Volume 231, number 2, 371–377 FEB 05671 April 1988

Modulation of HLA antigens in response to the binding of epidermal growth factor by A431 cells

Kazuo Hosoi*, Dilip S. Kittur+ and Michael Edidin

Department of Biology, The Johns Hopkins University, Charles and 34th Streets, Baltimore, MD 21218, USA

Received 11 December 1987

In a previous study [(1984) J. Cell Biol. 98, 725 731] we showed that the level of human MHC, HLA antigens on A431 carcinoma cells is reduced after these cells bind epidermal growth factor (EGF). Here we use flow cytometry to determine the effects of various doses and times of EGF treatment on HLA expression. We then show that the reduction in HLA expression is associated with a reduction in the level of phosphorylation of immunoprecipitable surface HLA antigens, although longer exposure of cells with EGF increased both surface HLA expression and their phosphorylation levels. Lateral diffusion of HLA antigens is lower in EGF-treated than in control cells. The lower diffusion coefficients measured may be causally related to the decreased phosphorylation of HLA antigens.

HLA antigen; EGF receptor; Phosphorylation; Lateral diffusion

1. INTRODUCTION

The binding of epidermal growth factor (EGF) to specific receptors initiates a number of metabolic events. Among these is phosphorylation of tyrosine on the cytoplasmic portion of the receptor itself, as well as a number of other membrane integral and cytoplasmic proteins [2]. Further consequences of EGF binding include alterations in activity of other protein kinases [3]. Thus EGF binding may alter the phosphorylation of many membrane-associated proteins, in turn leading to changes in their activity or in their metabolism.

In a previous study on the interrelationship between major histocompatibility complex (MHC), MHC antigens, HLA antigens of human cells, and the EGF receptor we noted that binding of EGF to its receptor effected a reduction of approx. 25% in the level of binding of anti-HLA antibodies to the cells [1]. We have investigated some aspects of this reduction further and find that EGF binding not only alters number of anti-HLA antibodies bound to treated cells, but also affects the level of phosphorylation of the HLA antigens in these cells, and the lateral diffusion of these antigens.

Correspondence address: M. Edidin, Department of Biology, The Johns Hopkins University, Charles and 34th Streets, Baltimore, MD 21218, USA

* *Present address:* K. Hosoi, Department of Oral Physiology, Josai Dental University, Sakado-shi, Saitama 350-02, Japan

\+ *Present address:* Department of Surgery, Francis Scott Key Medical Center, The Johns Hopkins University, School of Medicine and The Johns Hopkins Hospital, Baltimore, MD 21224, USA

2. MATERIALS AND METHODS

2.1. *Cells, antibodies and other reagents*

A431 cells were the kind gift of Dr Joseph DeLarco, Frederick Cancer Research Center. They were cultured in Dulbecco's modified medium, containing 10% fetal calf serum. KE-2, a monoclonal antibody against a monomorphic determinant of HLA antigens was the gift of Dr Roger Kennett, University of Pennsylvania. Its specificity and the preparation and labeling of Fab fragments have been described [1]. In the experiments described here we used KE-2 Fab labeled with 5-DTAF [5-(4,6-dichlorotriazinyl)aminofluorescein] (Molecular Probes, Eugene, OR), labeled following the manufacturer's instructions. BBM-1 [4], a hybridoma producing monoclonal antibody to human beta-2 microglobulin, was obtained from the American Type Culture Collection, Washington, DC. Epidermal growth factor, purified from mouse submaxillary glands by the method of Savage and Cohen [5] was the kind gift of Dr Akira Komoriya, Meloy Laboratories, Springfield, VA. Phorbol myristate acetate (TPA) was obtained from Sigma Chemical Co.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.2. *EGF treatment of cultured cells and labeling of HLA antigens*

