EGF effects on p53 in MDA-468 human breast cancer cells: implications for G₁ arrest

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Abstract. EGF, in pharmacological concentrations, inhibits cell proliferation of the MDA-468 human breast cancer cell line. Previously, we have demonstrated that this was characterized by a reversible cell cycle arrest at the G₁–S boundary, concomitant with downregulation of mRNA levels for p53 (a point mutant, p53^{273.His}). Since p53^{273.His} is regarded as a gain-of-function mutant and acts to enhance cell proliferation, we hypothesized that the G₁ arrest induced by EGF might be mediated by p53^{273.His}. In this study, we report an EGF-dependent altered conformation as indicated by immunofluorescence, while no significant immediate effects of EGF-treatment on p53^{273.His} protein levels and synthesis were observed. These experiments demonstrated a decreased PAb 240 (mutant-specific) reactivity of nuclear p53^{273.His} in EGF-treated cells, while that of PAb 1620 (wild-type specific) was enhanced. Staining with PAb 1801 (pan specific), on the other hand, showed little change upon EGF treatment. Further studies indicated a decreased phosphorylation of nuclear p53^{273.His} in EGF-treated cells. These EGF-dependent events were detected early enough to be attributed as causative of cell cycle arrest. We suggest that EGF-mediated, phosphorylation-dependent conformational change in nuclear p53^{273.His}, and in turn altered p53 function, may be responsible for EGF-dependent growth inhibition MDA-468 cells.

Functional alteration of tumour suppressor p53, either by association with viral proteins, or by mutations is critical for cellular transformation in many human tumours (Levine & Momand 1990, Hollstein *et al.* 1991, Levine *et al.* 1991). p53 regulates entry of cells into S phase and progression of DNA replication in both normal and transformed cells (Deppert *et al.* 1990, Shaulsky *et al.* 1990, Steinmeyer *et al.* 1990, Levine *et al.* 1991). Consistent with this, nuclear localization of p53 in normal fibroblasts is observed prior to G₁/S transition and during S phase but not in mitosis (Shaulsky *et al.* 1990). The ability of p53 to either induce cell cycle arrest or promote transformation could be determined by its conformation. A temperature-sensitive mutant p53 protein, p53^{135.Val}, was transforming in conjunction with *ras* at 37°C (mutant conformation PAb 240 reactive), but suppresses transformation at 32.5°C (wild-type form, PAb 246 reactive) by inducing a late G₁ arrest (Ginsberg *et al.* 1991b, Martinez *et al.* 1991, Michalovitz *et al.* 1991). Cell cycle regulation by p53 operates in late G₁ phase, prior to the restriction point (R point (Lin *et al.* 1992)). In normal and tumour cells,

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loss of p53 function abrogates a check-point in late G₁ which is thought to be responsible for maintaining genomic integrity following DNA damage (Kuerbitz *et al.* 1992, Harvey *et al.* 1993, Tsukada *et al.* 1993).

MDA-468 human breast cancer cells contain an amplified EGF receptor gene leading to overexpression of EGF receptors ($\sim 1.5 \times 10^6$ receptors/cell) with characteristics similar to those for normal cells (Filmus *et al.* 1985, Filmus *et al.* 1987). While EGF in physiological (picomolar) concentrations is mitogenic, pharmacological (nanomolar) concentrations were shown to inhibit cell proliferation of MDA-468 cells (Filmus *et al.* 1985, Church & Buick 1988). We have earlier demonstrated that EGF-mediated growth inhibitory effect was characterized by a reversible G₁ arrest accompanied by a decreased level of mRNA for p53 (Prasad & Church 1991). MDA-468 cells contain a single allele for p53 with a point mutation at codon 273 (p53^{273.His}) resulting in substitution of arginine by histidine (Nigro *et al.* 1989, Bartek *et al.* 1990). p53^{273.His} is more analogous to wild-type p53 in terms of ability to bind SV40 T antigen, to transactivate heterologous promoters when expressed as a GAL4 fusion protein and to react with wild-type specific antibody PAb 1620 (Bartek *et al.* 1990, Ginsberg *et al.* 1991a, Milner *et al.* 1987). However, p53^{273.His} has a longer half-life (>7–8 h), reacts with mutant specific antibody, PAb 240, and most interestingly, possess transforming potential in co-operation with activated *ras* (Hinds *et al.* 1990; reviewed in Levine *et al.* 1991, Michalovitz *et al.* 1991). p53^{273.His} has reduced non-specific DNA binding and fails to bind some sequences that are recognized by wild-type p53, but retains the ability to transactivate through a consensus sequence identified by Funk *et al.* (1992) (Bargonetti *et al.* 1991, Kern *et al.* 1991, Funk *et al.* 1992). Thus, while wild-type p53 is regarded as a negative regulator of cell proliferation, there exists a definite possibility that some of the transforming mutants of p53, such as p53^{273.His}, remain active, inducing the expression of genes essential for cell cycle progression.

