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A kinase associated with chromatin that can be activated by ligand–p185$^{c\text{-}Neu}$ or epidermal growth factor–receptor interactions

(Cdk2 kinase/nuclear kinase/complex chromatin-associated protein kinase)

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ABSTRACT **Some growth factors transduce positive growth signals, while others can act as growth inhibitors. Nuclear signaling events of previously quiescent cells stimulated with various growth factors have been studied by isolating the complexed chromatin-associated proteins and chromatin-associated proteins. Signals from the plasma membrane are integrated within the cells and quickly transduced to the nucleus. It is clear that several growth factors, such as epidermal growth factor, transforming growth factor α (but not transforming growth factor β), and platelet-derived growth factor, utilize similar intracellular signaling biochemistries to modulate nucleosomal characteristics. The very rapid and consistent phosphorylation of nuclear p33, p54, and low molecular mass proteins in the range of 15–18 kDa after growth factor stimulation implies that there is a coordination and integration of the cellular signaling processes. Additionally, phosphorylation of p33 and some low molecular mass histones has been found to occur within 5 min of growth factor treatment and to reach a maximum by 30 min. In this study, we report that Neu receptor activating factor also utilizes the same signaling mechanism and causes p33 to become phosphorylated. In addition, both the tumor promoter okadaic acid (which inhibits protein phosphatases 1 and 2A) and phorbol ester (phorbol 12-tetradecanoate 13-acetate) stimulate phosphorylation of p33, p54, and low molecular mass histones. However, transforming growth factor β, which is a growth inhibitor for fibroblasts, fails to increase p33 phosphorylation. In general, p33 phosphorylation patterns correspond to positive and negative mitogenic signal transduction. p33 isolated from the complexed chromatin-associated protein fraction appears to be a kinase, or tightly associated with a kinase, and shares antigenicity with the cell division cycle-dependent Cdk2 kinase as determined by antibody-dependent analysis. The rapid phosphorylation of nucleosomal proteins may influence sets of early genes needed for the induction and progression of the cell cycle.**

The complex events of proliferating eukaryotic cells are coordinated and controlled by undefined regulatory systems. How a signal from the plasma membrane is transduced to the nucleus and initiates DNA replication is poorly understood. Stimulation with peptide growth factors initiates a series of signaling events—including tyrosine phosphorylation (1–5) and ligand-induced receptor heterodimerization and homodimerization—that activate receptor tyrosine kinases (3, 6, 7), phosphatidylinositol and phosphatidylcholine turnover (8, 9), monovalent and divalent ion fluxes, intracellular pH regulation (10), and other protein kinases. Kinases known to be affected include protein kinase C (11), Raf kinase (12), casein kinase II (13), MAP-2 kinase (14), and S6 kinase (15).

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The kinase cascade is extremely complex and only partially understood. It is known that GTP-bound p21^{ras} can interact with the serine/threonine kinase Raf (16). Raf kinase phosphorylates and activates the mitogen-activated protein (MAP) kinase kinase (also known as MEK or ERK, extracellular signal-regulated kinase). Activated MAP kinase can also phosphorylate other serine/threonine kinases such as p90^{rsk} and transcription factor p62^{TCF} or Elk1 (17). It is thought that protooncogenic transcription factors, including c-Jun, c-Fos, c-Myc, and c-Myb, are potential substrates for MAP kinase or p90^{rsk} (18). c-Fos and c-Jun are known to become transcriptionally activated within minutes of growth factor stimulation (19–22).

Growth factor receptors can be categorized into those that are clearly coupled to cytoplasmic structures involved in signaling and those that have not been convincingly shown to be (23). The latter group includes the p75 low-affinity receptor for nerve growth factor and the neurotrophins (24, 25), betaglycan [also known as transforming growth factor (TGF)-β type III receptor] and the related molecule endoglin (26–28), the type II receptor for insulin-like growth factor (29–31), and syndecan and other membrane proteoglycans whose heparan sulfate chains bind fibroblast growth factors (32, 33).

Another large group of growth factors act by binding to surface receptors that have intrinsic tyrosine kinase activity (1–7). Growth factor receptors with tyrosine kinase activity have been further classified into subgroups based on sequence similarities and typical structural features. The epidermal growth factor (EGF) receptor, the receptor for TGF-α (2), and the receptor for Neu (also known as c-erbB-2 and HER2) (2, 34) belong to subclass 1 of this receptor family.