One day before treatment, A431 cells were plated at 2.5×10^5 cells per well of a 24-well Falcon plate. After approx. 30 h incubation EGF was added to the wells to give final concentrations ranging from 0.15 to 150 nM. Cells were cultured with EGF for a further 16–17 h. The same concentrations of EGF were added to another set of wells in the plate for 4 h. At the end of the incubation with EGF all wells were washed with phosphate-buffered saline (PBS), pH 7.4, 0.1% in bovine serum albumin (BSA; Sigma) and cells were removed from each well with trypsin/EDTA. The suspended cells were washed twice with BSA/PBS and labeled with 25 μl/tube of fluorescein-conjugated KE-2 at a final concentration of approx. 50 μg/ml. After 40 min incubation at 0°C, the cells were washed 3 times in BSA/PBS and fixed in 1% paraformaldehyde in PBS. The concentration and time of incubation were chosen to effectively saturate the HLA antigens of A431.

2.3. *Analysis of HLA expression of treated cells*

The labeled cells were examined in a Coulter 752 Flow Cytometer, exciting DTAF at 488 nm. Data were collected for approx. 25000 cells per sample, using a linear amplifier. The linearity of the amplifier was checked with beads conjugated with known numbers of fluorescein molecules (Flow Cytometry Systems Corp., Research Triangle Park, NC).

2.4. *Radio-phosphate labeling of A431 cells and isolation of phosphorylated HLA antigens*

Cells, plated at 2.5×10^5 per well were grown in the presence or absence of EGF before labeling. After washing three times in Hepes-buffered isotonic NaCl, 0.1% BSA, the cells were incubated in 0.5 ml phosphate-free Dulbecco's modified medium, supplemented with 2.4 mg/ml glucose and 1 mg/ml BSA and containing 0.1 mCi ^{32}P as sodium phosphate. In one experiment, the effect of EGF binding on phosphorylation was examined by adding EGF at intervals from 0 to 3 h after adding sodium [^{32}P]phosphate. In a second experiment, cells were first incubated in EGF for either 4 or 17 h, and then the phosphate label was added for 3 h. All incubations were at 37°C in a 5% CO_2 atmosphere.

After labeling, the cells were chilled and washed three times with cold BSA/PBS. They were then lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% BSA, 10 μg/ml trasylol, 2 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 1% NP-40) at 0°C for 20 min and the detergent insoluble residue pelleted by centrifugation at $10000 \times g$ for 20 min. The detergent-soluble supernatant was cleared of materials non-specifically binding to protein A by incubation with 50 μl per sample of fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) for 30 min at 4°C, followed by centrifugation at $\sim 1500 \times g$ for 10 min. The cleared supernatant was mixed with either KE-2, BBM.1 or goat anti-mouse Ig antibodies (specificity control), at equivalent concentrations together with 100 μl of 10% fixed *S. aureus* and incubated for 2 h at 4°C. The antigen–antibody–*S. aureus* complexes were pelleted by centrifugation at $\sim 1500 \times g$ for 10 min, and the pellets washed 4 times in NDS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.05% SDS, 0.05% Na-deoxycholate). The washed pellets were boiled in sample buffer (2% SDS, 10% glycerol, 0.25% 2-mercaptoethanol and 0.25% bromophenol blue in 0.0625 M Tris-HCl buffer, pH 6.8) and run on 5–15% acrylamide gels. The gel was first subjected to autoradiography to visualize the labeled HLA. Bands were then cut from the same gel and their radioactivity determined by liquid scintillation counting. Radioactivity was normalized to number of cells per well since long exposure to high concentrations of EGF inhibits growth of A431 cells. This procedure evaluates phosphorylation of all cellular HLA antigens, surface and internal. Incorporation of [^{32}P]phosphate into total detergent-soluble protein was measured in trichloroacetic acid precipitates of cell lysate prepared as described above.

The phosphorylation of surface HLA antigens was determined by adding KE-2 antibody to intact ^{32}P-labeled cells before lysis. 500 μl of KE-2 (at 250 μg/ml) were added to labeled cells that had been washed with BSA/PBS and the mixture incubated for 2 h at 4°C. In order to suppress exchange of antibody with labeled intracellular HLA molecules, the intact cells were then mixed with unlabeled cell lysate in BSA/PBS (1:4) and incubated for a further 1 h at 4°C. Following this the cells were lysed and HLA immunoprecipitated without further addition of antibody.

