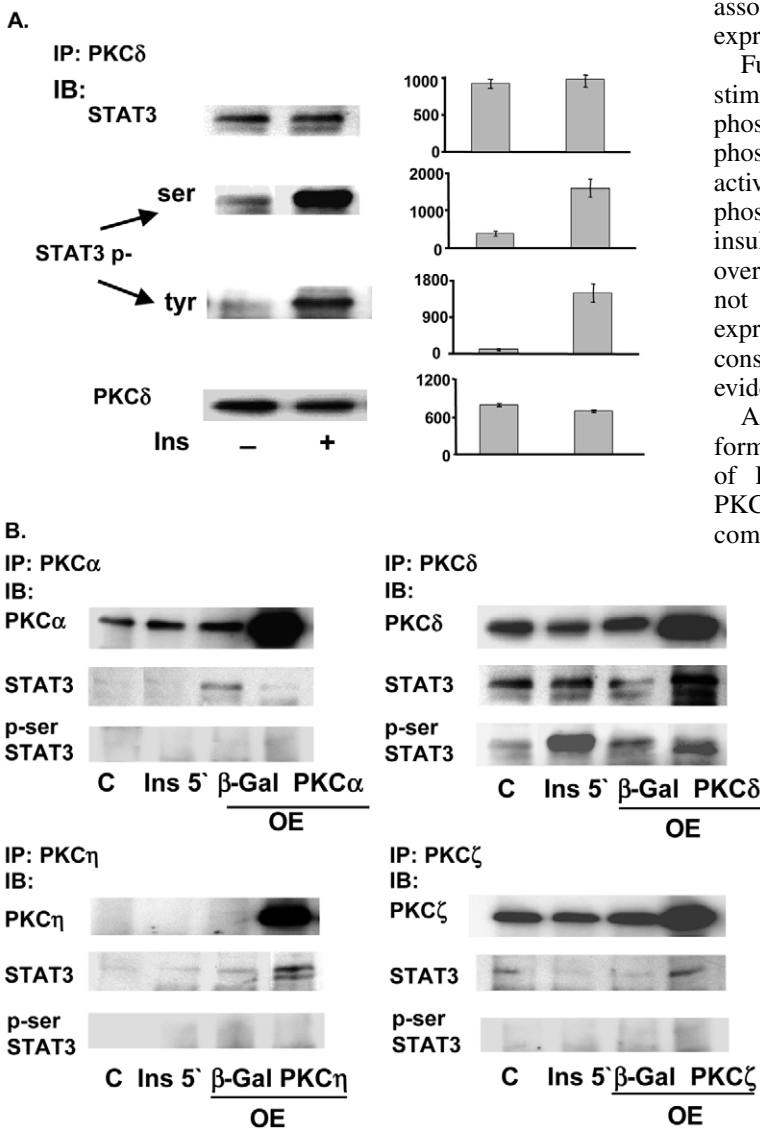


Fig. 3. Insulin-induced PKC δ activation regulates STAT3 association and phosphorylation. (A) Keratinocytes were untreated (-) or treated with insulin for 5 minutes (+). PKC δ immunoprecipitates were subjected to western blot analysis using antibodies against STAT3, anti-phosphotyrosine-705-STAT3 (p-tyr), anti-phosphoserine-727-STAT3 (p-ser) and anti-PKC δ . Relative optical densities of the blots are presented in arbitrary units. Relative optical density of four blots is presented in arbitrary units (mean \pm s.d.). (B) Primary keratinocytes were either untreated or infected for 1 hour with isoform-specific PKC recombinant adenovirus, or with β -galactosidase (β -Gal) adenovirus as control. Control cells were either untreated (C) or stimulated with insulin for 5 minutes (Ins 5'). Cells were extracted and immunoprecipitated (IP) with isoform-specific PKC antibodies. The immunoprecipitates were subjected to western blot analysis using anti-PKCs, anti-STAT3 or anti-phosphoserine-727-STAT3 (STAT p-ser) antibodies. Experiments were repeated three times.

and serine phosphorylation of STAT3 following insulin stimulation (Fig. 4B,C). Similar results were obtained when we inhibited PKC δ activity using the PKC δ inhibitor, rottlerin (results not shown). Collectively, these results confirm the link between insulin-induced PKC δ activation and the STAT3 phosphorylation state.

PKC δ -STAT3 association also depends on the activation of STAT3

Using a STAT3 wild-type construct and a dominant-negative STAT3 mutant (STAT3-705-tyrosine mutant), efficient expression of STAT3 could be achieved in keratinocytes, at levels 5- to 20-fold above basal expression (Fig. 5A). As expected, overexpressed wild-type STAT3 was constitutively phosphorylated on tyrosine residues and was found complexed with PKC δ . Similarly to the endogenous protein, insulin stimulation for 5 minutes further increased tyrosine phosphorylation of the overexpressed STAT3 (Fig. 5B). The importance of tyrosine residue 705 to the insulin-induced activation of STAT3 was further verified using a STAT3 tyrosine mutant in which Tyr705 was exchanged with phenylalanine. Following overexpression of the dominant-negative STAT3 tyrosine (705) mutant, insulin could no longer induce STAT3 tyrosine phosphorylation (Fig. 5B). This confirmed STAT3 Tyr705 to be essential for STAT3 activation by insulin (Fig. 5B). PKC δ and wild-type STAT3 were physically associated, and stimulation with insulin further augmented this association (Fig. 5C). Interestingly, overexpression of the tyrosine-inactive STAT3 mutant did not abrogate constitutive PKC δ -STAT3 complex formation. However, insulin-induced association was abolished, suggesting that STAT3 activation is not a prerequisite for complex formation with PKC δ . Similarly to the endogenous STAT3 protein, in keratinocytes overexpressing wild-type STAT3, inhibition of PKC δ activity by overexpression of a dominant-negative PKC δ decreased STAT3 tyrosine phosphorylation as well as its association with PKC δ (Fig. 5D). Similar results to those obtained by overexpressing a dominant-negative form of PKC δ were obtained using rottlerin, a specific inhibitor of PKC δ activation (results not shown). However, the STAT3 activation state also contributed to insulin-induced activation of PKC δ , as demonstrated in PKC activity assays. As early as 5 minutes following insulin stimulation, PKC δ activity was increased in PKC δ immunoprecipitates (Fig. 6A) and PKC activity was found to reside within STAT3 immunoprecipitates (Fig. 6B). Furthermore, dominant-negative STAT3-inhibited insulin-induced activation of endogenous and overexpressed PKC δ (Fig. 6A,B). Overall, these results suggest that the active form of PKC δ is required for the formation of the insulin-induced STAT3-PKC δ complex, and emphasize the crosstalk between PKC δ and STAT3 in regulating the activation state of the PKC δ -STAT3 complex.