The p185$^{c\text{-}Neu}$ receptor is a developmentally regulated tyrosine kinase and is thought to play an important role in neurogenesis (35). Neu receptor activating factor (NAF) increases *in vitro* and *in vivo* tyrosine phosphorylation, receptor dimerization, and growth only in cell lines expressing Neu receptors (36, 37). Neu receptors have unusually complex activation pathways, since they can associate as homodimers and heterodimers (3, 6, 7).

EGF, platelet-derived growth factor (PDGF), TGF-α, and NAF generally transduce positive mitogenic signals, while TGF-β can transduce negative growth signals. In the present study we defined certain molecular features of positive and negative growth signal transduction to the nucleus. We used sequential extraction and isolation of chromatin-associated proteins (CAP) and complexed CAP (CCAP) (38, 39) of ^{32}P-labeled quiescent cells stimulated with a variety of molecules, including EGF and NAF.

Abbreviations: MAP, mitogen-activated protein; TGF, transforming growth factor; NAF, Neu receptor activating factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CAP, chromatin-associated proteins; CCAP, complexed CAP; TPA, phorbol 12-tetradecanoate 13-acetate.

MATERIALS AND METHODS

Cell Lines. The creation and properties of the transfected murine fibroblast cell lines PN-NR6, NE19, M1, and DHFR-G8 have been described elsewhere (3, 6, 7, 36).

[^{32}P]Phosphate Labeling of Cells and Extraction of Cellular Proteins. The procedures described by Mahadevan *et al.* (38, 39) were followed with some slight modifications. Transfected cells were rendered quiescent by incubation in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% serum for 48 hr and then labeled for 3 hr in phosphate-free DMEM containing carrier-free [^{32}P]phosphate (NEN) at 0.5 mCi/ml (1 mCi = 37 MBq). Cells were treated with EGF [Boehringer Mannheim (BMB)], TGF-α (BMB), NAF obtained from ATL-2 serum-free medium (37), TGF-β (BMB), or PDGF (Sigma) at 50 ng/ml, phorbol 12-tetradecanoate 13-acetate (TPA; 100 nM), or okadaic acid (1 μM from BRL) for 30 min at 37°C. After stimulation, the cell monolayer was washed twice with phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 dissolved in 1 liter of water, pH 7.4) and cells were lysed by exposure to Triton-DOC buffer for 30 min at 4°C with rotation. The buffer was composed of 20 mM Hepes at pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 100 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 100 mM sodium molybdate, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, aprotinin at 10 μg/ml, pepstatin at 10 μg/ml, and leupeptin at 10 μg/ml. The sequential extraction was carried out as described by Mahadevan *et al.* (38, 39). The lysate was microcentrifuged at 16,000 $\times$ *g* for 15 min. Aliquots of Triton-DOC supernatant were then centrifuged at 100,000 $\times$ *g* for 30 min to produce pellets containing CCAP. The first Triton-DOC pellet after microcentrifugation was extracted with 9 M urea/1% Nonidet P-40/100 mM dithiothreitol to remove the bulk of the cytoskeletal proteins. The residue was then resuspended in 150 ml of 0.3 M hydrochloric acid and rotated at 4°C for 30 min, after which the insoluble material was removed by centrifugation for 15 min in a Microfuge (Beckman). Hydrochloric acid-extracted proteins in the supernatant were precipitated by adding 1 ml of acetone at −70°C for 2 hr and then were recovered by centrifugation for 30 min in a Microfuge. These proteins are called CAP.

Characterization of p33. In vitro *kinase assay.* CCAP were prepared from growth factor-stimulated but unlabeled cells. CCAP were redissolved in Triton-DOC buffer and immunoprecipitated with anti-Cdc2 kinase monoclonal antibody (raised against the PSTAIR region, which is common to Cdc2 and Cdk2 kinases). The immunoprecipitated proteins were washed three times and the *in vitro* H1 kinase assay was carried out with histones H1 and H3 as exogenous substrates (40). The *in vitro* kinase assay was also performed with electroeluted p33 and p54.

