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c-Myc-Enhanced S Phase Entry in Keratinocytes is Associated With Positive and Negative Effects on Cyclin-Dependent Kinases

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Abstract The function of the c-*myc* proto-oncogene in cell cycle progression remains unclear. In order to examine the role c-*myc* may play in cell cycle progression, we have expressed the hormone-inducible MycER protein in the nontransformed, EGF-dependent mouse keratinocyte cell line BALB/MK. We have found that activation of MycER, but not a mutant MycER, Gal4ER, or FosER, leads to an EGF-dependent and hormone-dependent increased incorporation of labeled thymidine only during the S phase of the cell cycle in BALB/MK cells. A possible explanation for the increase in thymidine incorporation comes from flow cytometric analyses that reveal that activation of MycER leads to an increase in the total number of cells that enter S phase after EGF restimulation. Investigation of the intracellular effects of Myc activation shows that the expression of several putative Myc-sensitive proteins, cyclins A, E, and D1, and the E2F-1 protein are unaffected by Myc induction. Interestingly, we find that the histone H1 kinase activity associated with an E2F-1 complex containing Cyclin A and Cdk-2, but not that associated with Cyclin E, in late G_1 and early S phases is increased in cells containing hormone-activated MycER, but not FosER. Although the mechanism for this Myc-dependent effect on E2F-1-associated kinase activity is still unknown, it does not appear to involve dissociation of the Cdk inhibitor $p27^{Kip1}$ from the complexes as suggested by others. However, we have also found that hormone-treated cells actually show more $p16^{INK4A}$ inhibitor associated with another kinase, Cdk-4, as the cells are entering S phase. Altogether, the data suggest that the presence of excessive Myc protein in keratinocytes can stimulate otherwise noncycling cells to enter the cell cycle, and that this effect of Myc involves both positive effects on E2F-1-associated Cdk-2 and negative effects on Cdk-4 in late G_1. J. Cell Biochem. 70:528–542, 1998.

Key words: c-Myc; Cdk; Cdk inhibitors; keratinocytes; cell cycle

The mechanisms by which c-*myc* contributes to cell proliferation, differentiation, and tumorigenesis are not yet understood. Expression of the gene is rapidly induced following mitogenic stimulation of resting cells, peaking within a short time and then decaying to a suprabasal level throughout following cell cycles [Tavtigian et al., 1994; Greenberg and Ziff, 1984; Kelly et al., 1983]. Expression is down-regulated in quiescent and differentiating cells, and many tumor types show deregulated expression of c-*myc* due to activating translocations or amplifications of the c-*myc* locus [Tanaka et al., 1989; Kazumoto et al., 1990; Koskinen et al., 1993; Marcu et al., 1992; Alitalo et al., 1987, and references therein]. The requirement for c-*myc* in the cell cycle derives from experimental data based primarily on antisense approaches in which loss of c-*myc* expression leads to an antiproliferative effect, most likely during the G_1 phase [Münger et al., 1992; Pietenpol et al., 1990; Gai et al., 1990]. This result is supported by other studies that used a gene targeting method to disrupt one allele of c-*myc* leading to a slower growth rate [Shichiri et al., 1993; Hanson et al., 1994]. Introduction of an exogenous c-*myc* transgene reversed this growth defect and appeared to accelerate cell growth relative to normal diploid cells.

Abbreviations: BALB/MK, BALB mouse keratinocytes; Cdk, cyclin-dependent kinase; E_2, estradiol; TK, thymidine kinase; 4-OHT, 4-hydroxytamoxifen.

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Experimental evidence has led to the general consensus that c-Myc functions most likely as a transcription factor during G_1 phase [Meichle et al., 1992; Dang, 1991; Marcu et al., 1992; Bishop et al., 1991]. The observations that c-Myc contains basic/helix-loop-helix/leucine zipper motifs common to the bHLH-LZ family of transcription factors and can bind DNA in a sequence-specific manner as a heterodimer with an unrelated bHLH-LZ protein, Max, strongly support a role for c-Myc in transcriptional regulation [Fisher et al., 1993; Amati et al., 1993]. Studies have also suggested a direct correlation between transformation by Myc and the ability of Myc to regulate gene expression [Meichle et al., 1992]. Mutation of the bHLH-LZ regions as well as at least one highly conserved amino-terminal domain of Myc, Myc box 2, results in both a transcriptionally defective protein as well as a transformation defective protein [Steiner et al., 1995; Meichle et al., 1992]. Several target genes (ODC, CAD, and α-prothymosin) of c-*myc* have been suggested, although the roles of these genes in cell cycle progression have not yet been elucidated [Benvenisty et al., 1992; Eilers et al., 1991; Miltenberger et al., 1995; Bello-Fernandez and Cleveland, 1992; Peña et al., 1993, 1995; Tavtigian et al., 1994; Packham and Cleveland, 1994]. The expression of key cell cycle proteins, cyclins A, E, and D1, has also been shown, at least indirectly, to be influenced by c-Myc expression [Jansen-Dürr et al., 1993; Shibuya et al., 1992; Philipp et al., 1994; Hanson et al., 1994; Roussel et al., 1995], and recent studies have shown that expression of cyclin A, in particular, may link c-Myc to transformation and perhaps to other c-Myc-induced effects on cell growth [Barrett et al., 1995; Hoang et al., 1994].

To investigate the function of c-*myc* in cell proliferation we have expressed the hormone-inducible chimeric MycER protein [Eilers et al., 1989] in the nontransformed, EGF-dependent BALB mouse keratinocyte (BALB/MK) cell line [Weissman and Aaronson, 1983]. We have shown previously that induction of MycER in early G1 prevents TGFβ1 inhibition of cell cycle progression [Alexandrow et al., 1995]. Here we report that hormone activation of MycER causes an EGF-dependent superincorporation of labeled thymidine during the replicative S phase of the cell cycle. The results suggest that overexpression of Myc in keratinocytes causes more EGF-restimulated keratinocytes to enter the cell cycle, an effect that may arise in part from both positive and negative effects on Cdk function.

MATERIALS AND METHODS

Antisera

Anti-Myc and anti-Fos polyclonal antisera were obtained from Stephen R. Hann (Vanderbilt University). Polyclonal antiserum raised against $p50^{cyclin\ E}$ was obtained from Jim Roberts (Fred Hutchinson Cancer Institute). Monoclonal antisera against $p50^{E2F\text{-}1}$ (sc-251) and $p34^{cyclin\ D1}$ (sc-450), polyclonal antisera against $p50^{E2F\text{-}1}$ (sc-193), $p60^{cyclin\ A}$ (sc-160), $p27^{Kip1}$ (sc-528), $p16^{INK4A}$ (sc-1207), $p15^{INK4B}$ (sc-1429), $p67^{Cdc25A}$ (sc-97), $p33^{Cdk\text{-}2}$ (sc-163), and $p34^{Cdk\text{-}4}$ (sc-601), and immunogenic Cdc25A peptide (sc-97P) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) Additional anti-$p27^{Kip1}$ polyclonal antiserum (where indicated) was obtained from Tony Hunter (Salk Institute).