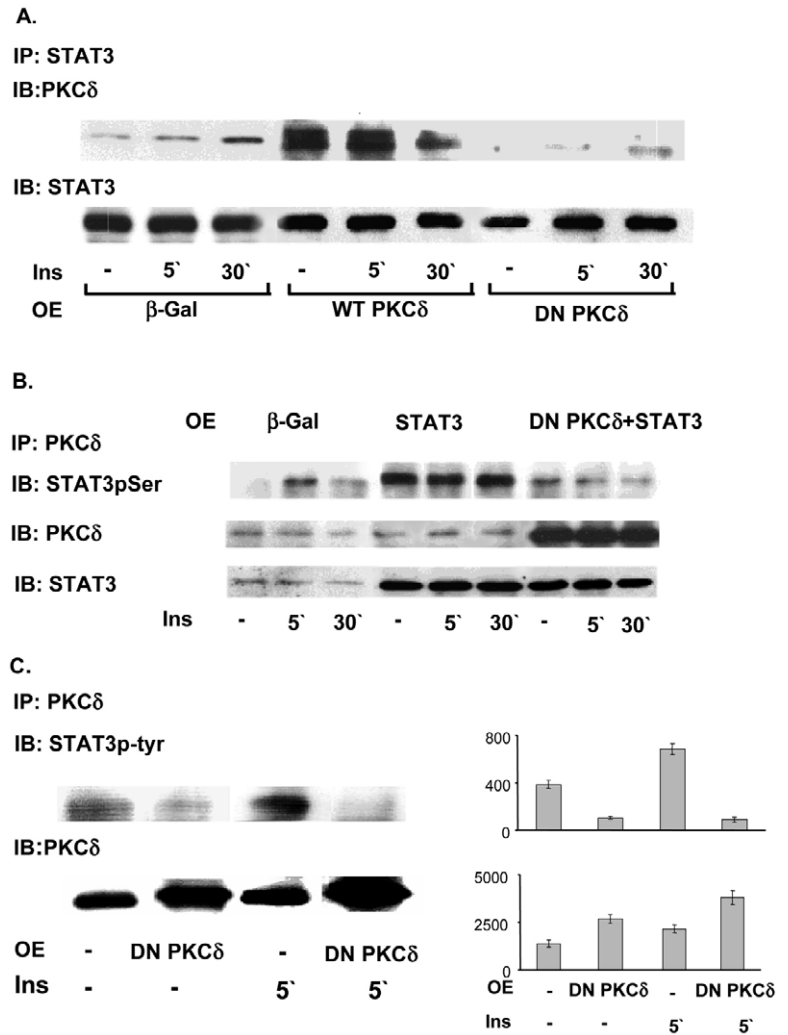


Fig. 4. Insulin-induced PKC δ -STAT3 association and activation depends on PKC δ activity. (A) Keratinocytes were infected (OE) for 1 hour with recombinant β -Gal (control), wild-type (WT) PKC δ and dominant-negative PKC δ (DN PKC δ) adenovirus constructs. Following infection, cells were incubated for 18 hours, and left untreated (-) or stimulated with insulin for 5 and 30 minutes. STAT3 immunoprecipitates were subjected to western blot analysis and probed with anti-PKC δ and anti-STAT3 antibodies. (B) Keratinocytes were either infected for 1 hour with β -Gal or WT STAT3 adenovirus constructs, or double infected with DN PKC δ followed by WT STAT3 recombinant adenovirus infection. Following infection, cells were incubated for 24 hours and overexpressing cells were untreated (-) or stimulated with insulin for 5 and 30 minutes. PKC δ immunoprecipitates were subjected to western blot analysis and probed with anti-phosphoserine-727-STAT3 (STAT3-p-ser), anti-PKC δ and anti-STAT3 antibodies. (C) Keratinocytes were either uninfected (-) or infected (OE) for 1 hour with recombinant dominant-negative PKC δ (DN PKC δ) adenovirus. After 24 hours, cells were left untreated (-) or treated with insulin for 5 minutes. PKC δ immunoprecipitates were subjected to western blot analysis and probed with anti-phosphotyrosine-705-STAT3 (STAT3-p-tyr) and anti-PKC δ antibodies. Relative optical density of three blots are presented in arbitrary units (mean \pm s.d.). Experiments were repeated at least three times.