Western blotting. In EGF- and TGF-β-stimulated cells, CCAP and CAP were prepared by sequential extraction as described above. Both the CCAP and CAP fractions were dissolved in sample lysis buffer and separated by SDS/10% PAGE. After electroblotting into nitrocellulose the blots were developed with anti-Cdk2 antibody by the ECL method (Amersham) using horseradish peroxidase-conjugated secondary antibody. The blots were stripped and reblotted with a variety of antibodies.

RESULTS

Growth Factors Stimulate p33 Phosphorylation. We examined the earliest nuclear signaling events induced by NAF and PDGF on quiescent DHFR-G8 cells, which express both p185$^{c\text{-}Neu}$ and PDGF receptors but do not express EGF receptors. After growth factor stimulation for 30 min at 37°C,

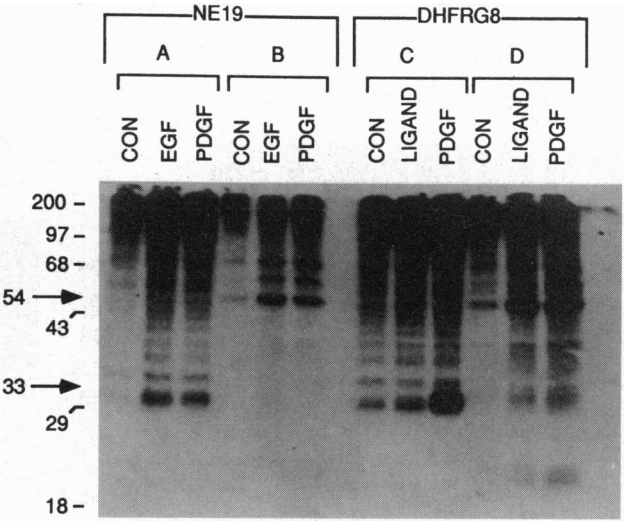

FIG. 1. EGF, NAF, and PDGF stimulate P33 and p54 phosphorylation in CCAP and CAP. Quiescent [^{32}P]phosphate-labeled cells (for 3 hr) were stimulated with PDGF at 50 ng/ml, 1% NAF (Ligand), or EGF at 50 ng/ml for 30 min; CON, unstimulated control cells. CCAP and CAP were separated by sequential extraction. Numbers on left are mass in kDa. p33 phosphorylation in CCAP (*A* and *C*) and p54 phosphorylation in CAP (*B* and *D*) are shown.

the cells were subjected to the sequential extraction procedure described by Mahadevan *et al.* (38, 39). Isolated CCAP and CAP fractions were dissolved and run separately in SDS/PAGE. NAF and PDGF both increased the p33 phosphorylation in CCAP (Fig. 1*C*). In CAP, p54 phosphorylation was increased by both growth factors (Fig. 1*D*) in comparison with the unstimulated cells. Stimulation of NE19 cells, which do not express p185$^{c\text{-}neu}$ but express both EGF and PDGF receptors, by EGF or PDGF also increased p33 phosphorylation in the CCAP fraction (Fig. 1*A*). In Fig. 1*B*, the phosphorylation of p54 in the isolated CAP fraction with growth factor stimulation was also evident.

We next studied the effect of different growth factors in the murine fibroblast cell line M1, which expresses EGF, PDGF, p185$^{c\text{-}Neu}$, TGF-α, TGF-β, and protein kinase C receptors as well as other normal fibroblast cell receptors. There was significant phosphorylation of p33 in response to EGF, TGF-α, NAF, or PDGF stimulation (Fig. 2*A*). TGF-β failed to induce p33 phosphorylation in the CCAP fraction. However the phosphorylation of p54 in the CAP fraction increased in response to TGF-β (Fig. 2*B*). The phosphorylation of low molecular mass proteins in the range of 15–18 kDa was also noted (data not shown) after EGF, NAF, or TGF-β treatment.

Kinetics of Signaling. It appears that p33 phosphorylation may correlate with certain types of transducing signals. We examined the kinetics of the growth factor-mediated nuclear signaling events in M1 (p185$^{c\text{-}Neu+}$, EGF receptor$^+$) cells. Quiescent M1 cells were stimulated with EGF for different time periods. The phosphorylation of p33 was extremely rapid, with the appearance of the p33 phosphorylated band in CCAP occurring within 5 min of growth factor treatment (Fig. 3*A*). The phosphorylation of low molecular mass proteins in the CAP fraction in the range of 15–18 kDa (presumably histones) also increased over the time course of stimulation (Fig. 3*B*).