2.5. *Measurement of lateral diffusion of HLA antigens*

Lateral diffusion of HLA antigens was compared in control and EGF-treated cells using the method of fluorescence recovery after photobleaching [6–8]. The instrument used for these measurements and the approaches to labeling have been described [6,7]. Cells were grown on coverslips and labeled with FL-KE-2 Fab for 15 min at 0°C. Diffusion was measured on 10 to 15 cells for each treatment group, and for the same number of cells in each control group.

3. RESULTS

The fluorescence of a population of F1-KE-2-labeled A431 cells approximates a normal distribution. In control populations, the peak of this distribution is approximately at channel 60. Under optimum conditions of treatment with EGF, 1.5 nM EGF for 4 h, the peak is shifted to channel 30. Calibration of the flow cytometer with standard fluorescein beads indicates that this shift represents a 50% decrease in fluorescent molecules bound per cell (fig.1).

The reduction in HLA binding after 4 h was only marked at one concentration of EGF. Concentrations 10-fold less or 10-fold greater had a small effect on KE-2 binding after 4 h, while no effect was seen if 100-times the optimum dose, 150 nM EGF, was used. The relative response to doses of EGF was the same in cells treated for 16–17 h as in cells treated for 4 h. The least KE-2 was bound to cells treated with 1.5 nM EGF, while the most bound to cells treated with 150 nM EGF. However, the absolute levels of KE-2 binding to cells treated for 17 h were very different from

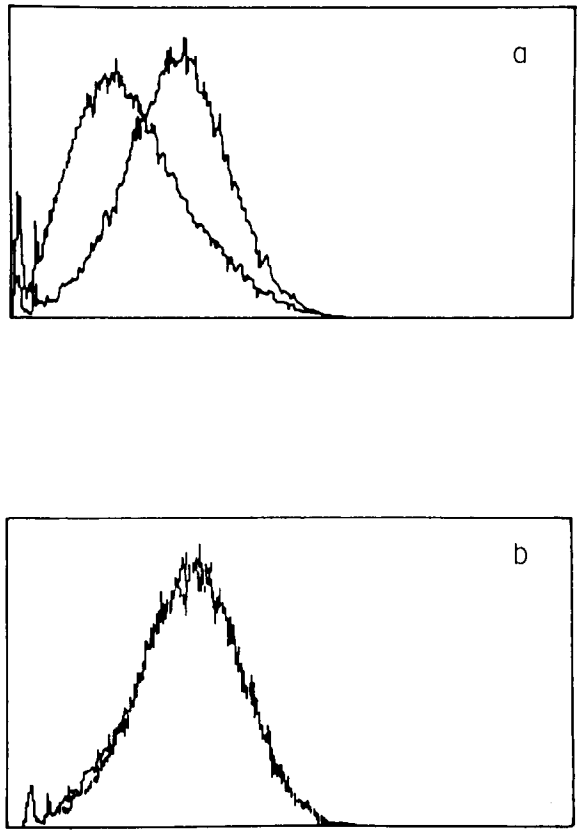

Fig.1. Distribution of fluorescence in populations of A431 cells labeled with fluorescent KE-2, anti-HLA antibodies. Ordinate, number of cells; abscissa, relative fluorescence on a scale of 256 channels. (a) Cells treated with 1.5 nM EGF for 4 h, right-hand peak, compared to untreated cells. (b) Cells treated with 150 nM EGF for 4 h compared to untreated cells.

those cells treated for 4 h. The depression in KE-2 binding after 17 h in 1.5 nM EGF was approx. one-third that induced by 4 h exposure to this concentration of EGF. Furthermore, 17 h exposure to 15 or 150 nM EGF resulted in an increase in the levels of KE-2 bound to approx. 125% of control values (fig.2). As has been reported [9,10], continued incubation in EGF inhibited the growth of A431 at all concentrations tested. Thus the levels of KE-2 bound did not correlate with the extent of inhibition of cell growth by EGF.