Nuclear translocation of STAT3

STAT3 activation is characterized by induction of tyrosine phosphorylation and dimerization of STAT3 followed by

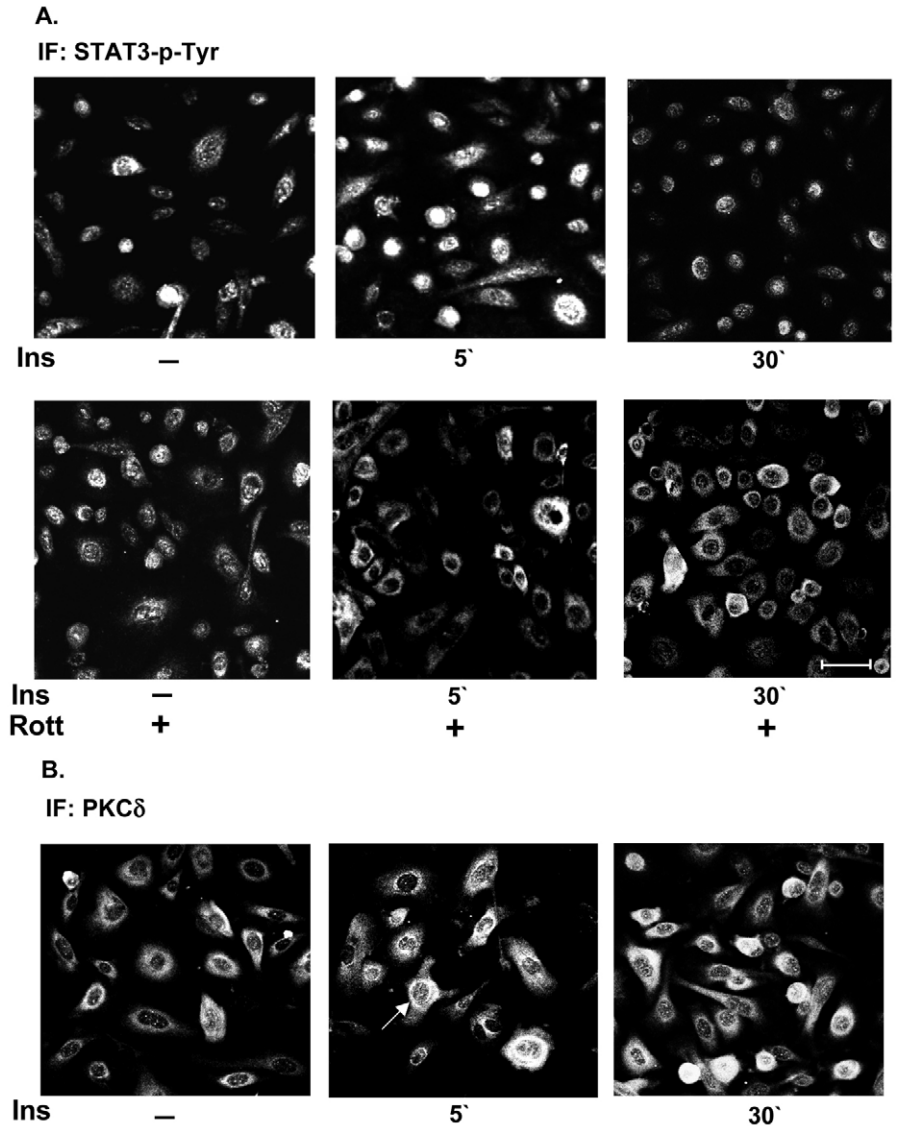


Fig. 7. Insulin-induced STAT3 nuclear translocation is abrogated by PKC δ inhibition. Primary keratinocytes were plated on glass slides and maintained for 5 days in low-Ca²⁺ MEM (0.05 mM) until they reached 80% confluence. (A) Cells were left untreated (upper panel) or pre-treated with 5 μ M rottlerin (Rott) for 7 minutes (lower panel), followed by 10⁻⁷ M insulin for 5 and 30 minutes (Ins). Cells were fixed in methanol, washed and air-dried. Cultures were analyzed by immunofluorescence using anti-phosphotyrosine-705-STAT3 (STAT3-p-tyr) antibody, followed by FITC-conjugated secondary antibody. Slides were viewed by confocal microscopy. (B) Cells were untreated (–) or treated with insulin (Ins) for 5 and 30 minutes. Following treatment, cells were fixed in methanol and analyzed by immunofluorescence using anti-PKC δ antibody followed by FITC conjugated secondary antibody and scanned by confocal microscope. Arrow in middle panel indicates translocation of PKC δ to the perinuclear membranes. Experiments were repeated at least three times. Bar, 20 μ m.

STAT3 could be detected in the cytoplasm of insulin-stimulated cells (Fig. 7A, lower panel). Interestingly, following insulin stimulation, the nuclear translocation of STAT3 was not accompanied by nuclear translocation of PKC δ . Under basal conditions, PKC δ expression was detected mostly in the cytoplasm, whereas in some cells, a perinuclear distribution was noticed. Following insulin stimulation, PKC δ distribution was shifted to the perinuclear area including the nuclear membrane but no protein was seen within the nucleus (Fig. 7B). Inhibition of PKC δ activity by pretreatment with rottlerin prior to insulin stimulation, completely abolished redistribution of PKC δ expression following insulin stimulation (data not shown).