The effect of TPA and okadaic acid was next studied in M1 cells. Treatment with either TPA or okadaic acid or the combination of both increased the phosphorylation of p33 in the CCAP fraction (Fig. 4*A*). Phosphorylation reached a maximum with the combination of TPA and okadaic acid. p54 phosphorylation in the isolated CAP fraction was also remarkable (Fig. 4*B*).

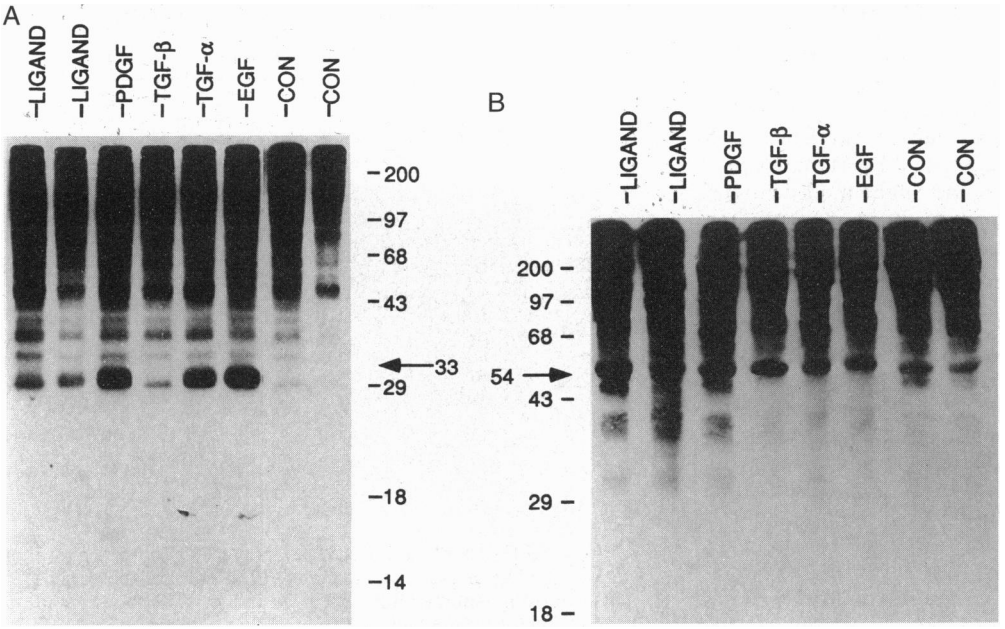

FIG. 2. (*A*) CCAP and CAP phosphorylation in M1 cells treated with EGF, TGF-α, NAF (Ligand), PDGF, or TGF-β. Quiescent M1 cells, labeled for 3 hr in [^{32}P]phosphate, were treated with different growth factors for 30 min; medium was removed and the cells were lysed; and sequential extraction was carried out as described earlier. The phosphorylation of p33 in CCAP was compared with that in untreated cells (CON). (*B*) The CAP fraction was separated by sequential extraction from M1 cells treated with growth factors EGF, TGF-α, TGF-β, NAF (Ligand), or PDGF. p54 phosphorylation is shown, along with that in control (CON) cells.

Cdc2/Cdk2 Kinase. To characterize the p33 species found in CCAP after EGF stimulation, the CCAP fraction was separated by ultracentrifugation, dissolved in Triton-DOC buffer, and immunoprecipitated with anti-Cdc2 kinase antibody. An *in vitro* immune complex kinase assay was carried out with histone H1 as the exogenous substrate. As shown in Fig. 5*A*, in addition to H1, we noted that p33 itself undergoes kinase-mediated phosphorylation. p33 phosphorylation indicates it may be a kinase or be tightly associated with a kinase.

To characterize p33 and p54, we electroeluted the p33 and p54 bands from the gel, denatured them with 6 M guanidine hydrochloride, and subjected the proteins to dialysis. We next carried out the *in vitro* kinase assay using H1 as the substrate. Once again, the p33 species was found to mediate kinase functions or be associated with a kinase activity (unpublished data), while the p54 has no kinase function.