EGF may directly or indirectly stimulate phosphorylation of membrane proteins. The cytoplasmic domain of HLA antigens contains several serine residues that may be phosphorylated in vivo [11]; a tyrosine in this domain of purified

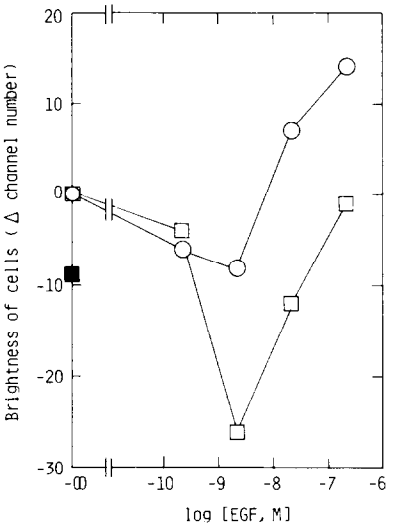

Fig.2. Effects of EGF on KE-2 binding to the HLA antigens of A431 cells. (□) 4 h treatment with EGF; (○) 17 h treatment with EGF; (■) 4 h treatment with 100 ng/ml of phorbol myristate acetate (TPA).

antigen is phosphorylated in vitro by pp60^{src} [12]. We therefore examined the effect of EGF on the phosphorylation of HLA antigens precipitated by KE-2 or by antibody to the beta-2m light chain. Phosphorylated HLA antigens could be specifically precipitated from detergent extracts of cells incubated for 1–4 h in [^{32}P]phosphate-containing medium. Addition of EGF to cultures reduced the

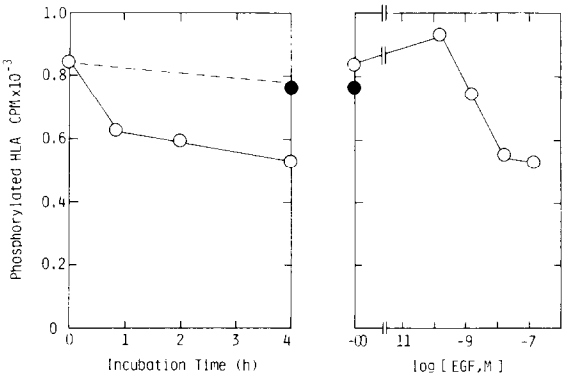

Fig.3. Phosphorylation of specifically precipitated HLA antigens after treatment of cells with EGF (○) or with 100 ng/ml TPA (●). (a) Time course of phosphorylation changes. (b) Dose-dependence of phosphorylation changes. [^{32}P]Phosphate and various ligands were simultaneously added to cells and incubated for the time indicated.

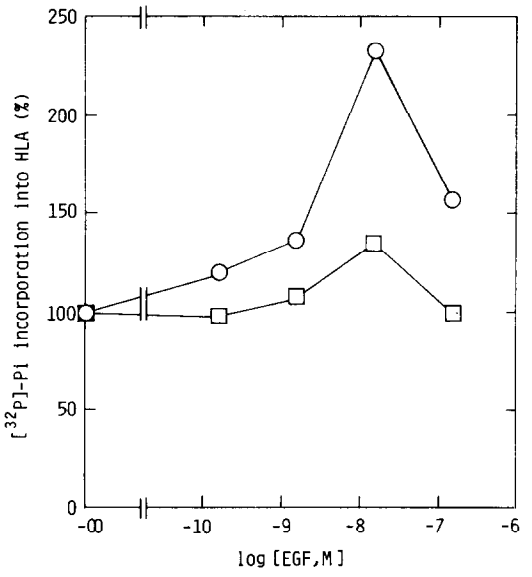

Fig.4. Phosphorylation of specifically precipitable HLA antigens labeled after incubation of cells with EGF for 4 (■), or 16 h (○) followed by washing and addition of ^{32}P for 4 h.

level of precipitable phosphorylated HLA, as a function both of time of incubation, and of EGF concentration (fig.3a,b). Phorbol myristate acetate, which activates protein kinase C [13], had no effect on HLA phosphorylation, though it had a small effect on binding of KE-2 to the cells (fig.2).