Indeed, several studies have indicated a role for p53^{273.His} in proliferation and tumour-igenicity of MDA-468 cells. These studies demonstrated that exogenously introduced wild-type p53 abolished the focus formation ability and tumourigenicity of MDA-468 cells (Casey *et al.* 1991, Wang *et al.* 1993). On the other hand, downregulation of p53^{273.His} levels by a flavonoid compound, quercetin, resulted in cell cycle arrest (Avila *et al.* 1994). These and the observation that EGF-treated MDA-468 cells displayed lower levels of mRNA for a mutant p53 suggested that p53 may play a role in growth inhibition. To examine this possibility and to understand the molecular mechanism involved, we initiated an analysis of EGF effects on p53 in MDA-468 human breast cancer cells, including protein synthesis, steady state protein levels, sub-cellular localization, and phosphorylation status of p53.

MATERIALS AND METHODS

Antibodies used

The following mouse monoclonal antibodies against p53 were obtained from Oncogene Science (Manhasset, NY). PAb 1801 (Ab 2), a human-specific antibody, reactive with both wild-type and mutant p53 (Banks *et al.* 1986), PAb 240 (Ab 3), a mutant-specific and conformation-dependent antibody (Gannon *et al.* 1990), and PAb 421 (Ab 1), a mammalian p53-specific antibody reactive with both wild-type and mutant forms of p53 (Harlow *et al.* 1981). PAb 1620, human wild-type p53-specific antibody (Milner *et al.* 1987), was provided as a gift from Dr Heinz-Kurt Hoechkoppel, Ciba-Geigy, Switzerland. A mouse monoclonal antibody, IgG2a, raised against an MHC antigen (a gift from Dr G. Carayanniotis, Memorial University of Newfoundland, Canada), was used as the antibody control in Western blot.

immunoprecipitation and immunofluorescence experiments. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse, F(ab)'2-specific IgG, for use in immunofluorescence was obtained from Jackson Immunochemicals/Bio-Can Scientific, Mississauga, Ontario, Canada.

Cell culture

MDA-468, a human breast cancer cell line, was maintained in Leibovitz-15 modified medium (ICN/Flow) supplemented with 10% FBS (ICN/Flow and Gibco Labs), 50 IU/ml penicillin and 50 µg/ml streptomycin (ICN/Flow). Cells were routinely grown in 100 mm plates and or 75 cm² flasks. A concentration of 1×10^{-8} M of EGF (Collaborative Res. Inc.) was employed in our studies. It has been demonstrated by several groups that this concentration (1×10^{-8} M) of EGF produces optimum growth inhibition (Gill & Lazar 1981, Barnes 1982, Lifshitz *et al.* 1983, Filmus *et al.* 1985, Filmus *et al.* 1987).