Cell Culture and Retrovirus Production

Noninfected BALB mouse keratinocytes (BALB/MK) were maintained in low-calcium MEM (Gibco, Grand Island, NY) supplemented with 8% dialyzed fetal calf serum (JRH Biosciences, Lenexa, KS) in 7% CO_2 incubators. The retroviral packaging line Ψ-2 [Stuhlmann et al., 1984] was maintained in 5% CO_2 in normal DMEM containing 10% newborn calf serum (Gibco). After transfection of Ψ-2 cells with the retrovirus-carrying plasmids pMV7-MycER [Eilers et al., 1989; Alexandrow et al., 1995], pMV7-ΔMycER [Eilers et al., 1989], pMV7-FosER [Superti-Furga et al., 1991; Reichmann et al., 1992], and pMV7-Gal4-ER-VP16 [Braselmann et al., 1993], the packaging lines were then cultured in phenol red-free DMEM containing charcoal-stripped newborn calf serum [Alexandrow et al., 1995]. After retroviral infection and G418 (Geneticin, Gibco) selection as described previously [Alexandrow et al., 1995], the BALB keratinocytes (MK/MycER, MK/ΔMycER, MK/FosER, and MK/Gal4-ER-VP16) were maintained in 5% CO_2 in low calcium, phenol red-free Medium-154 (Cascade Biologics, Portland, OR) containing charcoal-stripped, dialyzed fetal calf serum.

Cell Synchronization and Kinetic Assays

Keratinocytes (after reaching approximately 70% confluency) were synchronized in 100 mm plates (or 24-well plates for kinetic analyses) in

a quiescent state by depriving them of EGF for 72 h. After EGF starvation, EGF-containing medium (4 ng/ml) was added to the monolayer keratinocytes (approximately 5 x 10^6 cells per 100 mm plate at time of EGF restimulation) to restimulate entry into G_1, and synchrony was verified in each experiment by pulsing with 5 μCi/ml of [^{3}H]thymidine (5 Ci/mmol) for 1 h at the time of EGF restimulation (time 0) and 18 h after EGF restimulation (S phase time point) followed by analysis of TCA-insoluble material [Shipley et al., 1985] and comparison of time 0-h and time 18-h incorporations. For kinetic analyses, 5 μCi/ml of [^{3}H]thymidine was added at the times indicated in the figures to samples cultured under various conditions as described in each legend. After 1 h incubations with the labeled thymidine at each time point, samples were fixed with ascorbic acid and scintillation analyses of TCA-insoluble material were performed. Samples were analyzed in triplicate and results were plotted ± 1 standard deviation (s.d.).

Flow Cytometric Analyses

Synchronized keratinocytes were released into G_1 in the presence and the absence of 2 μM 4-hydroxytamoxifen (4-OHT; Research Biochemicals International, Natick, MA). At the times indicated in the figure, cells (one 100 mm plate per time point/condition) were scraped into calcium and magnesium-free phosphate-buffered saline (CMF-PBS, pH 7.4) and rapidly frozen in a dry ice/methanol bath and stored at −80°C. After all of the samples were collected, cells were resuspended in 500 μl Hepes-EDTA buffer (50 mM Hepes, 10 mM EDTA, pH 8.0), and incubated on ice for 30 min until cells had separated into single-cell distributions as visualized by microscopic examination. Hepes-EDTA buffer containing RNAse A (10 μg/ml, 375 μl) was added, followed by incubation on ice for 15 min and the addition of 375 μl of Hepes-EDTA buffer containing propidium iodide (PI) such that the final volume contained 50 μg/ml PI. After incubation in the dark at 4°C for 1 h, samples were analyzed by flourescent-activated cell sorting (FACS).

Radio-Immunoprecipitations

Logarithmically growing MK/ΔMycER, MK/FosER, and MK/Gal4-ER-VP16 cells (~4 x 10^6 cells/100 mm plate) were washed twice with PBS (pH 7.4) and starved of methionine for 1 h in methionine-free DMEM (warmed to 37°C) and labeled with 250 μCi of [^{35}S]methionine per ml (1 Ci = 37GBq) in fresh methionine-free DMEM for 1 h prior to lysis. Cells were washed twice with PBS and lysed in antibody lysis buffer [ALB; 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 0.5% SDS, 50 mM NaF, 100 μM $NaVO_4$, 75 μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.1TIU (trypsin inhibitor units) of aprotinin per ml, 1 μg/ml of leupeptin, 8 mM iodoacetamide] followed by ultrasonication. Lysates were normalized for total protein using the Bradford assay (Bio-Rad, Richmond, CA) and then precleared with protein-A Sepharose (Pharmacia, Gaithersburg, MD) prior to addition of anti-c-Myc or anti-c-Fos polyclonal antisera. Following incubation at 4°C for 4 h, antibody-antigen complexes were precipitated with protein-A Sepharose beads and washed five times with RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM NaF, 100 μM $NaVO_4$, 75 μg/ml PMSF, 0.1TIU/ml aprotinin, 1 μg/ml leupeptin, 8 mM iodoacetamide]. Samples were suspended in 1× Laemmli buffer, boiled, and separated by 10% SDS-PAGE. Gels were enhanced with 2,5-diphenyloxazole (PPO; Fisher Biotech, Orangeburg, NY), dried, and exposed to X-ray film. Coomassie-blue staining of the gels prior to enhancement verified that equal amounts of antisera were added to each lysate (data not shown).

Histone and RP-A Kinase Assays

Synchronized keratinocytes were released into G_1 in the presence and the absence of 2 μM 4-OHT or β-estradiol (Sigma). At the times indicated in the figures, cells (one 100 mm plate per time point/condition) were scraped into PBS and rapidly frozen in a dry ice/methanol bath and stored at −80°C until all time points were collected. Cell were lysed and sonicated in TNN buffer [50 mM Tris (pH 7.4), 250 mM NaCl, 0.1% NP-40], normalized to total protein using Bradford assays, and precleared with protein-A Sepharose beads. Anti-cyclin E polyclonal antiserum (3 μl/ml) or anti-E2F-1 monoclonal antiserum (1.0 μg/ml) was added to the lysates and rocked at 4°C for 2 h. Following immunoprecipitation with protein-A Sepharose, pellets were washed 4× with TNN buffer and 2× with kinase buffer [KB; 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 100 μM $NaVO_4$,

2.5 mM EGTA] containing 0.1 mg/ml bovine serum albumin. Pellets were resuspended in 40 µl of KB containing 30 µM cold ATP and 5 µCi of γ-[^{32}P]ATP and 10 µg of histone H1 (Sigma) or 0.8 µg of RP-A (provided by B. Stillman, Cold Spring Harbor Laboratory) per reaction. Samples were incubated at 37°C for 30 min and reactions stopped by adding 40 µl of 2× Laemmli buffer. Labeled histone bands were separated by SDS-PAGE and visualized by autoradiography. Incorporation into histone bands was quantitated using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Coomassie-blue staining of the gels verified that equal amounts of antisera and histone or RP-A substrate were present in each lysate and kinase reaction (data not shown).

Immunoprecipitation-Western Analyses

For Western detection of proteins co-immunoprecipitated with the anti-E2F-1 monoclonal antiserum (1.0 µg/ml), anti-Cdk-2 polyclonal antiserum (1.0 µg/ml), or anti-Cdk-4 polyclonal antiserum (1.0 µg/ml), lysates were prepared and immunoprecipitations with the appropriate antisera were performed as described above for the kinase reactions. After washing the pellets 4× with TNN buffer and 2× with KB, 1× Laemmli buffer was added and proteins were separated by SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were blocked for one hour at room temperature in PBS-T [phosphate-buffered saline (pH 7.4), 0.1% Tween-20] containing 5% nonfat dry milk. After washing for 1 h with excess PBS-T, membranes were incubated with the indicated amount of primary antisera in PBS-T at room temperature for 1 to 2 h. After a 1 h wash with excess PBS-T, the membranes were then incubated at room temperature for 1 h with the indicated amount of appropriate horseradish peroxidase (HRP) conjugated secondary antibody in PBS-T for 1 h at room temperature. Following a final wash in excess PBS-T, membranes were subjected to enhanced chemiluminescence (ECL) analysis as per the protocol of the manufacturer (Amersham, Arlington Heights, IL). The size of the recognized bands was determined by comparison to the mobility of prestained protein standards (Gibco). Recognition of the primary immunoprecipitating IgG heavy chain by the HRP-conjugated secondary antibody was seen in all immunoprecipitation-western analyses, and is indicated by an arrow in some figures. This was used to verify equal gel loading and that equal amounts of immunoprecipitating antisera were added to the lysates.