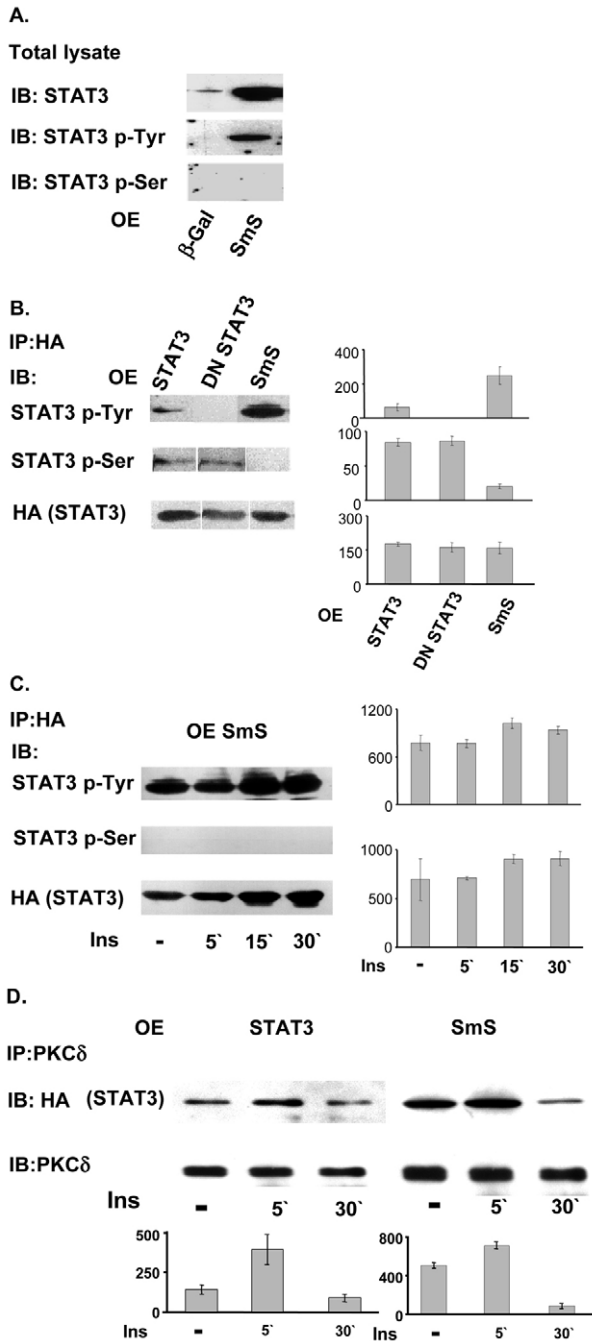
Role of STAT3 serine phosphorylation

To directly study the functional contribution of STAT3 serine phosphorylation to insulin-induced PKC δ activation in keratinocyte proliferation, we next constructed a HA-tagged STAT3 Ser727 to Ala mutant. Ser727 located at the Pro-X-Ser-Pro motif was previously shown to contribute to transcriptional activation of STAT3 (Chung et al., 1997; Jain

et al., 1999; Novotny-Diermayr et al., 2002). This mutant (SmS) was introduced into an adenovirus construct and used to follow the STAT3 activation state in response to insulin stimulation. STAT3 SmS was efficiently expressed in keratinocytes (Fig. 8). No serine phosphorylation was observed in the overexpressing keratinocytes, in either the basal state (Fig. 9B) nor in response to insulin stimulation (Fig. 8C), confirming that the Ser727 residue is uniquely phosphorylated in response to insulin. However, surprisingly, in the basal state, constitutive tyrosine phosphorylation of the serine mutant STAT3 was increased at least five- to tenfold above levels observed with wild-type STAT3 (Fig. 8B), and the STAT3 serine mutant was localized to the nucleus of unstimulated, overexpressing cells (results not shown). Furthermore, the STAT3 serine mutant was constitutively associated with PKC δ , but no further increase in STAT3 tyrosine phosphorylation and association with PKC δ was seen in response to insulin stimulation (Fig. 8C).

The role of STAT3 in keratinocyte proliferation

In previous studies, we identified a pathway which links PKC δ



activation by insulin to keratinocyte proliferation (Shen et al., 2001). Therefore, we next studied the involvement of STAT3 in this pathway. Overexpression of PKC δ and overexpression of STAT3 were able to induce keratinocyte proliferation to a similar degree (Fig. 9A). No proliferation was induced when cells were infected with dominant-negative constructs of STAT3 and PKC δ as well as by overexpression of the STAT3 serine mutant (Fig. 9A, SmS). These results further confirmed that STAT3 serine phosphorylation is required for induction of keratinocyte proliferation. Moreover, abrogation of PKC δ activity by infection with a DN PKC δ construct inhibited proliferation induced by STAT3 overexpression (Fig. 9A). We next investigated the role of STAT3 serine and tyrosine

Fig. 8. Overexpression of serine mutant STAT3 using recombinant adenovirus. (A) Keratinocytes were infected for 1 hour using recombinant adenoviruses encoding β -Gal and STAT3 serine mutant (SmS). After 18 hours, total cell lysates were subjected to western blot analysis utilizing anti-STAT3 (top panel), anti-STAT3-p-tyr (middle panel) and STAT3-p-ser (bottom panel) antibodies. (B,C) Keratinocytes were infected for 1 hour using recombinant adenoviruses encoding WT STAT3, DN STAT3 and STAT3 serine mutant (SmS). After 24 hours, cells were (B) left untreated or (C) stimulated with insulin for the designated time periods (0, 5, 15 or 30 minutes). HA immunoprecipitates were subjected to western blot analysis and probed with anti-STAT3-p-tyr (top panel), anti-STAT3-p-Ser (middle panel) and anti-STAT3 (bottom panel) antibodies. Relative optical density of three representative blots is presented in arbitrary units (mean \pm s.d.). (D) Keratinocytes were infected for 1 hour using recombinant adenoviruses encoding WT STAT3 and STAT3 serine mutant (SmS). Cells were stimulated with insulin for 5 and 30 minutes. Lysates were immunoprecipitated with PKC δ antibody and analyzed by western blotting, using anti-HA. Equal loading of gels was confirmed by reblotting with PKC δ antibody. Relative optical density of blots is presented in arbitrary units (mean \pm s.d.). Experiments were repeated at least three times.

phosphorylation and the activation state of PKC δ , in the regulation of insulin-induced keratinocyte proliferation. Following overexpression of WT STAT3, DN STAT3, SmS and kinase active (WT) and kinase inactive (DN) PKC δ , only inactivation of PKC δ significantly inhibited insulin-induced keratinocyte proliferation (Fig. 9B). These results suggest an imperative regulatory role for PKC δ , in mediating insulin signaling via the STAT3-PKC δ pathway in insulin-induced keratinocyte proliferation. However, STAT3 mutants were unable to abrogate insulin-induced keratinocyte proliferation suggesting a lesser role for STAT3 compared with PKC δ in the regulation of insulin action in keratinocytes,

The role of PKC δ and STAT3 in inducing keratinocyte proliferation was also confirmed by FACS analysis (Fig. 9C and results not shown). Increase in the percentage of actively proliferating S-phase cells was detected following overexpression with WT PKC δ and WT STAT3 recombinant adenoviruses whereas infection with DN PKC δ , DN STAT3 and SmS did not induce cell proliferation (Fig. 9C). Interestingly, by following the Sub-G1 population we identified that in comparison to β -Gal overexpressing cells, only overexpression of WT PKC δ but not overexpression of DN PKC δ , WT STAT3 or STAT3 mutants induced keratinocyte apoptosis (Fig. 9C, A-apoptotic cells). Collectively, the results suggest that PKC δ plays a specific role in regulation of STAT3 727 serine phosphorylation and mediation of insulin-induced keratinocyte proliferation.