Western blot analysis

Subconfluent cells were treated with EGF (1×10^{-8} M) for 0 to 48 h followed by trypsinization. After washing once with PBS, cells were resuspended with cell homogenization buffer (20 mM Tris/HCl (pH 7.4), 5 mM EGTA, 1 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenyl-methylsulfonylfluoride (PMSF)) (all from Sigma, Oakville, Ontario, Canada). Cell suspensions were then subjected to five cycles of ultrasonification (30 sec on and 30 sec off) on ice. Complete cell lysis was confirmed by microscopic examination. Protein concentrations were then determined by Lowry's colourimetric assay (Lowry *et al.* 1951). One hundred micrograms of total protein was run on an 8% SDS-PAGE, transferred to nitro-cellulose (Optibind, Mandel Sci. using a semi-dry transfer method (Hoefer/Fischer Sci. Ltd, Ontario, Canada). The nitro-cellulose filter was incubated overnight in 10% dried milk membrane blocking agent at 4°C with constant rotation. Western blot analysis was then performed with PAb 1801, followed by an alkaline-phosphatase detection system as per the manufacturer's instructions (Amersham, Oakville, Ontario, Canada). The primary anti-p53 antibody, PAb 1801 was used at 10 µg/ml and the second antibody, biotinylated goat anti-mouse IgG, was used at 1:250 dilution.

Immunofluorescence experiments

Cells were grown on chambered glass slides with serum containing media under conditions described in the figure legend (Figure 1). At the indicated intervals, monolayers were washed three times with PBS, and fixed with a methanol:acetone (1:1) mixture at -20°C for 10 min. After fixing, slides were either air dried and stored at -70°C or stained immediately. Cells were washed three times with PBS and blocked with 3% bovine serum albumin in PBS (PBS/BSA) for 30 min at room temperature. After rinsing three times with PBS, cells were incubated at RT for 60 min with either a control antibody (anti-MHC) or an anti-p53 antibody (PAb 1801 or PAb 240) at 5 µg/ml concentration in PBS/BSA. After three washes with PBS, slides were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (F(ab)'2 specific) at 1:50 dilution in PBS/BSA for 30 min at room temperature. Following staining, cells were washed extensively with PBS followed by a final rinse with double distilled water, mounted with 50% glycerol and observed under a fluorescent microscope. Photographs were taken using Kodak Ektachrome 400 film. For PAb 1620 staining, cells were prepared essentially in an identical manner except that blocking was carried out with 3% PBS/BSA at 37°C. For the experiments with aphidicolin (5 µg/ml final concentration; Sigma), cells were treated for 24 h prior to fixing.

Metabolic labelling

Cells (1×10^6 cells/100 mm plates) cultured $\pm$ EGF (1×10^{-8} M) for indicated durations, were labelled with 3.7 MBq/ml ^{35}S -methionine in methionine-free medium or $\text{H}_3(^{32}\text{P})\text{O}_4$ ((carrier free; specific activity 10555 GBq/mg) ICN, Montreal, Canada) in phosphate-free DMEM media with 20 mM Hepes (pH 8.0) during the last 2 h of incubation prior to harvesting. At the end of the labelling period, cells were washed three times with ice-cold PBS followed by scraping with 10 mM EDTA (pH 8.0) in PBS. Cells were pelleted by centrifugation at 800 g at 4°C. Nuclear extract from these cells were prepared as detailed below. For phosphorylation experiments (Figure 3c), cells (1×10^6 cells/100 mm plates) were incubated with 9.26 MBq/ml of $\text{H}_3(^{32}\text{P})\text{O}_4$ in phosphate-free DMEM media with 20 mM Hepes (pH 8.0) during the final 3 h of epidermal growth factor (EGF) treatment. At the end of labelling, cells were lysed and immunoprecipitated using the appropriate antibody as described below.

Immunoprecipitation from nuclear extracts

The cell pellet was resuspended in buffer (20 mM Hepes (pH 7.6), 20% glycerol, 0.5 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF (Lassar *et al.* 1991)) at 50 $\mu\text{l}/\text{pellet}$ from each 100-mm plate. The suspension was gently rocked at 4°C for 1 h followed by centrifugation at 10000 g for 5 min at 4°C. The supernatant was subjected to scintillation counting. Equivalent counts in 40 μl volume (volume adjusted with buffer), from each sample of extracts (see below), were incubated with 350 μl of nuclear extract immunoprecipitation buffer (10 mM Hepes (pH 7.6), 250 mM NaCl, 0.25% NP-40, 5 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF (Lassar *et al.* 1991), 1.0 μg of anti-p53 antibody (PAb 1801) and 25 μl of 50% protein A-Sepharose. The incubation was carried out at 4°C for 90 min with gentle rocking. The immunoprecipitates were then recovered by centrifugation at 10000 g for 2 min at 4°C. The Sepharose pellet was washed four times with RIPA buffer (10 mM Tris.HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF (Lassar *et al.* 1991)). The samples were then boiled with SDS digestion buffer for 5 min and centrifuged. The supernatant was separated on a 10% SDS-PAGE. The gels were dried at 80°C for 1 h and exposed to Kodak XAR film at -70°C .