Immunoblotting

Synchronized keratinocytes were released into G_1 in the presence or absence of 2 µM OHT or estradiol. At the times indicated in the figures, cells (one 100 mm plate per time point/condition) were scraped into PBS (pH 7.4) and frozen in dry ice/methanol and stored at −80°C until all time points were collected. Frozen cell pellets were thawed on ice and resuspended in 200 µl of ALB followed by ultrasonication. Samples were normalized using Bradford assays and 200 µl of 2× Laemmli buffer was added followed by resolution of proteins by 10% or 15% SDS-PAGE and transfer to nitrocellulose membranes. Membranes were blocked at room temperature with PBS-T containing 5% dry milk. Subsequent steps were identical to those described above for the immunoprecipitation-western analyses. The size of the recognized bands was determined by comparison to the mobility of prestained protein standards (Gibco). Where indicated, immunogenic Cdc25A blocking peptide was incubated with the primary antiserum (as recommended by the manufacturer) on ice for 2 h prior to incubation with the filters.

RESULTS

Activation of Myc Leads to An Increased Incorporation of Labeled Thymidine Only During S Phase

Others have shown that overexpression of c-*myc* alone causes quiescent Rat1a cells to progress into S phase in the absence of serum [Eilers et al., 1989; Jansen-Dürr et al., 1993]. Although studies from our laboratory have shown that EGF-deprived, quiescent BALB/MK cells expressing the hormone-inducible MycER fusion protein (MK/MycER) are unable to progress into S phase in the presence of hormone-activated MycER [Alexandrow et al., 1995], we have found that activation of MycER does affect the level of radio-labeled thymidine that is incorporated into DNA during the replicative period of EGF-restimulated cells. MK/MycER cells treated with the natural hormone estradiol (estrogen), or the synthetic hormone 4-hydroxytamoxifen (4-OHT), incorporate greater levels of [^{3}H]thymidine than untreated

control cells (Fig. 1A). Uninfected BALB/MK cells are unaffected by treatment with estrogen indicating that the increased incorporation of thymidine is not due to cellular effects of the hormone or carrier (Fig. 1B). In addition, BALB/MK cells carrying a retrovirally-transduced fusion construct consisting of the Gal4 DNA-binding domain fused to the ER domain and the herpesvirus VP16 transactivation domain (MK/Gal4ER-VP16) do not show an increase in thymidine incorporation in response to estrogen or 4-OHT treatment (Fig. 1C). This suggests that neither the simple presence of an exogenous hormone-inducible fusion protein nor the process of retroviral infection nor the existence of the retroviral sequences are contributing to the effect on thymidine incorporation.

To determine if the effect of MycER activation on thymidine incorporation is specific for the Myc proto-oncogene, we retrovirally infected BALB/MK cells with recombinant viruses carrying hormone-inducible FosER (MK/FosER) and mutant MycER (MK/ΔMycER) proteins, pooled G418-resistant cells, and tested them for their response to estrogen and 4-OHT. Radio-immunoprecipitation analyses verified that expression of both fusion proteins was achieved (Fig. 2A). Treatment of both the MK/ΔMycER (Fig. 2B) and MK/FosER cells (data not shown; see Fig. 2 legend) with estrogen or 4-OHT did not result in any increase in thymidine incorporation and may actually have been inhibitory toward cell growth. This indicates that the increased incorporation of thymidine is dependent on induction of an intact Myc protein and is specific for the Myc protein because another proto-oncogene product, Fos, cannot substitute for Myc. As the domain deleted in the ΔMycER protein has been shown to be required for the putative transactivation function of Myc, the effect of Myc on thymidine incorporation may be mediated by transcriptional activation of genes downstream of Myc.

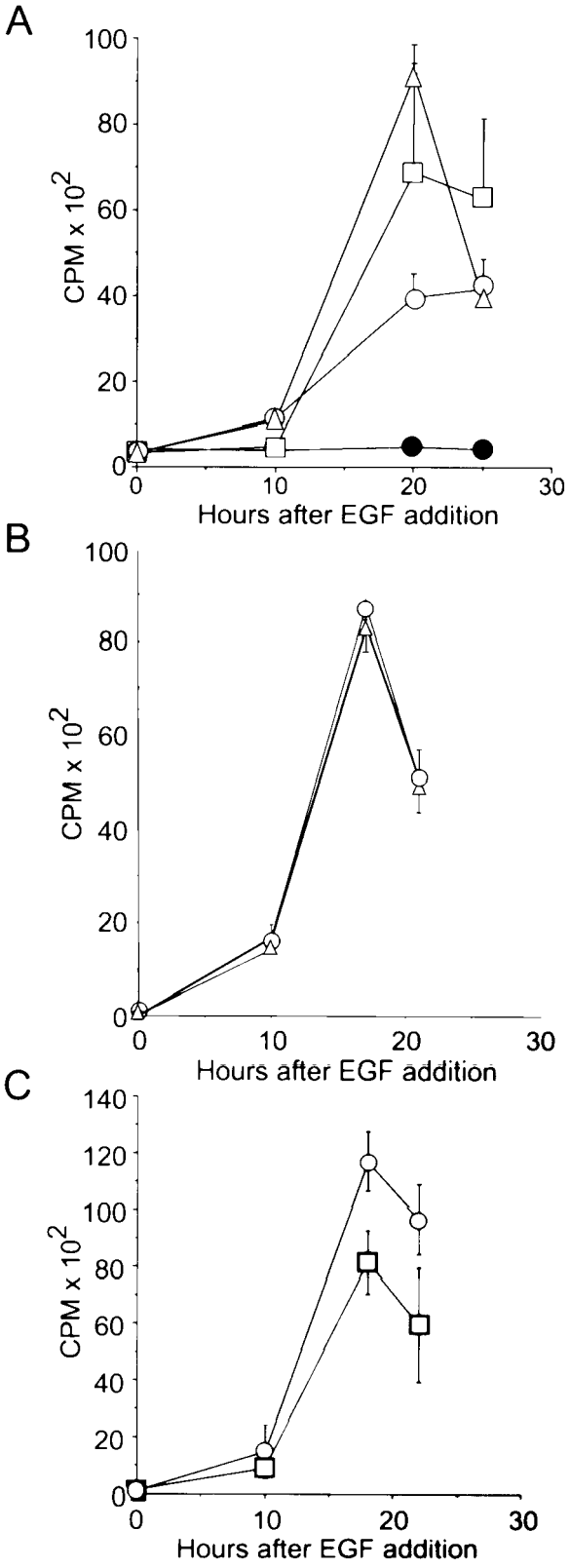
Myc Activation Causes an Increase in the Number of EGF-Restimulated Cells Entering S Phase and Reduces the Length of G_1

We next hypothesized that the increase in thymidine incorporation could be the result of an increase in the number of EGF-restimulated keratinocytes progressing into S phase upon MycER activation. To address this question, we performed flow cytometric analyses (FACS) on the keratinocytes at many time points during the cell cycle following EGF restimulation. The FACS analyses in Figure 3A revealed that in the presence of activated MycER, but not FosER (data not shown; see Fig. 3 legend), more keratinocytes were entering S phase after EGF-restimulation. This result could be sufficient to explain the observed elevated incorporation of labeled thymidine upon MycER activation as more cells in S phase would produce a greater incorporation of thymidine. An interpretation of these data might also be that the presence of excessive Myc protein in the keratinocytes is supplying additional signals, or signals that are lacking, in keratinocytes in which the EGF signal alone is not sufficient for progression into S phase.