Discussion

The STAT family of proteins are transcriptional activators, which can be activated in response to a variety of stimuli including cytokines, growth factors and hormones (Calo et al., 2003; Heim, 1999). However, the mechanism of STAT activation is unique, compared with that of other transcriptional activators. The presence of an SH2 domain and a C-terminal tyrosine residue, which becomes phosphorylated upon stimulation, provides the basis for the interaction of STATs with several signaling complexes. Such signaling pathways can directly regulate subsequent homo- and

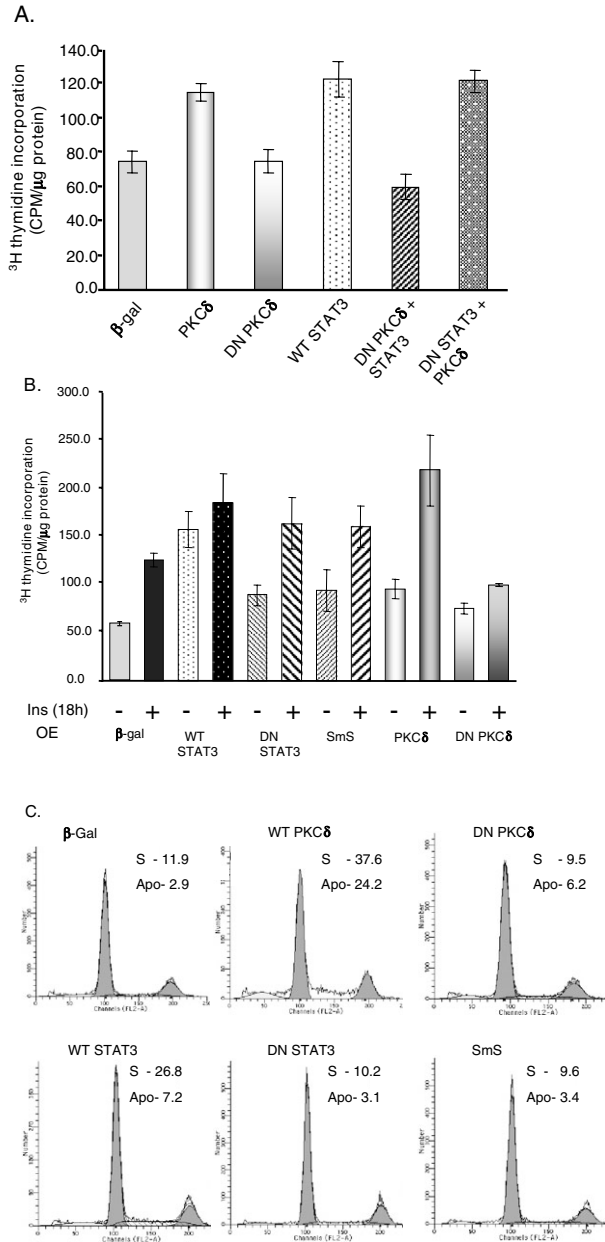


Fig. 9. Effects of overexpression of PKC δ and STAT3 on keratinocytes proliferation and cell death. Keratinocytes were infected for 1 hour with (A) recombinant adenoviruses β -Gal, PKC δ , WT STAT3, DN STAT3 or double infected with DN PKC δ followed by WT STAT3 or with DN STAT3 followed by WT PKC δ infection. (B) with recombinant adenoviruses β -Gal, PKC δ , DN PKC δ , WT STAT3, DN STAT3 and STAT3 serine mutant (SmS) and un-treated (-) or treated (+) with insulin. Twenty-four hours following infection, cell proliferation was analyzed by [3 H]thymidine incorporation as described in Materials and Methods. Results are presented as cpm/ μ g protein. Each bar represents the mean of three determinations in a plate from the same culture. Experiments were repeated at least three times. (mean \pm s.d.). Significant differences were observed between β -gal control values and those for WT STAT3 ($P < 0.001$) and PKC δ ($P < 0.0005$). (C) Keratinocytes were infected with recombinant adenoviruses encoding β -Gal, PKC δ , WT STAT3, DN STAT3 and STAT3 serine mutant (SmS). Following infection (24 hours) FACS cell-cycle analysis of propidium-iodide-stained keratinocytes was performed. Apoptotic cell death was defined as the sub-G1 population (Apo, percentage of sub-G1 population) and proliferation was identified as the population of S-phase cells (S, percentage of S-phase population).