A short time (4 h) exposure of A431 cells with 1.5 or 15 nM EGF significantly reduced the phosphorylation of HLA (fig.3). The reduction is well correlated with the changes of the level of cell surface HLA (fig.2). However, if the phosphorylation of HLA was observed after long time treatment (17 h) of EGF, it was significantly increased; the effect was maximum at 15 nM with small increase even at 1.5 nM (fig.4). These changes again well correlated with the changes of surface HLA levels (fig.2); a treatment with 15 nM EGF increased surface HLA whereas 1.5 nM reversed a marked decrease seen when cells were treated with the hormone for 4 h. The apparent discrepancy seen between the data of total surface HLA and phosphorylation of HLA after long time-exposure

Table 1

Effects of EGF treatments on the level of phosphorylated HLA with A431 cells

Treatments	^{32}P in HLA			^{32}P in trichloro-acetic acid-insoluble fraction (cpm/well) $\times 10^{-6}$ [B]	[A]/[B] (%)
	(cpm/band per well)[c] [A]	(cpm/band per 10^6 cells)	(%)		
Total HLA					
None	1868	1893	(100)	9.33	(100)
EGF (4 h)[a]	1388	1554	82	8.48	82
EGF (16 h)[b]	1993	2623	138	6.18	161
Surface HLA					
None	1529	1490 (79)[d]	100	6.28	100
EGF (4 h)[a]	1027	1185 (76)	79	6.24	68
EGF (16 h)[b]	1833	2500 (95)	168	5.56	135

[a] Labeling with [^{32}P]phosphate for 4 h in the presence of 15 nM EGF

[b] Labeling with [^{32}P]phosphate for 4 h after incubation of cells with 15 nM EGF for 16 h, followed by washing

[c] These values are normalized by the background activity (535 cpm) of the gel

[d] % of total HLA-associated radioactivity in surface HLA antigens

After the labeling with [^{32}P]phosphate, phosphorylated HLA antigens were immunoprecipitated from either whole cell extracts after lysis (total HLA) or from extracts prepared with cells which had been immunoreacted with antibody before lysis (membrane bound HLA), and isolated by SDS gel electrophoresis

with 1.5 nM EGF may be explained by the fact that the total HLA was measured at exactly 17 h after the hormone treatment whereas the radioactive phosphate incorporation took an extra 4 h for labeling after 17 h EGF treatment. The effects of EGF to stimulate the phosphorylation seem to be initiated roughly around 4 h after the addition of EGF since the labeling after this period for 4 h (fig.4) increased the phosphorylation but the labeling before 4 h in the presence of EGF (fig.3) decreased it.

In control cells, 80% of the ^{32}P incorporated into HLA antigens was associated with surface HLA (table 1, column B). This proportion remained roughly the same when the cells were treated with EGF for 4 h and increased to 95% after 17 h. It can also be seen from table 1 that the ratio of specifically precipitable radioactivity (column A) to trichloroacetic acid-precipitable radioactivity (column B) is not constant. Thus the changes in phosphorylation of HLA were not due to changes in sizes of metabolic pools affecting all phosphoproteins.

These results suggest that short time treatments of A431 cells with EGF evoke the reduction of both surface HLA and their phosphorylation whereas long time exposures of the cells with the hormone increase them. The EGF concentrations effective in the reductions and increments seem approx. 1.5 nM and 15 nM respectively.

The lateral diffusion of HLA antigens is constrained in most cells studied presumably by interactions between the HLA antigens and some molecules of the cytoplasm. Phosphorylation could regulate such interactions [14]. Since our results indicate that most phosphorylated HLA molecules are at the cell surface, it was of interest to see if the changes in phosphorylation were correlated with changes in lateral mobility of HLA antigens. As is the case in other cells, lateral diffusion of HLA antigens was approx. 1/10 that calculated from the molecular mass of the antigens and the apparent viscosity of lipid bilayers. About 60% of the labeled molecules were mobile in the plane of the membrane. Though the mobile fraction of HLA antigens in cells treated with EGF did not differ significantly from that in controls (table 2), the diffusion coefficients of the HLA antigens were significantly lower in EGF-treated cells than