The FACS analyses also showed a slight reduction in the length of G_1 phase (compare hormone-treated vs. control cells at time points 9.5 through 14 h in Fig. 3A) as reported by other groups [Shichiri et al., 1993; Hanson et al., 1994; Karn et al., 1989]. Premature entry into G_1 phase combined with an overall increase in the number of EGF-restimulated cells entering S phase might be predicted to lead to an increase in total cell number over time. However, in disagreement with this prediction, keratinocytes treated with hormone for 3 days showed no significant changes in total cell number when compared to control cells (Fig. 3B). As 3 days is sufficient for the keratinocytes to undergo more than two complete cell cycles (one cell cycle is approximately 30 h; see FACS analysis above), it is evident that any predicted increase in the total number of cells is not present after several cell cycles. Although the reason for this apparent loss of cycling cells remains to be shown, one likely explanation for this observation could be that there is an increase in Myc-induced apoptosis, an effect that has been seen in other studies involving overexpressed Myc protein in cells [Evan et al., 1992; Wagner et al., 1994; Gibson et al., 1995; Shi et al., 1992; Bissonnette et al., 1992; Milner et al., 1993].

Abundance of Putative Myc-Sensitive Cyclins Are Unaffected by Myc Activation

It was apparent that overexpression of Myc in the keratinocytes was causing otherwise non-cycling cells in the population to enter the cell cycle, as indicated by both a greater incorporation of labeled thymidine and an increase in the



number of cells entering S phase subsequent to EGF re-stimulation. We hypothesized that activation of MycER was altering the cell cycle machinery and promoting cell cycle progression of non-cycling cells in the keratinocyte population. Based on these ideas, we examined the effects of MycER activation on several aspects of the cell cycle machinery.

Previous studies have shown that the expression of several G_1 cyclins is affected by Myc expression in different cell types [Jansen-Dürr et al., 1993; Shibuya et al., 1992; Philipp et al., 1994; Hanson et al., 1994; Roussel et al., 1995]. It has been shown that cyclins A and E increase in total levels after hormone treatment of Rat1a cells carrying the MycER protein [Jansen-Dürr et al., 1993], while other studies have demonstrated a dependence of the expression of the same cyclins on Myc in hematopoietic cells [Shibuya et al., 1992]. In contrast, cyclin D1 expression has been suggested to be a target of downregulation by Myc [Philipp et al., 1994; Solomon et al., 1995].

To determine if the Myc-dependent effects on thymidine incorporation and enhanced S phase entry were in part mediated by altered regulation of G_1 cyclin expression, we performed Western analyses on cells cultured in the presence or absence of hormone to examine the effect of Myc activation on the total levels of these proteins. We chose to examine time points in late G_1 for two primary reasons. The cyclins under question are normally expressed and regulate Cdk activity during this time, and data presented above suggested that activation of Myc

Fig. 1. Activation of MycER in keratinocytes leads to increased incorporation of labeled thymidine during S phase. MK/MycER (**A**), uninfected (**B**), and MK/Gal4ER-VP16 cells (**C**) were synchronized at the beginning of G_1 phase in 24-well plates using EGF deprivation and restimulation as described in Materials and Methods. At the time of restimulation with EGF (time 0), 2 μM estradiol or 4-hydroxytamoxifen (4-OHT) was added to appropriate wells and remained throughout the course of the experiment. Beginning at the hours indicated for each time point in the curves, triplicate wells were pulsed for one hour with ^{3}H-thymidine and incorporation of labeled thymidine was determined for each condition as described in Materials and Methods. A: EGF alone (open circles), no EGF (closed circles), EGF and 4-OHT (open squares), EGF and estradiol (open triangles). B: EGF alone (open circles), EGF and estradiol (open triangles). C: EGF alone (open circles), EGF and 4-OHT (open squares). Values on curves are means of triplicate counts ± 1 s.d. For A, the data are representative of greater than three independent experiments performed in triplicate with the mean increases in thymidine incorporation following hormone treatment varying from two- to four-fold above control samples.

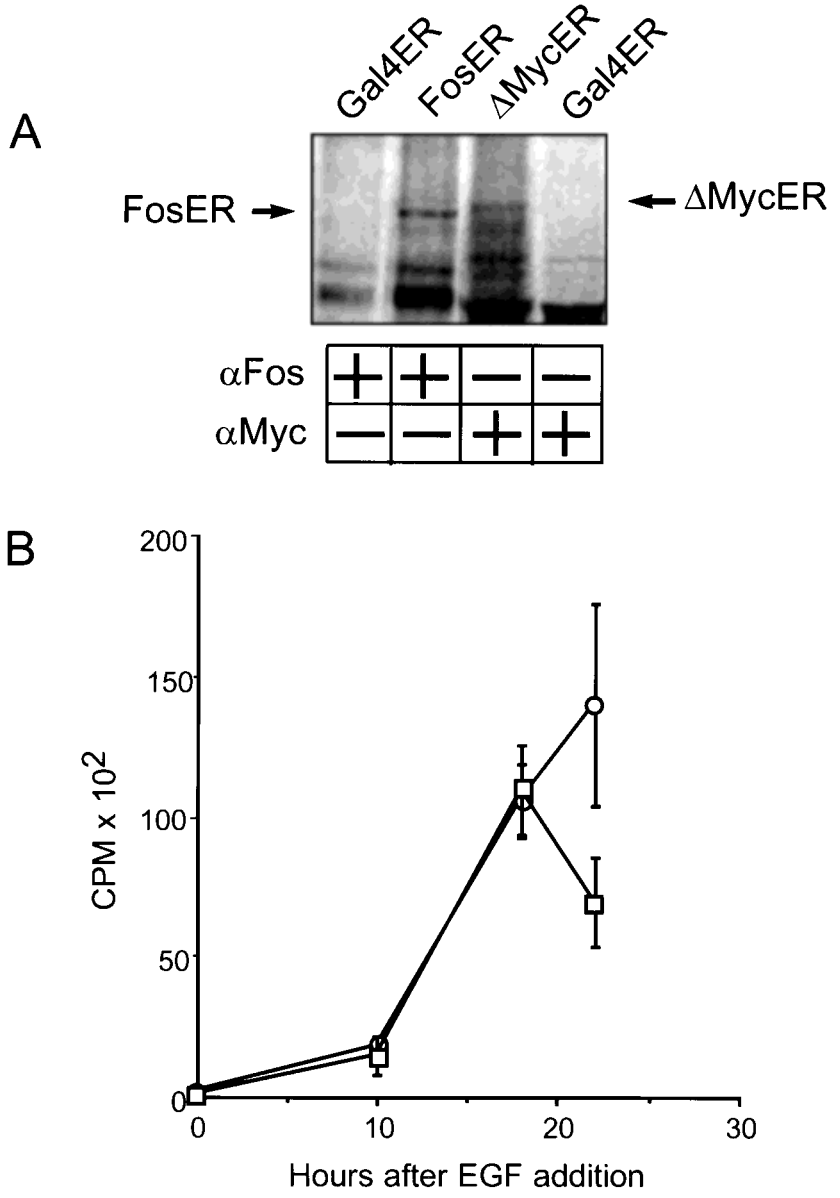
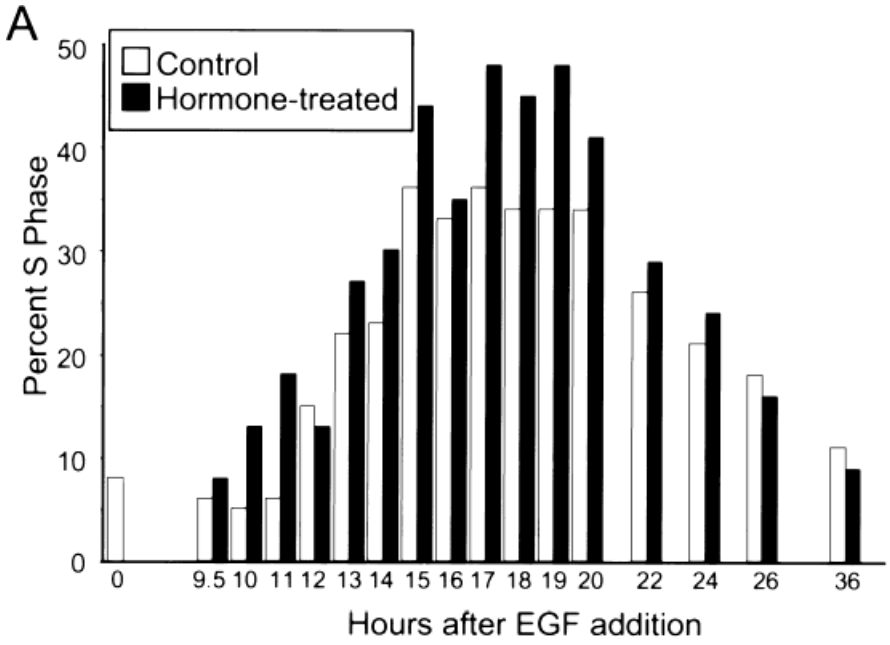