Immunoprecipitation from whole cell lysates

Cells were washed twice with PBS before harvesting. $\text{H}_3(^{32}\text{P})\text{O}_4$ labelled cells were harvested by scraping with 0.5 ml lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM

Figure 1. Immunofluorescent studies on sub cellular localization of p53 in response to EGF. MDA-468 cells cultured under conditions indicated below for varying durations. Cells were fixed with methanol/acetone (1:1), blocked with 3% BSA/PBS, and incubated with the anti-p53 antibodies (5 $\mu\text{g}/\text{ml}$) followed by staining with FITC-conjugated anti-mouse immunoglobulin (1:50). The p53 localization was detected by fluorescent microscopy at $\times 40$ magnification. Panels a, b, c, g and i are control/untreated cells; panels d, h and j are 24 h EGF (1×10^{-8} M)-treated cells; panel e, 24 h after EGF was withdrawn from 48 h EGF-treated cells; panel f, cells treated with aphidicolin (5 $\mu\text{g}/\text{ml}$) for 24 h. Primary antibodies: panels a, secondary antibody alone, panel b, control antibody, panels c to f, PAb 240 (mutant-specific); panels g and h, PAb 1801 (pan-specific); and panels i and j, PAb 1620 (wild-type specific).

PMSF, 200 μM Na_3VO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 100 mM NaF (Margolis *et al.* 1989) for 30 min on ice, and centrifuged 30 min at 10000 g in a microfuge at 4°C. The supernatant was recovered and treated with 1 μg of p53 antibody (PAb 1801) for at least 1 h at 4°C followed by 30 μl of 50% protein A-Sepharose (Amersham) with rotation at 4°C. The immunoprecipitates were then recovered by centrifugation for 30 s at 10000 g , washed three times with 0.5 ml of wash buffer (20 mM Hepes (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4 (Margolis *et al.* 1989)) followed by a final wash in PBS and resuspension in 30 μl of ten times SDS-digestion buffer. The samples were then boiled for 5 min, pelleted, and the supernatant was recovered and electrophoresed on SDS-PAGE. The samples were always adjusted for equal radioactivity. Experiments in which sample standardization was done based on equal cell numbers yielded similar results. At the end of electrophoresis, gels were dried, and autoradiographed using Kodak XAR film at -70°C .

RESULTS

Western blot analysis

The effect of growth inhibitory concentrations of EGF on p53 protein levels was examined by Western blot analysis using a human-specific antibody, PAb 1801. In addition, EGF effect on p53 protein synthesis was investigated by immunoprecipitation of p53 with PAb 1801, after brief metabolic labelling of cells with ^{35}S -methionine. Although immunoprecipitation experiments indicated marked downregulation of p53 synthesis by 3–6 h (data not shown), Western blot analysis revealed little alteration in steady state levels for p53 protein even after 24 h of EGF treatment (Figure 2, lanes 3 to 8). However, after 48 h of EGF treatment, p53 protein levels were considerably lower compared to untreated cells (Figure 2, lanes 9 and 10).

Immunofluorescence studies

The cellular site of action for p53, a transcription factor, is nuclear. Hence, we examined the sub-cellular localization of p53 in MDA-468 cells, in cycling and EGF-treated conditions. To this end, we made use of three anti-p53 antibodies in immunofluorescence studies as detailed in the Materials section. All these antibodies are known to react with the p53 protein in MDA-468 cells in immunoprecipitation procedures to various extents, and to produce a strong cellular staining in immunofluorescence reactions (Milner *et al.* 1987, Bartek *et al.* 1990).