We further characterized p33 in EGF- and TGF-β-stimulated cells by sequential isolation and fractionation of CCAP and CAP fractions followed by immunoblotting with a commercially available anti-Cdk2 kinase antibody. In both the CCAP (Fig. 5*B*) and CAP (Fig. 5*C*) fractions, Cdk2 kinase antibody recognized a protein of molecular mass 33 kDa. We then stripped and reblotted the blots with anti-Cdc2 kinase antibody. A very weak band was also observed with another commercially available anti-Cdc2 kinase antibody (data not shown). p33 may represent a Cdk2-type kinase that undergoes very rapid phosphorylation with growth factors and tumor promoters.

At time zero in Fig. 5 *B* and *C* the relative abundances of p33 forms in the CCAP and CAP fractions are indeed different as observed by Western blotting. The p33 form in the CCAP fraction is less than that seen in the CAP fraction. However,

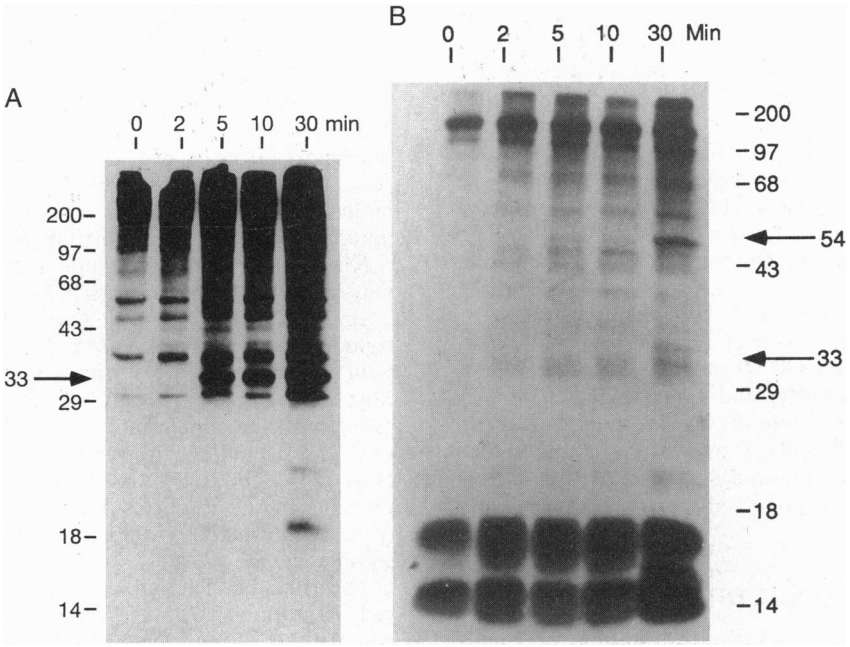

FIG. 3. (*A*) Kinetics of p33 phosphorylation in EGF-treated M1 cells. [^{32}P]Phosphate-labeled quiescent M1 cells were treated with EGF at 50 ng/ml for the indicated time periods. Cells were lysed and sequential extraction was performed. (*B*) Kinetics of phosphorylation of low molecular mass proteins in the range of 15–18 kDa. Phosphate-labeled cells were prepared as described for *A*, and the CAP fractions were isolated after treatment with EGF (50 ng/ml) for different time periods.