Fig. 2. Cells carrying FosER or ΔMycER chimeric proteins do not show any effect of hormone on the level of tritiated-thymidine incorporation. **A**: Asynchronous MK/FosER, MK/ΔMycER, and MK/Gal4ER-VP16 cells were labeled with ^{35}S-methionine and radio-immunoprecipitations performed as described in Materials and Methods. Expression of FosER and ΔMycER is shown in the appropriate cell types, but both proteins are absent in the MK/Gal4ER-VP16 cells. **B**: MK/ΔMycER cells were synchronized at the beginning of G_1 phase and pulse-labeled with ^{3}H-thymidine for 1 h at the indicated times as described in the legend for Figure 1. EGF alone (open circles), EGF and 4-OHT added at the time of EGF restimulation (open squares). Values on curves are means of triplicate counts ± 1 s.d. An almost identical result was found with cells carrying the FosER protein (data not shown).

in the keratinocytes might be affecting rate-limiting events in late G_1 leading to premature and enhanced entry of the population into S phase. Figure 4 shows that activation of Myc early in G_1 did not produce any significant effect on the abundance of cyclins A, E, and D1 in late G_1. Cyclins D2 and D3 were also unaffected by Myc activation (data not shown).

Histone Kinase Activity of E2F-1-Associated Cyclin A/Cdk-2 Is Increased Following Myc Activation

Recent studies have suggested that the presence of excessive Myc protein in cells is accompanied by an increase in the histone kinase activity associated with cyclin E/Cdk-2 and that



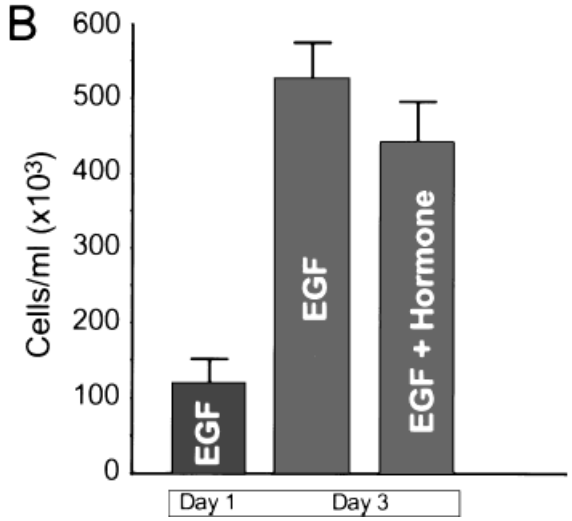


Fig. 3. Activation of MycER in the keratinocytes causes an increase in the number of EGF-restimulated cells entering S phase and reduces the duration of G_1. **A**: MK/MycER cells were synchronized at the begining of G_1 as described in the legend to Figure 1. For hormone-treated samples, 2 μM 4-OHT was added to the cells at the time of EGF restimulation (time 0). At the times indicated below each pair of columns, 2 x 10^6 cells were collected for each condition and processed for FACS analysis as described in Materials and Methods. The results of this analysis (percent of cells in S phase only) are shown and are representative of three similar experiments with nearly identical results. For each time point shown, an equal number of cells were analyzed. Analysis of MK cells carrying the FosER protein showed a FACS pattern identical to control cells shown above (data not shown). **B**: Asynchronous MK/MycER cells were treated or untreated with 2 μM estradiol beginning on day 1 and allowed to culture for 3 days. For each column shown, cells were trypsinized to single-cell condition and samples counted using a haemacytometer. The means of triplicate counts ± 1 s.d. are plotted.