In control untreated cells, antibodies PAb 240 and PAb 1801 displayed a characteristic nuclear signal along with generalized cytoplasm staining (Figures 1c and g). However, the addition of EGF to the media elicited differential staining patterns with the two antibodies. With PAb 1801, EGF treatment did not significantly alter the nuclear signal (Figures 2g and h). The cells were examined for up to 72 h in the presence or absence of EGF, and no significant shifts in the intracellular distribution were observed during this period (data not shown). On the other hand, PAb 240, a mutant-specific antibody, weakly detected nuclear signals in EGF-treated cells while continuing to produce strong signals in the cytoplasm (Figure 1, compare d with c). This intriguing lack of nuclear staining became apparent as early as 6 h post-EGF treatment in about 25% to 30% of cells and was observed in 70% to 75% of the population within 24 h of EGF treatment. Untreated control cells continued to display the characteristic nuclear signal along with cytoplasmic staining (Figure 1c).

Withdrawal of EGF from the media after 48 h of EGF treatment resulted in re-appearance of nuclear signal in 18–24 h (Figure 1e) consistent with the previously observed resumption of DNA synthesis following EGF withdrawal and the reversible nature of the cell cycle arrest

Figure 2. Effect of EGF on p53 steady state protein levels. Total protein (100 μ g/lane) isolated from MDA-468 cells cultured +/- EGF (10^{-8} M), was separated on an 8% SDS-PAGE followed by Western blot analysis. Lanes 1 and 3, control/untreated at 0 h; lanes 2 and 10, +EGF for 48 h; lane 4, +EGF for 3 h; lane 5, +EGF for 6 h; lane 6, +EGF for 9 h; lane 7, +EGF for 12 h; lane 8, +EGF for 24 h; lane 9, untreated cells after 48 h. Lanes 1 and 2 were probed with a control non-specific antibody (anti-MHC), while the remaining lanes were immunoblotted with PAb 1801 (anti-p53). The arrow indicates the specific p53 band.

(Prasad & Church 1991). To test whether this alteration in staining pattern with PAb 240 is specific to EGF or is an indirect result of growth inhibition, another G_1 arrest-inducing agent, aphidicolin, was employed. Aphidicolin is a specific inhibitor of DNA polymerase- α and induces a late G_1 block in the cell cycle. In MDA-468 cells, treatment with aphidicolin (5 $\mu\text{g/ml}$) for 24 h resulted in an 80% drop in DNA synthesis, indicating that a majority of cells were cell cycle arrested. Immunofluorescence of such growth arrested cells with PAb 240 did not reveal any significant shift in the p53 nuclear staining pattern (Figure 1f). This observation confirmed that the loss of PAb 240 reactivity in response to EGF was not a non-specific reaction to G_1 arrest but specific to the EGF treatment.

We wished to determine whether another conformation-specific antibody, PAb 1620, would react with the nuclear p53 in EGF-treated cells. PAb 1620, stained nuclei and in particular the nucleoli with high intensity in the absence of EGF (Figure 2i) as reported earlier (Slingerland *et al.* 1993). EGF treatment did indeed cause an increase in overall nuclear staining (Figure 1j). Taken together, the immunofluorescence data demonstrated that EGF-treated cells continued to carry nuclear p53 that is reactive with PAb 1801 and more so with PAb 1620 but not with PAb 240.

Phosphorylation studies

The immunofluorescence data indicated an EGF-induced shift in the conformation of nuclear p53, or, alternatively, decreased nuclear translocation of a population of p53 molecules that are reactive with PAb 240. Protein phosphorylation is believed to be a molecular mechanism that may mediate both altered protein conformation and nuclear translocation (Addison *et al.* 1990, Barford 1991). Therefore, the effect of EGF on the phosphorylation status of p53 was studied. $\text{H}_3(^{32}\text{P})\text{O}_4$ -labelled nuclear extracts were immunoprecipitated with PAb 1801 (Figure 3a). Interestingly, nuclear p53 was phosphorylated to a lesser extent upon EGF-treatment compared to that from untreated cells. Phosphorylation of p53 was about 65% of control, within 2 h of EGF addition (Figure 3a, compare lanes 3 and 4); it was 43% of control (lanes 5 and 6) and about 30% of control after 4 and 6 h of EGF