this interaction was not determined. Our study establishes a direct link between STAT3 serine phosphorylation and the activation and regulation of keratinocyte proliferation in response to insulin signaling in the skin. Our previous studies suggested that the activation of PKC δ by insulin is unique and is not induced by other factors such as EGF, PDGF, IGF or KGF (Shen et al., 2001). This study extends these results to identify STAT3 activation and serine phosphorylation as downstream regulators of insulin-mediated signaling in keratinocyte proliferation. Moreover, the physical linkage between PKC δ and STAT3 was shown to be specific and does not involve other PKC isoforms expressed in the skin. Upon insulin-induced activation of PKC δ , STAT3 becomes phosphorylated on both serine and tyrosine residues, leading to subsequent STAT3 nuclear localization and activation. Abrogation of PKC δ activity was able to inhibit the physical association of STAT3 with PKC δ as well as to inhibit STAT3 phosphorylation, nuclear translocation and activation. Furthermore, abrogation of either PKC δ activity or serine phosphorylation of STAT3-blocked PKC δ and STAT3-mediated keratinocyte proliferation. However, although abrogation of PKC δ activity was able to inhibit STAT3-induced keratinocyte proliferation, a dominant-negative form of STAT3 was not able to efficiently inhibit PKC δ -induced proliferation. These results suggest that serine phosphorylation of STAT3 by PKC δ is indispensable for the downstream STAT3 signaling in mediation of keratinocyte proliferation. Furthermore, STAT3 mutants were not able to inhibit insulin-induced proliferation. These results could be associated with the inefficient physical link between PKC δ and STAT3 mutant forms (SmS and DN STAT3) allowing the activation of endogenous STAT3 by insulin-induced PKC δ activation. However, other possible explanations could be related to the STAT3 mechanism of action. STAT3 as a transcription activator acts downstream of various signaling pathways including signaling by IGF-1 and Leptin. The convergence of IGF-1 and Leptin pathways with the insulin signaling pathway can lead to indirect effects of insulin via alternative pathways following the blockade of insulin-STAT3

heterodimerization of the protein to direct its localization to the nucleus. In the nucleus, STATs bind DNA to induce transcription of specific genes (Chatterjee-Kishore et al., 2000; Imada and Leonard, 2000; Levy and Darnell, 2002). In this study, we describe a unique pathway in skin keratinocyte proliferation, which involves STAT3 activation downstream of insulin-mediated PKC δ activation. The association between STAT3 serine phosphorylation and PKC δ was previously established in other STAT3-mediated pathways including stimulation with IL6, LIF and EGF (Jain et al., 1999; Novotny-Diermayr et al., 2002; Schuringa et al., 2001). In addition to PKC δ , several other kinases including Erk, p38, and JNK have been shown to directly or indirectly induce phosphorylation of STAT3-Ser727 (Abe et al., 2001b; Chung et al., 1997; Jain et al., 1998; Jain et al., 1999; Kuroki and O'Flaherty, 1999; Lim and Cao, 1999; Sengupta et al., 1998; Xu et al., 2003). However, the specific physiological role for

activation (Kim et al., 2000; Xu et al., 2005). Therefore, chronic effects of insulin on cell proliferation measured 18-24 hours following stimulation does not reflect directly just acute activation of PKC δ and STAT3 but rather is the consequence of the overall compensatory mechanisms. Finally, although our results clearly show that insulin does not phosphorylate STAT3 mutants, constitutive complex formation of STAT3 with PKC δ and nuclear localization of the STAT3 mutant protein suggest alternative mechanisms distinct from protein phosphorylation to transmit insulin signaling in cell proliferation. Although the role of tyrosine phosphorylation as an essential step for activation of STAT3 is well established (Darnell et al., 1994; Levy and Darnell, 2002), the precise role of a single serine phosphorylation event, such as the one associated with the activation of Ser727 in STAT3, was not known. Indeed, in several studies, serine phosphorylation was shown to coincide with and contribute to transcriptional activation following stimulation *in vitro* (Ceresa and Pessin, 1996; Decker and Kovarik, 2000; Gotoh et al., 1996). However, several recent studies suggested that STAT3 serine phosphorylation following stimulation with EGF, NGF or IL-6 has a regulatory role distinct from that of STAT tyrosine phosphorylation and activation (Abe et al., 2001a; Kovarik et al., 1999; Lim and Cao, 1999). In keratinocytes, tyrosine and serine phosphorylation of STAT3 seem to coincide following insulin stimulation. However, in cells expressing a STAT3 Ser727 mutation, serine phosphorylation was completely abrogated whereas STAT3 tyrosine phosphorylation was significantly increased. These results are in agreement with recent results obtained in transgenic mice expressing a form of the STAT3 Ser727 mutant (Shen et al., 2004). Although fibroblasts carrying the serine mutation are defective in their transcriptional response *in vitro*, fibroblasts induced by oncostatin M exhibit intact tyrosine phosphorylation of STAT3. Furthermore, in the serine-mutant mouse, many of the downstream signaling pathways associated with STAT3 remain intact (Shen et al., 2004). Interestingly, in the serine-mutant mouse, STAT3 serine phosphorylation was found to specifically affect levels of members of the insulin like growth factor (IGF1) family and to influence growth-hormone-mediated signaling. Furthermore, the phenotype observed with this mutant was associated with marked growth retardation, reduced thymic epithelial cell number and induction of apoptotic cell death (Shen et al., 2004). These results are in agreement with our study supporting a role for STAT3 activation and serine phosphorylation in the physiological proliferation of keratinocytes under the regulation of another member of the insulin family of growth factors, insulin. Overall, these results suggest that rather than transcription *per se*, serine phosphorylation is involved in directing the specificity of the signaling pathway. Indeed, serine phosphorylation could be associated with a variety of mechanisms regulating cell signaling leading to calibration of the signal and a specific physiological outcome. These mechanisms include the induction of conformational changes, induction of specific complex formation, and phosphorylation and regulation of other downstream effectors by PKC δ and STAT3. The unique ability of a transcription factor such as STAT3 to physically interact via an SH2 domain with various signaling protein complexes would also provide a mechanism whereby a downstream element such as STAT3 could affect

PKC δ activation via phosphorylation or direct interaction with various components of the signaling complex.

The association of STAT3 with cell proliferation was established in several model systems. STAT3 was found to be activated and constitutively phosphorylated in many tumor cells *in vitro* and *in vivo* (Bromberg et al., 1999; Frank, 1999; Takeda et al., 1998; Turkson and Jove, 2000) including the induction of the oncogenic phenotype in Src-transformed fibroblasts (Bromberg et al., 1998; Silva, 2004; Turkson et al., 1998). In addition, specifically in skin, targeted deletion of STAT3 abrogates skin remodeling and is associated with a blockage of hair-cycle progression and impaired wound healing (Akira, 2000; Sano et al., 1999; Sano et al., 2000).