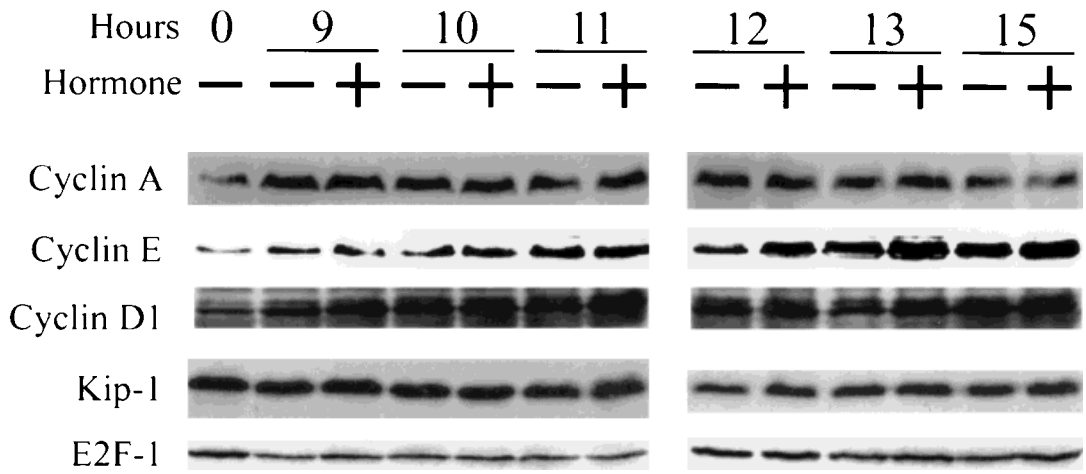
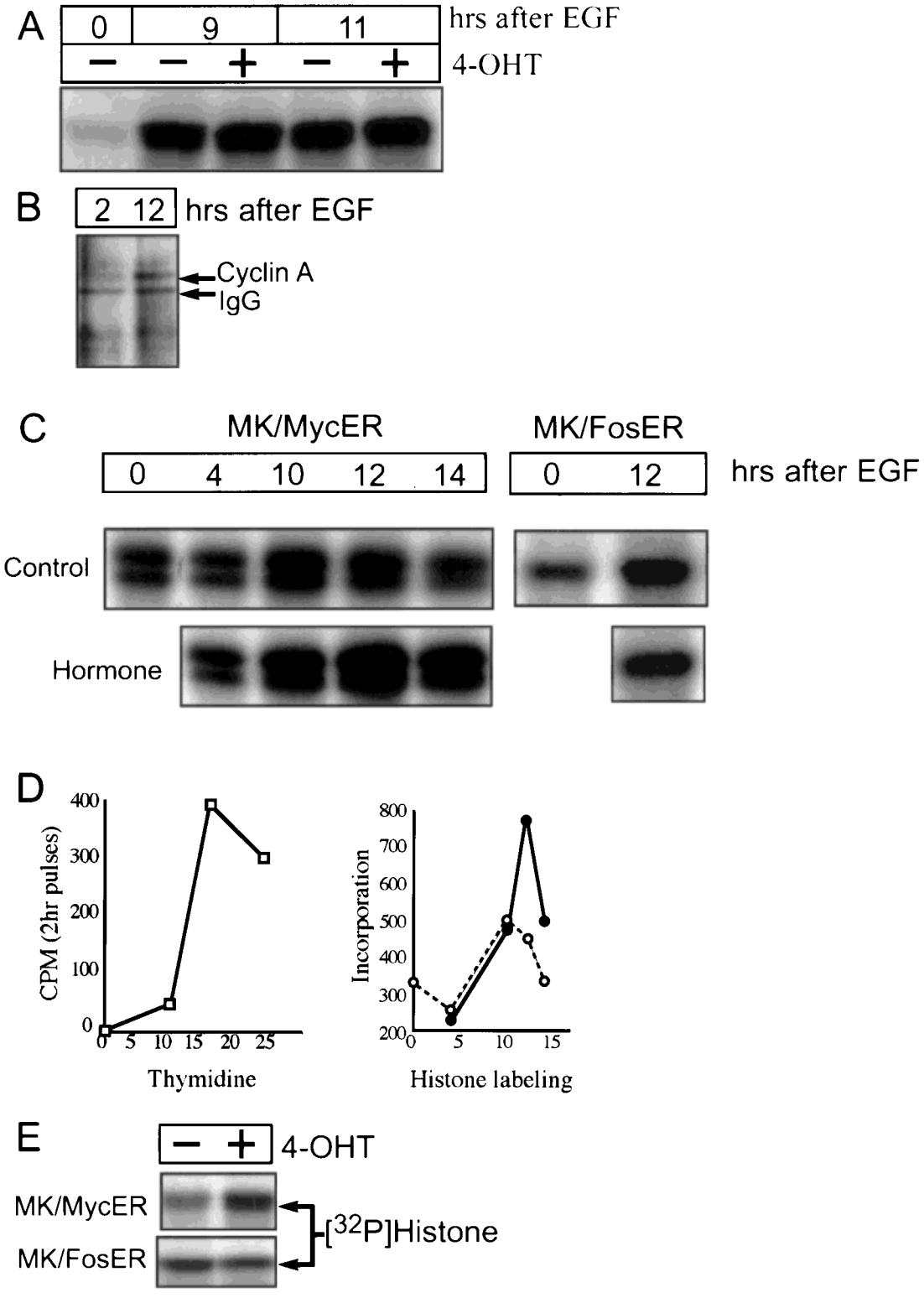


Fig. 4. Activation of MycER in the keratinocytes does not affect the expression of G_1 cyclins, the Kip1 inhibitor protein, or the E2F-1 protein. MK/MycER cells were synchronized at the beginning of G_1 phase as described in Materials and Methods, and re-entry into G_1 and S phases, and the MycER-induced superincorporation of labeled thymidine, were verified using the same methods shown in Figure 1A (data not shown). For samples treated with hormone inducer, 2 μM estradiol was added at the time of restimulation with EGF (time 0). At the times following EGF addition indicated above the protein bands, cells were collected and Western analyses performed using the antisera shown as described in Materials and Methods. The concentrations of antisera used on the filters were 0.5 μg/ml for anti-cyclin A, anti-cyclin E, anticyclin D1, and anti-E2F-1, and 0.5 μl/ml for anti-Kip1 (obtained from T. Hunter). The dilution of appropriate secondary in all cases was 1:10,000.

the mechanism for this effect involves dissociation of the $p27^{KIP1}$ protein from the enzyme complex [Steiner et al., 1995; Vlach et al., 1996]. Based on these results, we hypothesized that activation of MycER in the keratinocytes might also be affecting the activity of the cyclin E/Cdk-2 complex, leading to a shortening of G_1 and a greater percentage of cells entering S phase. However, examination of the activity of Cdk-2 associated with cyclin E in late G_1 showed that activation of MycER had no effect on the histone H1 kinase activity of this complex (Fig. 5A).

Another Cdk-2-containing complex that has been shown to form in late G_1 and early S phases is that of the E2F-1/DP-1/cyclin A/Cdk-2 holocomplex [Shirodkar et al., 1992; Wu et al., 1995; Lees et al., 1992; Cao et al., 1992]. Although endogenous cell cycle-regulated enzymatic activity of this complex has not yet been shown, it is predicted that, upon formation, the complex becomes active toward substrates important in the transition into S phase [Lees et al., 1992]. Immunoprecipitation-western analyses showed that cyclin A and Cdk-2 were indeed

Fig. 5. Activation of MycER in the keratinocytes leads to an increase in the histone H1 kinase activity associated with the Cyclin A/Cdk-2/E2F-1 complex late in G_1. MK/MycER or MK/FosER cells were synchronized at the beginning of G_1 as described in Materials and Methods, and the ability of MycER to cause superincorporation of labeled thymidine was verified using the approach shown in Figure 1A (data not shown). For samples treated with hormone inducer, 2 μM 4-OHT (A and E) or estradiol (C) was added at the time of restimulation with EGF (time 0). In each case, the numbers above the data indicate the times after EGF restimulation when the samples were collected, and "+" and "−" indicate that the cells were cultured in the presence or absence of hormone, respectively. **A**: MK/MycER lysates were incubated with anti-cyclin E polyclonal antiserum followed by analysis of co-immunoprecipitated histone H1 kinase activity as described in Materials and Methods. **B**: MK/MycER lysates were subjected to Western analyses after immunoprecipitation with anti-E2F-1 monoclonal antiserum to verify association of Cyclin A with the E2F-1 protein late in G_1 (time 12 h) in the keratinocytes. 0.5 μg/ml of anti-Cyclin A antiserum was used, and the secondary antibody was diluted 1:10,000. **C**: MK/MycER and MK/FosER lysates were incubated with anti-E2F-1 monoclonal antisera followed by analysis of co-immunoprecipitated histone H1 kinase activity as described in Materials and Methods. Results shown are from the same experiment and are representative of three independent experiments with similar results. **D**: At the times indicated, incorporation of [^{3}H]thymidine was determined for control cells as described in Materials and Methods, and the means of triplicate counts were plotted to show cell synchronization and rate of entry into S phase during collection of lysates for kinase analyses. Relative levels of ^{32}P incorporation into histone bands was determined for each time point shown in (C) using PhosphorImager analysis of labeled bands, and the data were plotted (right panel). **E**: EGF-deprived MK/MycER or MK/FosER lysates were analyzed as described above for E2F-1-associated histone H1 kinase activity following 12 h of hormone treatment in an EGF-deprived state. PhosphorImager analysis showed that there was approximately a two-fold increase in histone labeling in the presence of hormone in lysates from MK/MycER cells (data not shown).