Figure 3. (a) Effect of EGF on phosphorylation of nuclear p53. MDA-468 cells (1×10^6 cells/100 mm plate) were treated with $\pm$ EGF (1×10^{-8} M) for the indicated periods. Nuclear extracts from $\text{H}_3(^{32}\text{P})\text{O}_4$ (9.26 MBq/ml for the final 2 h of EGF treatment) labelled cells were used for immunoprecipitation with PAb 1801 (lanes 2–8) or control antibody (lane 1) and were separated on a 10% SDS-PAGE. Lanes 1 and 2, 0 h (labelled for 2 h prior to the commencement of EGF-treatment); lanes 3, 5 and 7, untreated, corresponding to 2 h, 4 h and 6 h controls, respectively; lanes 4, 6 and 8, EGF-treated 2 h, 4 h and 6 h, respectively. The arrowhead indicates the specific band corresponding to p53. (b) Effect of EGF on nuclear *de novo* synthesized p53. MDA-468 cells (1×10^6 cells/100 mm plate) were treated with EGF (1×10^{-8} M) for the indicated periods. Cells were labelled with ^{35}S -methionine (3.7 MBq/ml) during the final 2 h of EGF-treatment. Immunoprecipitates from the nuclear extracts with PAb 1801 (lanes 2–8) and control antibody (lane 1) were separated on a 10% SDS-PAGE. Lanes 1 and 2, 0 h (labelled for 2 h prior to the commencement of EGF-treatment); lanes 3, 5 and 7, untreated, corresponding to 2 h, 4 h and 6 h controls, respectively; lanes 4, 6 and 8, EGF-treated 2 h, 4 h and 6 h, respectively. The arrowhead indicates the specific band corresponding to p53. (c) Effect of EGF on p53 phosphorylation. MDA-468 cells (1×10^6 cells/100 mm plate) were treated with EGF ($\times 10^{-8}$ M) for the indicated periods. Cells were labelled with $\text{H}_3(^{32}\text{P})\text{O}_4$ (9.26 MBq/ml) for the final 3 h of EGF treatment. Immunoprecipitates with PAb 1801 (lanes 2–5) and control antibody (lane 1) were separated on a 10% SDS-PAGE. The arrowhead indicates the specific band corresponding to p53. Lanes 1 and 2, control/untreated 3 h; lane 4, control/untreated 6 h; lanes 3 and 5, EGF-treated 3 h and 6 h, respectively. (d) Comparative laser densitometric scanning analysis of panels a, b and c. $\square$, total extract – $^{32}\text{PO}_4$ (c); ▨ , nuclear extract – $^{32}\text{PO}_4$ (a); and $\blacksquare$, nuclear extract – ^{35}S (b).

treatment, respectively (lanes 7 and 8), as revealed by the laser densitometric scanning analysis (Figure 3d).

To test whether EGF induces a block in nuclear translocation, p53 immunoprecipitations with PAb 1801 from nuclear extracts prepared from cells labelled with ^{35}S -methionine during the last 2 h of EGF-treatment were performed. The results (Figure 3b) demonstrated no significant differences in the nuclear content of newly synthesized, and therefore labelled, p53

protein up to 4 h after EGF-treatment. We interpret this to indicate no significant changes in the nuclear translocation of the newly synthesized p53 in the presence of EGF. After 6 h of EGF treatment, there was a decrease in the nuclear translocation of newly synthesized p53 (which was about 60% of control), whereas phosphorylated p53 levels were much lower at the same interval of EGF treatment (about 30% of control) indicating that the differences observed with the phosphorylation of p53 were significant (Figure 3d).

Metabolic labelling of cells with $H_3(^{32}P)O_4$ and subsequent immunoprecipitation of p53 from whole cell extracts with PAb 1801 also revealed significant differences in the phosphorylation levels of p53 in response to EGF (Figure 3c). EGF caused a dramatic reduction in p53 phosphorylation within 3 h to about 25% of control (Figure 3c, lanes 2 and 3), however, at 6 h post-treatment, EGF-treated cells displayed similar p53- ^{32}P content (about 86% of control) as compared to untreated control cells (Figure 3c, lanes 4 and 5). This may be due to increased phosphorylation of cytoplasmic p53 protein masking the changes in nuclear p53. Increased phosphorylation of cytoplasmic p53 further indicates that the cellular kinases in general are not inactivated in the presence of EGF and that overall cellular phosphorylation is not affected in response to EGF.