Table 2

Effect of EGF on lateral diffusion of HLA antigens in A431 cells

Treatment	No.	D cm^2/s ($\times 10^{10}$) ± SE		R (%)
		Arithmetic	Geometric	
None	105	5 ± 0.4	4 ± 0.2	61 ± 2
15 nM EGF				
4 h	46	4 ± 0.4	3 ± 0.2	54 ± 2
17 h	39	4 ± 0.5	3 ± 0.2	59 ± 2
1.5 nM EGF				
4 h	44	4 ± 0.5	3 ± 0.2	62 ± 2
17 h	41	4 ± 0.4	3 ± 0.2	59 ± 2

All diffusion coefficients for HLA antigens in EGF-treated cells differ significantly from that for HLA antigens in control cells ($P < 0.01$)

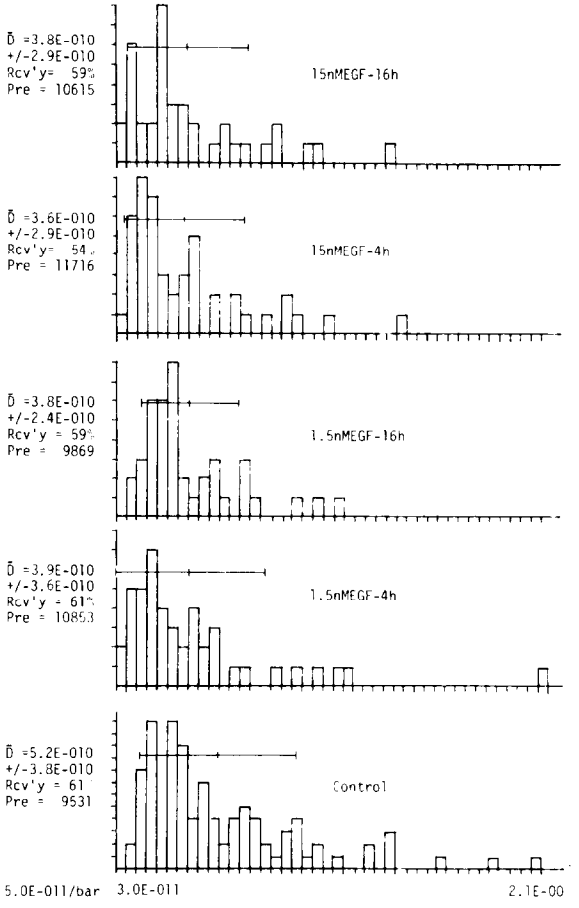

Fig.5. Diffusion coefficients measured for HLA antigens on control and EGF-treated cells.

in controls (table 2). This is also evident in the histograms of diffusion coefficients of fig.5. Even concentrations of EGF which do not greatly affect phosphorylation of total cell HLA do appear to affect lateral diffusion of the antigens.

The values for diffusion coefficients are not normally distributed. This distribution distorts both the mean diffusion coefficients and the standard error of these means. Accordingly, in addition to the arithmetic means, we also took the geometric means of the diffusion coefficients. The log-normal distribution upon which these means are based has a reduced variance, and, perhaps more importantly, down-weights outlying values of D. The geometric means of the diffusion coefficients, given in table 2, while lower than the arithmetic means also differ significantly between control and EGF-treated cells.

4. DISCUSSION

This report extends our previous observation that fewer anti-HLA antibodies bind to EGF-treated A431 cells than to control cells [1]. Using flow cytometric measurements of fluorescent antibody binding, we find that the reduction in antibody binding by prior EGF treatment is at a maximum when the concentration of EGF used is 1.5 nM, which is sufficient to saturate high affinity EGF receptors, but that higher concentrations of EGF may reverse the effect, or even enhance binding of anti-HLA antibodies. The kinetics of the effect we observed here are slower than those reported earlier [1]. However, we did not use the same A431 cells and growth and culture conditions also were slightly different from those used earlier. We have some preliminary evidence (Hosoi and Edidin, unpublished) that the kinetics of the reduction in KE-2 binding may vary with culture conditions.