A
0
9
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Control
Hormone
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400
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0 5 10 15 20 25
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Histone labeling
E
− +
4-OHT
MK/MycER
MK/FosER
[32P]Histone

Figure 5.

complexed with E2F-1 in the keratinocytes in late G_1/early S phase as predicted from evidence in the literature (Fig. 5B, and data not shown). Examination of the histone H1 kinase activity associated with E2F-1 in late G_1 and early S phases revealed that as cells progress into S phase the kinase activity increases and peaks shortly after entry into the DNA-replicative period (Fig. 5C,D). In addition, the kinetics of kinase activity appear to correlate with the ability of the E2F-1 protein to bind DNA as shown by others [Dynlacht et al., 1994; Xu et al., 1994; Krek et al., 1994]. Interestingly, the histone kinase activity associated with E2F-1 in late G_1 and early S phases was increased following activation of MycER, but not FosER (Fig. 5C,D). Although the amount of increase in E2F-1-associated H1 kinase activity was only approximately two-fold, this increase was nonetheless proportional to the observed increase in thymidine incorporation and was greater than the increase in the number of cells entering S phase (see FACS analyses above). In addition, as there are at least four other E2F family members in cells, there is the potential for Myc-related effects on complexes containing other E2F proteins. However, we have been unable to demonstrate this due to a lack of availability of appropriate antisera.

We also tested whether the E2F-1-associated Cdk-2 showed increased kinase activity toward another putative substrate, replication protein A (RP-A), which is required for initiation of DNA replication and becomes phosphorylated as cells enter S phase [Longhese et al., 1994; Dutta and Stillman, 1992; Dutta et al., 1991; Fotedar and Roberts, 1992]. Unfortunately, RP-A was not a substrate for Cdk-2 in vitro, although increased kinase activity toward histone was again observed (data not shown). This result was not surprising since studies from other laboratories have shown that, although phosphorylation of RP-A is dependent on cyclin A/Cdk-2 activity in late G_1 and early S phase, RP-A itself is a poor substrate for Cdk-2 in vitro [Pan et al., 1994; Fang and Newport, 1993; Brush et al., 1994].

Previous studies from our laboratory have shown that activation of MycER in the keratinocytes is not sufficient to stimulate EGF-deprived cells to enter the cell cycle, as has been shown with other cell types such as Rat1a cells [Alexandrow et al., 1995; Eilers et al., 1989; Jansen-Dürr et al., 1993]. Interestingly, activation of MycER in EGF-deprived (quiescent) keratinocytes also leads to an increase in the E2F-1-associated kinase activity after several hours (Fig. 5E). This result suggests that activation of MycER in the absence of EGF-derived signals is itself sufficient to cause the increase in E2F-1-associated kinase activity. It also suggests that the kinase activity of E2F-1-associated Cdk-2 is not sufficient to cause quiescent keratinocytes to progress into S phase.

Increase in E2F-1-Associated Kinase Activity Does Not Involve Dissociation of the Cdk Inhibitor $p27^{Kip1}$ Nor an Increase in E2F-1 Expression

Recent evidence has suggested that the positive effect of Myc expression on Cdk-2 activity involves dissociation of the Cdk inhibitor $p27^{Kip1}$ from the cyclin/Cdk complexes [Steiner et al., 1995; Vlach et al., 1996]. Based on this, we performed experiments to investigate whether there were changes in the amount of $p27^{Kip1}$ or another Cdk inhibitor, $p21^{Cip1}$, associated with E2F-1-containing complexes. Figure 6A shows that in the presence of activated MycER, no observable differences were seen in the basal levels of $p27^{Kip1}$ bound to E2F-1-containing complexes in late G_1. In addition, the amount of $p27^{Kip1}$ associated with total Cdk-2 in the cells (data not shown), as well as the total protein expression of the inhibitor (Fig. 4), were both unaffected by Myc activation. The $p21^{Cip1}$ protein was undetectable in these co-immunoprecipitation assays, but was recognized in total cell lysates and showed no significant changes in expression following MycER activation (data not shown). Probing of the anti-E2F-1 IP-Western filter with anti-E2F-1 antiserum verifed that E2F-1 was being immunoprecipitated in equal amounts from the cells (Fig. 6A).

The lack of an effect of Myc on Cdk-2-associated inhibitors prompted us to investigate the association of Cdk inhibitors with another member of the Cdk family, Cdk-4. Interestingly, when we examined the amount of the $p16^{INK4A}$ Cdk-4/6 inhibitor associated with Cdk-4 in Myc-activated populations, we actually found more $p16^{INK4A}$, but not $p15^{INK4B}$ (which appeared as a doublet), associated with Cdk-4 in late G_1 as cells were entering S phase (Fig. 6B). This was not due to an increase in abundance of $p16^{INK4A}$ following Myc activation since Western analysis showed no changes in total protein levels of the inhibitor (Fig. 6C).

We also examined whether Myc activation in the keratinocytes was affecting the abundance

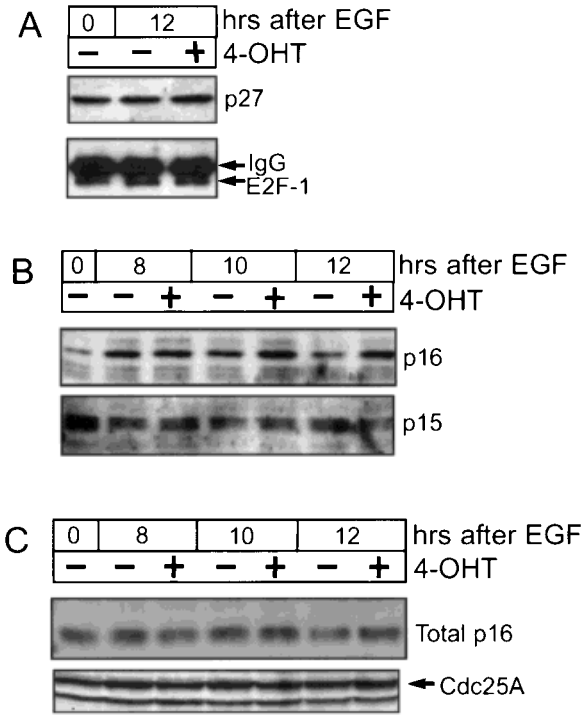

Fig. 6. Activation of MycER is associated with an increased level of complexing between Cdk-4 and the $p16^{INK4A}$ protein. MK/MycER cells were synchronized at the beginning of G_1 as described in Materials and Methods. For samples treated with hormone inducer, 2 μM 4-OHT was added at the time of restimulation with EGF (time 0). In each case, the numbers above the data indicate the times after EGF restimulation when the samples were collected, and "+" and "−" indicate that the cells were cultured in the presence or absence of hormone, respectively. **A**: Lysates were subjected to immunoprecipitation with anti-E2F-1 monoclonal antiserum followed by Western detection of co-immunoprecipitated proteins as described in Materials and Methods. 0.5 μg/ml of anti-$p27^{Kip1}$ and anti-E2F-1 polyclonal antisera were used, and the secondary antibody was diluted 1:10,000. **B**: Lysates were subjected to immunoprecipitation with anti-Cdk-4 polyclonal antisera followed by Western detection of co-immunoprecipitated proteins as described in Materials and Methods. 0.5 μg/ml of anti-$p16^{INK4A}$ and 1.0 μg/ml of anti-$p15^{INK4B}$ polyclonal antisera were used, and the secondary antibody was diluted 1:1,000 and 1:5,000, respectively. **C**: Cells were analyzed by Western analysis for the effects of hormone treatment on the total levels of $p16^{INK4A}$ and $p67^{Cdc25A}$. 0.5 μg/ml of anti-$p16^{INK4A}$ and anti-Cdc25A polyclonal antisera were used, and the secondary antibody was diluted 1:1,000. Four high molecular weight bands were detected by the anti-Cdc25A antiserum, but separate analyses in which immunogenic Cdc25A peptide was used to block the primary antiserum showed that only the 70 kDa band (indicated with the arrow) was specific for the antiserum in the assay (data not shown).