Interestingly, similarly to STAT3, PKC δ activation in several model systems was shown to mediate signaling by growth factors such as EGF, NGF, PDGF, insulin, TNF α and IL-6 (Gliki et al., 2001; Jackson et al., 2001; Jain et al., 1999; Kontny et al., 1999; Novotny-Diermayr et al., 2002; Robin et al., 2004; Shen et al., 2001). However, although STAT3 was shown to be involved in mediating cell growth and oncogenic potential, further studies suggest PKC δ to be primarily involved in cell differentiation and apoptosis; thus inhibition of PKC δ activity and loss of PKC δ during transformation are associated with cell growth and oncogenesis (Fujii et al., 2000; Li et al., 1999; Li et al., 1996b; Lucas and Sanchez-Margalet, 1995). Our results suggest possible explanations for the role of PKC δ in directing the specificity of signal activation in keratinocytes. Under physiological settings in normal primary keratinocytes, PKC δ activation is associated with STAT3 and linked to insulin signaling and keratinocyte proliferation. However, in cell transformation and during tumor progression, PKC δ expression is downregulated or inactivated (Geiges et al., 1995; Joseloff et al., 2002; Langzam et al., 2001). The resulting loss of STAT3-PKC δ complexes could contribute to the transformed phenotype by enabling STAT3 phosphorylation and activation to come under the regulation of a variety of other growth factors distinct from insulin (Bromberg et al., 1999; Calo et al., 2003; Chan et al., 2004). Alternatively, the fact that only PKC δ but not STAT3 overexpression could induce cell apoptosis suggests that PKC δ -mediated apoptosis is distinct from the proliferative pathway which is regulated by both PKC δ and STAT3 activation. Another possible explanation for the differences between PKC δ and STAT3 regulation could be based on intracellular localization. For example, our results in primary murine keratinocytes demonstrated PKC δ translocation to the perinuclear region following insulin stimulation to be independent of the nuclear translocation of STAT3. Furthermore, our study has confirmed that whereas serine phosphorylation is crucial for functional outcome, the serine mutation does not abrogate nuclear translocation. These results are in agreement with recent studies suggesting that the C-terminus trans-activating domain and not the N-terminal domain regulates STAT3 nuclear localization (Ma et al., 2003; Prana et al., 2004). Therefore, although STAT3 serine phosphorylation is coupled to PKC δ translocation to the nucleus in a variety of transformed cell lines, the perinuclear localization of PKC δ in primary keratinocytes could result in a different physiological outcome in response to STAT3 phosphorylation and transactivation (Buchner, 2000; Goodnight et al., 1995; Jain et al., 1999). Overall, these results suggest a specific role for PKC δ as a serine kinase of STAT3 and as a

regulator of insulin-mediated STAT3 signaling in normal skin physiology. Furthermore, the interaction of STAT3 with PKC δ facilitates PKC δ function and is essential for insulin-induced proliferation of skin keratinocytes. Future studies will be aimed at understanding the mechanisms that underlie the specificity of the signal induced by STAT3 serine phosphorylation versus tyrosine phosphorylation in keratinocyte physiology and the pathology of skin cancer.

Materials and Methods

Reagents and antibodies

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with a SuperSignal West Pico Chemiluminescent Substrate Kit purchased from Pierce. Monoclonal anti p-Tyr antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies to PKC isoforms, polyclonal anti-STAT3, were purchased from Santa Cruz Biotechnology (CA). Monoclonal antibodies to PKC isoforms and STAT3 were purchased from Transduction Laboratories (Lexington, KY). Antibodies against STAT3, anti-phospho-tyrosine-705-STAT3 and anti-phospho-serine-727-STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Horseradish-peroxidase-labeled anti-rabbit and anti-mouse IgG were obtained from Bio-Rad. Leupeptin, PMSF, β -mercaptoethanol, orthovanadate, and pepstatin were purchased from Sigma Chemicals (St. Louis, MO). Insulin (HumulinR-recombinant human insulin) was purchased from Lilly France (Fergersheim, France). The PKC delta inhibitor, rottlerin, was purchased from Calbiochem.

Isolation and culture of murine keratinocytes

Primary keratinocytes were isolated as described from newborn BALB/C mice (Dlugosz et al., 1995). Keratinocytes were cultured in Eagle's minimal essential medium (EMEM) containing 8% fetal bovine serum pretreated with Chelex-100 (BioRad). To maintain a proliferative basal cell phenotype, the final Ca^{2+} concentration was adjusted to 0.05 mM. Serum starvation was induced by maintaining keratinocytes in medium containing 0.05% serum for 24 hours. Experiments were performed 5-7 days after plating.

Total cell extracts and western blots

Whole-cell lysates were prepared by scraping cells on ice into 300 μ l lysis buffer containing 5% SDS, 20% 2- β -mercaptoethanol and 50% western upper gel buffer (0.5 M Tris-HCl, pH 6.8). Lysates were homogenized, boiled for 5 minutes, and centrifuged at 28,000 g for 20 minutes. Supernatant containing the total cell protein extracts was transferred to a fresh tube. Protein loading buffer was added to an equal volume of the total cell extracts, samples were boiled for 5 minutes and centrifuged. Supernatants were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Specific protein bands were detected by immunoblotting using specific antibodies and visualized by enhanced chemiluminescence.

Preparation of cell extracts in RIPA buffer

For crude membrane fractions, cells were washed with cold PBS three times, resuspended in RIPA buffer without SDS containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% deoxycholic acid, 1% Triton X-100, 0.25 mM EDTA (pH 8.0), and protease and phosphatase inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 200 μ M NaVO_4 and 5 mM NaF). Lysates were incubated for 30 minutes at 4°C on ice and centrifuged at 4°C for 30 minutes at 28,000 g. The supernatant containing the soluble protein fraction (cytoplasmic and membrane) was transferred to another tube and used for immunoprecipitation. Protein concentrations were measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). Western blot analysis of cellular protein fractions was carried out as described (Li et al., 1996a).