DISCUSSION

We have previously characterized EGF-mediated growth inhibition in MDA-468 cells as a reversible G_1 arrest (Prasad & Church 1991). Observation of lowered mRNA levels for p53 in EGF-treated cells and the documented G_1 regulatory role of p53, led us to hypothesize an involvement of p53 in EGF-mediated G_1 arrest. MDA-468 cells are homozygously deleted for another tumour suppressor, the retinoblastoma gene (Rb) and harbour a single allele for p53 with a point mutation at codon 273 (Nigro *et al.* 1989, Bartek *et al.* 1990). Therefore, it seemed quite conceivable that loss of negative regulation by Rb and wild-type p53, together with an added dominant oncogenic function of the persistent mutant p53 allele, may be driving the cell cycle of MDA-468 cells. Several studies did indeed underscore the importance of p53 in the cell cycle progression of MDA-468 cells (Casey *et al.* 1991, Wang *et al.* 1993, Avila *et al.* 1994). This portion of the study was initiated to investigate the possibility of p53 being the target for EGF in MDA-468 cells.

We have earlier observed downregulation of mRNA levels for p53 after about 18–24 h of EGF treatment (Prasad & Church 1991). However, an immediate EGF effect observed in MDA-468 cells was reduced overall protein synthesis, which was maximum within 12 h of EGF-treatment (Prasad & Church, unpublished observation). Therefore, the observed EGF-effect on p53 mRNA levels is unlikely to constitute a cause for growth inhibition. Our experiments indicate little alteration in p53 protein levels in response to EGF up until 24 h (Figure 2). This was a reasonable observation considering the apparent over-expression and longer half-life (7–8 h) of mutant p53 (Hinds *et al.* 1990). These results make it clear that any change in p53 judged a putative cause of growth inhibition must then be post-translational and would be expected to reverse the function of the native p53 protein. Here we report that such a change is reflected in an altered antibody-reactivity to nuclear p53, as detected by a mutant-specific antibody, PAb 240, in response to EGF (Figures 1c and d). Lack of PAb 240 nuclear staining occurs early enough to be attributed as causative, and is reversible upon withdrawal of EGF (Figure 2e). Moreover, this observation is specific to EGF-treatment and not a non-specific result of cell cycle arrest as indicated by immunofluorescence of aphidicolin-treated cells (Figure 2f). Considered alone, this might simply be interpreted as a block in nuclear translocation of mutant p53 by EGF, thereby removing an essential driving force

for cell cycle progression. However, detection of nuclear p53 with PAb 1801 in EGF-treated cells raised an important question as to whether or not p53 so detected was in the wild-type conformation, being non-reactive with mutant-specific PAb 240. Indeed, nuclear p53 was recognized and detected by a wild-type specific antibody, PAb 1620, in untreated cells and the staining was enhanced in EGF-treated cells (Figures 2i and j). Intriguingly, PAb 1620 stained preferentially the nucleoli in untreated cells with only faint staining of the rest of the nucleus. In EGF-treated cells, however, increased staining of the entire nucleus was apparent. It was clear that in EGF-treated cells, nuclear p53 retains only a wild-type specific (detected by PAb 1620) epitope and not a PAb 240 reactive one.