of the E2F-1 protein. Figure 4 shows that induction of Myc in early G_1 did not affect the late-G_1 abundance of the E2F-1 protein. Altogether, the data presented suggest that any effect of Myc on the E2F-1-associated kinase activity is not caused by a decrease in associated $p27^{Kip1}$ or $p21^{Cip1}$ levels, nor an increase in the abundance of the E2F-1 protein in the cells. Another plausible mechanism that has not been investigated is Myc-dependent regulation of Cdk-2 activity through alterations in the phosphorylation state of the enzyme, cyclin, and/or E2F-1 protein. Related to this idea, recent data would suggest that Myc is capable of upregulating the expression of the Cdc25A tyrosine/threonine phosphatase, which likely acts to stimulate Cdk kinase activity by dephosphorylating threonine 14 and/or tyrosine 15 sites in the amino terminus of Cdk-2 [Galaktionov et al., 1996; Gautier et al., 1991; Dunphy and Kumagai, 1991]. In contrast to the results seen in other cell types [Galaktionov et al., 1996], activation of MycER in the keratinocytes did not affect the total protein levels of the Cdc25A phosphatase during G_1 phase (Fig. 6C). However, it still remains to be shown whether excessive Myc in cells can affect the activity of Cdc25A or other phosphatases and kinases involved in cyclin/Cdk regulation.

DISCUSSION

In this report, we demonstrate a Myc-dependent and Myc-specific increase in the amount of labeled thymidine that is incorporated by a non-transformed mouse keratinocyte cell line following activation of the hormone-sensitive MycER protein. Data presented show that this effect on thymidine incorporation is likely attributable to an overall increase in the number of EGF-restimulated keratinocytes that are progressing into S phase and may also involve a reduction in the length of G_1. Examination of several putative Myc-sensitive target proteins reveals that activation of MycER in the keratinocytes has no effect on G_1 cyclin expression or cyclin E-associated histone kinase activity, as shown by others. However, the effect of Myc on cell cycle progression has been shown to be associated with a positive effect of Myc on the activity of Cdk-2 associated with the E2F-1 protein in late G_1 and early S phases. Although the mechanism responsible for this increase in E2F-1-associated Cdk-2 activity remains unknown, evidence presented suggests that there are no changes in the association of $p27^{Kip1}$ or $p21^{Cip1}$ and that there is no increase in the abundance of E2F-1 protein in the keratinocytes.

Several reports suggest an important biological link between Myc and E2F-1. Myc and E2F-1 have individually been shown to be capable of promoting entry into and progression through the cell cycle in serum-deprived cells after over-

expression of either protein [Kowalik et al., 1995; Eilers et al., 1989; Jansen-Dürr et al., 1993; Shan and Lee, 1994]. In experiments testing the ability of E2F-1 or Myc to block TGFβ1-induced growth arrest, overexpression of either Myc or E2F-1 renders cells insensitive to the inhibitory effects of TGFβ1 [Schwarz et al., 1995; Alexandrow et al., 1995; Selvakumaran et al., 1994]. In addition, recent data from Lee and colleagues [Shan et al., 1996] has shown that overexpression of E2F-1 in growing cell populations results in a higher number of cells in S phase, a result that is the same as that seen here upon activation of Myc in the keratinocytes. These correllations of the biological effects of Myc and E2F-1 suggest that both proteins may function in a common regulatory pathway.

The ability of Myc to positively influence downstream E2F-1/Cdk-2 kinase activity might explain some of the biological similarities observed following overexpression of Myc or E2F-1 in various cell types. The results presented here demonstrate a novel biochemical effect of deregulated Myc expression on Cdk-2 kinase activity associated with E2F-1 in late G_1, suggesting that at least one function of Myc may be positive regulation of kinase activity associated with E2F1. In this manner, overexpression of either Myc or E2F-1 may eventually produce the same biochemical effect in cells: E2F-1/Cdk-2 complexes become more active resulting in altered growth characteristics.

An unexpected effect of activating the MycER protein in the keratinocytes was an increase in the amount of $p16^{INK4A}$ associated with Cdk-4, likely followed by a decrease in Cdk-4-associated kinase activity, as cells were entering S phase. Interestingly, other reports show that negative regulation of cyclin D1 (and likely Cdk-4 function) is also attributed to elevated Myc levels in various cell types [Philipp et al., 1994; Solomon et al., 1995; Jansen-Dürr et al., 1993]. Such data appear contradictory to existing models of cell cycle progression in which cyclin D1-associated function *promotes* progression through G_1 phase; however, several studies suggest that deregulated expression of cyclin D1 can actually *prevent* progression into S phase. It has been shown that injection of cyclin D1protein into cells can block progression into S phase and, in a similar manner, overexpression of cyclin D1 can inhibit cell proliferation [Quelle et al., 1993; Philipp et al., 1994]. In addition, other data have shown that Myc alleles capable of downregulating cyclin D1 expression are associated with an increased number of cells in S phase, whereas alleles of Myc that cannot repress cyclin D1 expression do not cause enhanced S phase entry and actually produce a delayed progression into the replicative period [Philipp et al., 1994]. These results make it interesting to speculate that negative regulation of Cdk-4 function following Myc-induced repression of cyclin D1, as shown by others, or increased association of $p16^{INK4A}$ with Cdk-4, as shown here, may comprise a mechanism whereby a rate-limiting step in G_1 to S progression is removed.

Alternatively, the results presented here could also support a negative regulatory feedback model. Recent studies have shown that overexpression of E2F-1 leads to negative feedback regulation of cyclin D/Cdk-4 kinase activity [Khleif et al., 1996]. This effect of deregulated E2F-1 expression on Cdk-4 kinase activity is mediated by an increase in protein expression of $p16^{INK4A}$ and a concommitant decrease in Cdk-4 kinase activity [Khleif et al., 1996]. The investigators suggested that this effect of excessive E2F-1 protein is evidence for a negative feedback regulatory mechanism whereby in cells entering S phase after pRB has been inactivated by Cdk-4-catalyzed phosphorylation (releasing functional E2F-1), the presence of free E2F-1 protein upregulates $p16^{INK4A}$ leading to downregulation of growth-promoting Cdk-4 kinase activity toward pRB [Khleif et al., 1996]. In agreement with this model, the keratinocytes could be responding to the Myc-induced increase in S phase-promoting E2F-1/Cdk-2 kinase activity by increasing the association of $p16^{INK4A}$ with Cdk-4. This would lead to downregulation of pRB-kinase activity and prevent over-stimulation of G_1 growth signals.

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