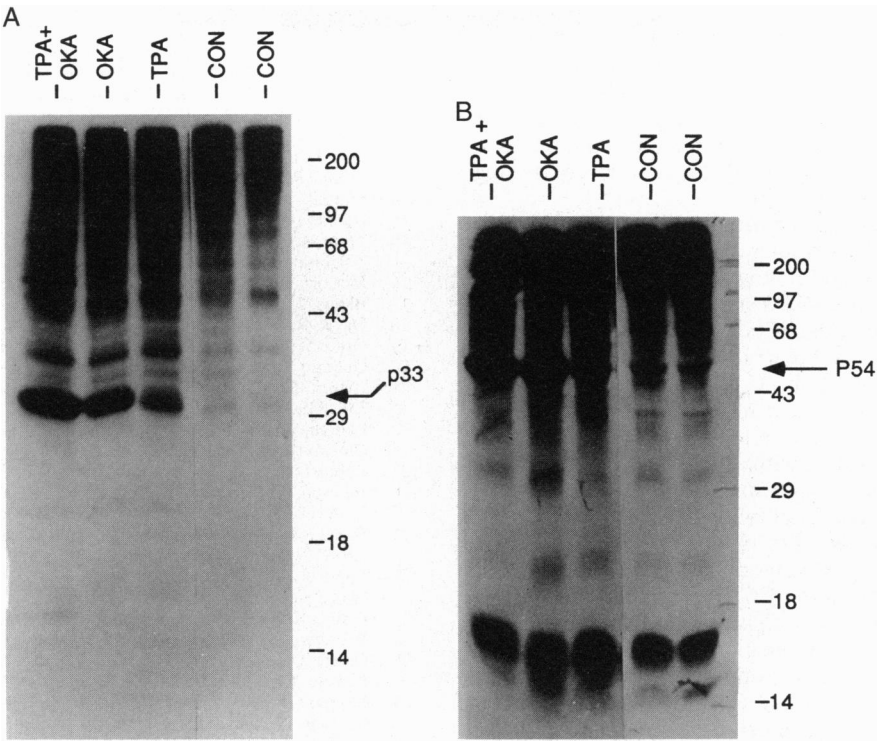

FIG. 4. Effect of tumor promoters TPA and okadaic acid on phosphorylation of p33, p54, and low molecular mass proteins. [^{32}P]Phosphate-labeled quiescent M1 cells were treated with TPA, okadaic acid, or a combination of both for 30 min, and after the removal of tumor promoters the cells were lysed and subjected to sequential extraction. The phosphorylation of p33 in CCAP is shown in *A*. The phosphorylation of p54 and low molecular mass proteins in the range of 15–18 kDa is shown in *B*.

in Fig. 3*A* the p33 form is not phosphorylated at time zero but undergoes rapid phosphorylation upon mitogenic stimulation. The p33 form becomes only minimally phosphorylated in the CAP fraction as seen in Fig. 3*B*. Despite the increased abundance of the p33 in the CAP fraction it is not phosphorylated with the same kinetics as the p33 in the CCAP fraction.

DISCUSSION

Several distinct polypeptide growth factors such as EGF or NAF elicit a common chromatin-associated nuclear phosphoprotein signal response in fibroblasts. These factors led to activation of p33 in the CCAP fraction. The p33 species appears to be a kinase and shares antigenicity with the Cdk2 kinase (as determined by polyclonal antibodies).

Progression through the cell cycle can be regulated by cyclin-dependent protein kinases (CDKs) (41–44). CDKs are thought to be essential for the start of the S phase and mitosis (45–47). A mitotic role of the CDKs has been identified for the prototypic CDK, Cdc2 kinase (48). Cdc2 kinase is positively regulated by cyclin B, which is synthesized during the S and G_2