EGF treatment of A431 cells also reduces phosphorylation of specifically precipitable HLA antigens, mainly those at the cell surface, in a dose-dependent manner. Though a concentration of EGF giving maximum inhibition of phosphorylation could not be defined in experiments in which cells are concurrently given the hormone and [^{32}P]phosphate, an optimum dose of EGF was defined in experiments in which cells are labeled with ^{32}P after incubation with EGF. This experiment also makes it evident that the reduction in HLA phosphorylation in EGF-treated cells is not due to cell damage or death. The optimum concentration for stimulation of phosphorylation defined by it is 10-fold higher than the optimum for suppression of anti-HLA binding. In both systems little or no effect of EGF is seen if its concentration is varied 10-fold higher or 10-fold lower than its optimum.

Recently, Cantrell et al. [15] reported a short-term effect of phorbol esters on the phosphorylation of HLA antigens and of the T_i/T_3 complex of T lymphoblasts. The phosphorylation stimulated by phorbol esters is associated with a reduction in surface levels of T3 antigens, but has no effect on the levels of HLA antigens. Feuerstein et al. [16] have also reported increases in HLA phosphorylation after exposure of cells to phorbol esters. In our experiments phorbol myristate acetate treatment also had little effect on levels of HLA at the cell surface and had no effect on HLA phosphorylation. However, we examined HLA phosphorylation at later times after phorbol ester treatment than were used by Cantrell et al. [15].

The site and mechanism of the changes in HLA phosphorylation induced by EGF are unclear. The long times used in our experiments obscure the immediate effects of EGF on the cells, and our observations could reflect the secondary action of kinases, other than the EGF receptor tyrosine kinase on the HLA antigens. If one considers the observed constancy of a serine phosphorylation site on MHC antigens [11], and the uncertainty whether or not the tyrosine of the cytoplasmic region is indeed phosphorylated in vivo, it seems likely that the HLA molecules are not in fact directly labeled by the EGF receptor kinase. We also cannot tell if the reductions in phosphorylation induced by EGF are due to reduced activity of a kinase or to enhanced activities of a phosphodiesterase.

There are several reports [17–19] that the recycling of surface receptors for insulin-like growth factors and transferrin is affected by the level of phosphorylation of the receptors. Reductions in receptor phosphorylation lead to longer residence times at the cell surface [19] while 'hyperphosphorylation' [18] seems to be associated with enhanced receptor internalization in coated vesicles. Unlike these other receptors, HLA an-

tigens seem to be marked for internalization by dephosphorylation, rather than by phosphorylation. However, endocytosis of HLA antigens and some other membrane proteins appears to take place outside of the usual pathway of coated pits and coated vesicles [20–22] (Wier and Edidin, unpublished). It might be that this rather different endocytic cycle uses phosphorylation as a control point but in a manner different to that in the cycle for transferrin receptors.

EGF treatment significantly affects the lateral diffusion of surface HLA antigens. While the differences in diffusion coefficients between HLA in control cells and HLA antigens of cells treated with 1.5 or 15 nM EGF are small, they are significant. Other work [23], comparing the lateral diffusion of mutant MHC antigens completely lacking cytoplasmic tails with the lateral diffusion of mutants bearing 2–4 cytoplasmic residues, one of which is a serine, suggests that dephosphorylation of MHC antigens results in smaller diffusion coefficients, possibly due to the molecules' aggregation [24]. Lateral diffusion coefficients vary as the log of the radius of the diffusing particles [25]. Therefore, a change of 20–25% in diffusion coefficient could reflect formation of aggregates of several hundred molecules. Since MHC antigens appear normally to exist as monomers [26] (Edidin, Zidovetski and Jovin, unpublished), aggregates of this size indicate considerable reorganization of the cell surface. The persistence of the effect on lateral diffusion at 17 h could reflect the persistence of a high proportion of unphosphorylated HLA molecules at the cell surface even after newly phosphorylated molecules arrive at the surface.

Acknowledgements: This work was supported by a grant, AI-19814, from the National Institutes of Health. This is contribution number 1397 from the Department of Biology.

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