Immunoprecipitation and western blot analysis

Protein cell lysates, prepared in RIPA buffer, were precleared by mixing 0.3 ml cell lysate with 25 μ l of Protein A/G Sepharose (Santa Cruz Biotechnology), and the suspension was rotated continuously for 30 minutes at 4°C. The preparation was then centrifuged at maximal speed at 4°C for 10 minutes. Supernatants were incubated with specific polyclonal or monoclonal antibodies to the individual PKC isoforms (diluted 1:100), anti-STAT3, anti-phosphotyrosine-STAT3, or anti-phosphoserine-STAT3 antibodies overnight at 4°C followed by incubation with protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 2 hours. The suspension was then centrifuged at maximal speed for 10 minutes at 4°C, the pellet was washed twice with RIPA buffer, twice with cold PBS and subjected to western blot analysis. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane (0.2 μ m) (BIO-RAD) and blotted with specific polyclonal or monoclonal antibodies.

Experiments were repeated several times, as indicated in the figure legends, and

blots of repeated experiments were analyzed by densitometry. Arbitrary units were normalized to a similar scale for all original figures maintaining the differential quantitative responses. Statistical analysis (mean \pm s.d.) was performed accordingly.

Recombinant adenovirus constructs

The recombinant adenovirus vectors were constructed as described (Miyake et al., 1996). A dominant-negative mutant of mouse PKC δ was generated by substitution of the lysine residue at the ATP-binding site with alanine (Ohba et al., 1998). Adenoviral vectors containing the genes for HA-tagged STAT3 and HA-tagged DN STAT3 (AxCASTAT3 and AxCAAdnSTAT3), which contained a CAG promoter (chicken β -actin promoter with cytomegalovirus enhancer), were prepared by homologous recombination in HEK293 cells by using pAxCA 1w cosmid cassette, as described previously (Miyake et al., 1996). The dominant-negative activity of STAT3F gene was demonstrated by the abrogation of its tyrosine phosphorylation. A serine phosphorylation STAT3 mutant adenovirus was generated by the COS-TPC method using cosmids and recombinant adenoviruses containing pEFHA-Stat3-S727A expression vector (Miyake et al., 1996). PEFHA-Stat3S-727A was prepared by introducing a point mutation at Ser727 to Ala (Abe et al., 2001a). Viral titer was adjusted by the TCID₅₀ method according to the manufacturer's protocol (Takara Shuzo, Japan) (Miyake et al., 1996). Briefly, a virus solution from HEK293 cell lysates was serially diluted into 96-well collagen-coated plates. A row of uninfected cells was used as controls. Complete cytopathic effect was judged by microscopic evaluation after 11-13 days of infection. Wells in which the virus induced a full cytopathic effect were counted and the 50% tissue culture infectious dose was calculated (TCID₅₀) in correlation with the number of plaque-forming units. More than 95% of keratinocytes in the culture dish expressed the transduced protein following recombinant adenovirus infection as analyzed by β -galactosidase adenovirus infection.

Overexpression of specific proteins by using of the recombinant adenovirus vectors

The culture medium was aspirated and keratinocyte cultures were infected with the viral supernatant containing PKC δ , DN PKC δ , WT STAT3 or DN STAT3 recombinant adenoviruses at a multiplicity of infection (MOI) of 12 for 1 hour. The cultures were then washed twice with PBS and re-fed low- Ca^{2+} MEM (0.05 mM) for 24 hours. β -Galactosidase adenovirus was used as a negative control to exclude possible deleterious effects of the vector itself. Following infection, cells were incubated for 24 hours, and then were left untreated or treated with insulin, and used for proliferation assays, activity assays, immunocytochemistry assays or for immunoprecipitation and western blotting.

PKC activity

Specific PKC activity was determined in freshly prepared immunoprecipitates from keratinocyte cultures following appropriate treatments. These lysates were prepared in RIPA buffer without NaF. Activity was measured with use of the SignATECT Protein Kinase C Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Neurogranin (AAKIQAS*FRGHMARKK) was used as the substrate for PKC activity in these studies.

Cell proliferation

Cell proliferation was measured by [³H]thymidine incorporation in six-well plates. Cells were pulsed with [³H]thymidine (1 μ Ci/ml) for 1 hour. After incubation, cells were washed five times with PBS, and 5% TCA was then added to each well for 1 hour. The solution was removed and cells were solubilized in 1 M NaOH. The labeled thymidine incorporated into cells was counted in the ³H window of a Tricarb liquid scintillation counter.

Immunofluorescence

Primary keratinocytes were plated on ProbeOn Plus glass slides. Keratinocytes after 5 days of culture were left untreated or treated with insulin for 5 minutes and 30 minutes with or without pre-treatment with rottlerin for 7 minutes, infected either with PKC δ , DN PKC δ , or DN STAT3 adenoviruses for 1 hour, washed twice with Ca^{2+} - and Mg^{2+} -free PBS and maintained in culture in 0.05 mM Ca^{2+} EMEM. After infection, keratinocytes were incubated for 24 hours, then fixed in methanol for 10 minutes followed by permeabilization with 0.2% Triton X-100 for 5 minutes, rinsed with PBS and incubated for 18 hours at 4°C, with specific anti-PKC, anti-phosphotyrosine STAT3 or anti-STAT3-phosphoserine antibodies diluted in 1% BSA in PBS. After incubation, slides were washed twice for 10 minutes with PBS and incubated with biotinylated secondary anti-rabbit antibody for 40 minutes, washed twice in PBS and incubated with streptavidin-FITC for 40 minutes. Following two washes in PBS and one wash with double-distilled water, slides were air dried and mounted with Vectashield mounting medium (Vector). Fluorescence was examined by laser scanning confocal imaging microscopy (MRC1024, Bio-Rad, UK).

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