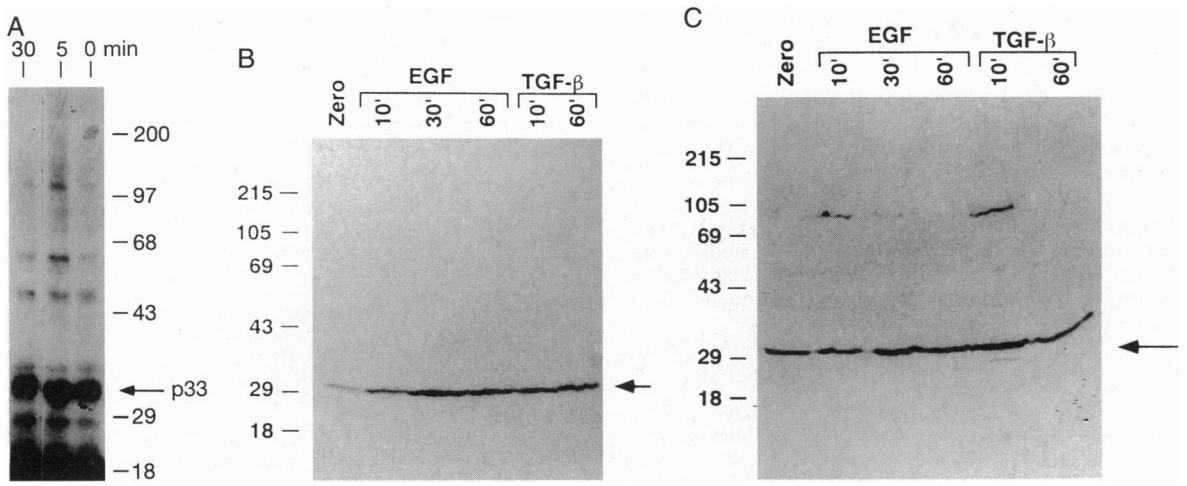

FIG. 5. (*A*) Characterization of p33 by *in vitro* kinase assay. Unlabeled M1 cells were treated with EGF at 50 ng/ml for different time periods as indicated. Cells were lysed and the CCAP fractions were separated by sequential extractions. The CCAP fraction was dissolved in Triton-DOC buffer and was immunoprecipitated with anti-Cdc2 kinase antibody. The immune complex was washed thrice and H1 kinase was assayed as described earlier (40), using H1 and H3 as exogenous substrates. (*B* and *C*) Characterization of p33 by Western blotting. Unlabeled M1 cells were treated with either EGF or TGF-β at 50 ng/ml for different time periods as indicated (in min). Cells were lysed and the CCAP fractions were separated by sequential extractions. The CCAP and CAP fractions were dissolved in sample lysis buffer and separated by SDS/10% PAGE, then transferred to a nitrocellulose membrane. The blots were probed with anti-Cdk2 kinase antibody and finally developed by the ECL method, using horseradish peroxidase-conjugated secondary anti-rabbit antibody. The p33 band is shown by arrows in the CCAP (*B*) and CAP (*C*) fractions.

phases (49–54). At least one additional family member, Cdk2, also functions during the S phase (53). Cdk2, like Cdc2, is regulated by its association with cyclins. Cdk2 associates in a complex with cyclin E in the middle of G_1 (55–58) and at the start of the S phase by formation of a complex with cyclin A (51, 52). Cyclin E–Cdk2 may function in controlling progression through G_1 (59), and cyclin A-Cdk2 may act in controlling the start of DNA synthesis (55, 56).

Little is known about the identity of the p33 involved in nuclear signaling. Previous studies showed that isolated p33 sequences do not correspond to any known protein sequence in the data bank (38). Since p33 is similar in molecular size to the cell cycle-dependent Cdk2/Cdc2 kinase (40, 53, 60), we used anti-Cdc2 kinase antibody (raised against the PSTAIR region, which is common to the Cdc2 and Cdk2 kinases) to precipitate protein from the redissolved CCAP fraction and then performed an *in vitro* kinase assay using H1 and H3 as exogenous substrates. Blotting with anti-Cdk2 kinase antibody was positive and indicates significant antigenic similarity between the p33 species associated with CCAP and the Cdk2 kinase. *In vitro* kinase assays also indicate that p33 may be a kinase. The results of kinase assays of electroeluted p54 in the CAP fraction and p33 from the CCAP fraction further demonstrated that p33 is a kinase or tightly bound to a kinase. We further characterized p33 from the unlabeled cells treated with growth factors and separated into the CCAP and CAP fractions. p33 appears to resemble the Cdk2 kinase, as judged by its enzymatic activity and antigenicity.

The mechanism by which phosphoproteins such as p33 become associated with the chromatin-enriched fraction in response to various mitogenic stimulations is not clear. It is likely that all these growth factors (except TGF-β) and tumor promoters transduce a very similar pattern of nuclear signaling. p33 phosphorylation may be needed for the transduction of a nuclear mitogenic signal. Since EGF, TGF-α, NAF, and PDGF all lead to the phosphorylation of p33, it appears that the p33 phosphorylation signal may represent a common mitogenic signal. However, our studies showed that TGF-β failed to cause phosphorylation of this protein in the CCAP fractions.

p33 appears to be located in the nucleus, and its phosphorylation may occur as a consequence of mitogen-stimulated activation of a nuclear kinase. It is possible that p33 is a nuclear kinase that undergoes rapid phosphorylation upon mitogenic stimulation of MAP kinases. Our data indicate that p33 resembles a form of the Cdk2 kinase or is a protein tightly associated with the Cdk2 kinase. These issues can be evaluated by studying *in vitro* phosphorylation of proteins isolated from CCAP and CAP fractions. Purifying p33 and p54 from CCAP and CAP, respectively, should allow elucidation of their precise role in mitogenic signaling.

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