These observations led us to hypothesize a molecular mechanism that may be responsible for EGF-induced growth inhibition in MDA-468 cells. EGF clearly induces a change in the conformation of nuclear p53, leading to decreased amounts of p53 molecules expressing the mutant-specific epitope. MDA-468 cells may therefore contain p53 molecules capable of two distinct conformations, both mutant and wild-type-like, despite the presence of a single, genotypically mutant allele. Evidence for the existence of two distinct populations of p53 in MDA-468 cells is inconclusive. PAb 240 reacts weakly with p53^{273.His} in immunoprecipitation experiments. While p53^{273.His} is reactive with the wild-type specific antibody, PAb 1620, sequential immunoprecipitation from nuclear extracts, in our hands, failed to bring down detectable amounts of protein with conformation-specific antibodies (both PAb 1620 and PAb 240), perhaps due to the higher salt concentrations employed for preparation of nuclear extracts. Alternatively, p53^{273.His} in MDA-468 cells may be in a distinct or unique conformation. This may explain its peculiar properties. p53^{273.His} might retain its wild-type conformation, as recognized by PAb 1620, but may also have an altered conformation relevant to other epitopes. This might result in a poorly recognizable PAb 240 epitope. The observed differences in PAb 240 immunofluorescence could be an indication of further change in the conformation of p53^{273.His}. However, loss of PAb 240 reactivity might have occurred simply as a result of epitope masking due to post-translational modifications such as phosphorylation. We do not think this is the case however, since EGF-treated cells demonstrated reduced, not increased, p53 phosphorylation, although we have no detailed information on the sites that are affected. Moreover, p53 can be immunoprecipitated from whole cell extracts with PAb 240, albeit poorly, from both untreated and EGF-treated cells. Although, we were unable to confirm either a 'two distinct population theory' or a 'unique distinct conformation theory', data reported here indicate that EGF is clearly tilting the balance of p53 conformation from mutant-like to more wild-type-like. However, DNA binding studies using p53 from MDA-468 cells and p53 responsive DNA sequences, provided clues in support of existence of conformationally distinct populations with unique DNA binding properties (Prasad & Church 1997). More studies are clearly needed to understand the molecular and functional interaction of p53 molecules and biological consequences.

This type of phenomenon is not altogether unprecedented. Evidence in support of an apparent conformational flexibility of wild-type p53 can be found as long as 12 years ago (Milner 1984) and confirmation has been accumulating in the literature over the past few years (Milner 1991, Milner & Medcalf 1991, Ullrich, Mercer & Appella 1992, Zerrahn *et al.* 1992, Fiscella *et al.* 1993, Halazonetis *et al.* 1993, Halazonetis & Kandil 1993, Ullrich *et al.* 1993). These studies, based on immunoreactivity of p53 molecules, were further strengthened by the knowledge gained by crystal structure of p53-DNA complexes (Arrowsmith & Morin 1996). These studies suggest a possible partial folding of mutant p53 molecules thereby exposing PAb 240 epitope. In wild-type molecules, however, PAb 240 epitope is cryptic because of extensive folding. Further, it was suggested that in a given cell p53 can remain in

both forms of alternate protein folding. Two forms can be interchanged based on the cellular environment. Protein folding can be altered by external factors such as temperature changes, phosphorylation, or interaction with other cellular proteins (Arrowsmith & Morin 1996). Indeed, work from several laboratories has convincingly demonstrated that phosphorylation plays an important role in regulating p53 function (Ullrich, Mercer & Appella 1992, Ullrich *et al.* 1992, Fiscella *et al.* 1993, Ullrich *et al.* 1993). Interestingly, our experiments indicated a considerable reduction in the levels of p53 phosphorylation upon EGF treatment (Figure 3). This, coupled with no changes in p53 protein levels as indicated by the Western blot analysis (Figure 2) and by ³⁵S-methionine labelled cell immunoprecipitations from nuclear fractions (Figure 3c), suggest that the decreased phosphorylation of p53^{273.His} is significant.

In light of this, our observations are important for two reasons. First, our evidence suggests conformational flexibility for a p53 mutant, where a dynamic equilibrium between two conformations in the nucleus would determine the G₁-S progression. Second, this is one of the few studies linking a growth factor signal transduction pathway to a biological role for p53. EGF-dependent signalling is envisioned to shift the p53 conformation equilibrium towards the growth suppressing wild-type form thereby inhibiting cell cycle progression. Consistent with this notion is our recent observation that MDA-468 cells carry functionally distinct populations of p53 molecules that are conformationally different (Prasad & Church 1997). Further studies are ongoing to identify the possible kinases involved in this EGF-induced phenomenon in order to delineate underlying molecular pathways leading to G₁ arrest.

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