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EPIDERMAL GROWTH FACTOR STIMULATES THE PHOSPHORYLATION OF A SPECIFIC NUCLEAR PROTEIN IN CHICK EMBRYO EPIDERMIS

Kenneth R. Huff and Gordon Guroff

Section on Intermediary Metabolism
Laboratory of Developmental Neurobiology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20014

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Summary

Epidermal growth factor treatment of chick embryo epidermis in culture media or of chick embryos *in vivo* results in increased phosphorylation of a specific nuclear protein electrophoretically identical to the f_1 histone. This effect is produced by concentrations as low as 0.01 μg/ml though it is maximal at 4 μg/ml; it is apparent as early as 1/2 hour after treatment *in vitro* and it is maximal at 2-4 hours. The effect of epidermal growth factor is mimicked by dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine, but not by nerve growth factor.

Introduction

Mouse epidermal growth factor (EGF) stimulates epithelial and endothelial cell proliferation in a variety of culture systems (1-5), is a very potent mitogen in other cell culture systems (6-11), and interacts with receptors on cells of different derivation from a number of species (12-16). As an example, human EGF, recently isolated, appears to be identical to the gastric acid secretion-inhibiting hormone, urogastrone, and has high structural and antigenic homology with mouse EGF (17-20). In some of these systems the pleiotypic effects of EGF, other than proliferation, have been analyzed (21-24) and include rapid transport of small molecules, increased synthesis of RNA and conversion of ribosomes to polysomes, and increased synthesis of protein. The final

Abbreviations: EGF, epidermal growth factor; ODC, ornithine decarboxylase; NGF, nerve growth factor; SDS, sodium dodecyl sulfate; dBcAMP, dibutyryl cyclic AMP; IBMX, 3-isobutyl-1-methyl xanthine.

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result in epidermal cultures is enhanced keratinization and thickening of the epidermis (25). Despite a range of responses crossing divergent cell types as well as species lines, the mechanisms of action of this hormone in producing its trophic effects have not been fully characterized.

The finding of a transient but marked EGF-dependent increase in ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17, ODC) activity (26), and the recent implication of a rapid cyclic nucleotide response (27,28), has led us to speculate on the similarity of these events to some of those which follow treatment by the related hormone nerve growth factor (NGF) of one of its responsive tissues, the superior cervical ganglion. In that tissue the response to NGF includes a rapid rise in cAMP levels (29), a marked increase in ODC activit (30), an increase in RNA polymerase activity (31), and, most recently demonstrated an increase in the phosphorylation of a specific nuclear protein (32), now known to be the f_1 histone (unpublished results). This latter finding has stimulated our investigation of a similar role of nuclear protein phosphorylation in the mode of action of EGF in the trophic response of epidermal tissue to this substance.

Materials and Methods

Sheets of 8 day chick embryo epidermis were prepared as described by Cohen (25) and equilibrated for 2 hours in BGJ$_b$ medium before treatment with mouse EGF or other agents for up to 4 hours. During the last hour of incubation (^{32}P) KH_2PO_4, 1.2 mCi/ml (ICN Pharmaceuticals, Inc.) was used to label the tissue. SDS slab gel electrophoresis and autoradiography were carried out on extracts of purified nuclei prepared from the tissue, as described previously (32), and optical density scans were then done on the autoradiograms. Epidermal growth factor was prepared by the method of Savage and Cohen (33); 2.5S nerve growth factor was prepared according to the procedure of Bocchini and Angeletti (34).

Results

Extracts of epidermis treated with EGF *in vitro* showed a darker band on the autoradiograms from SDS-gel electrophoresis and a higher peak on the optical density tracing made from the films (Fig. 1A and B) indicating an increased phosphorylation of a material with a molecular weight of about 30,000. This material has the same migration as f_1 histone standard and has an identical position with the nuclear protein preferentially phosphorylated following NGF

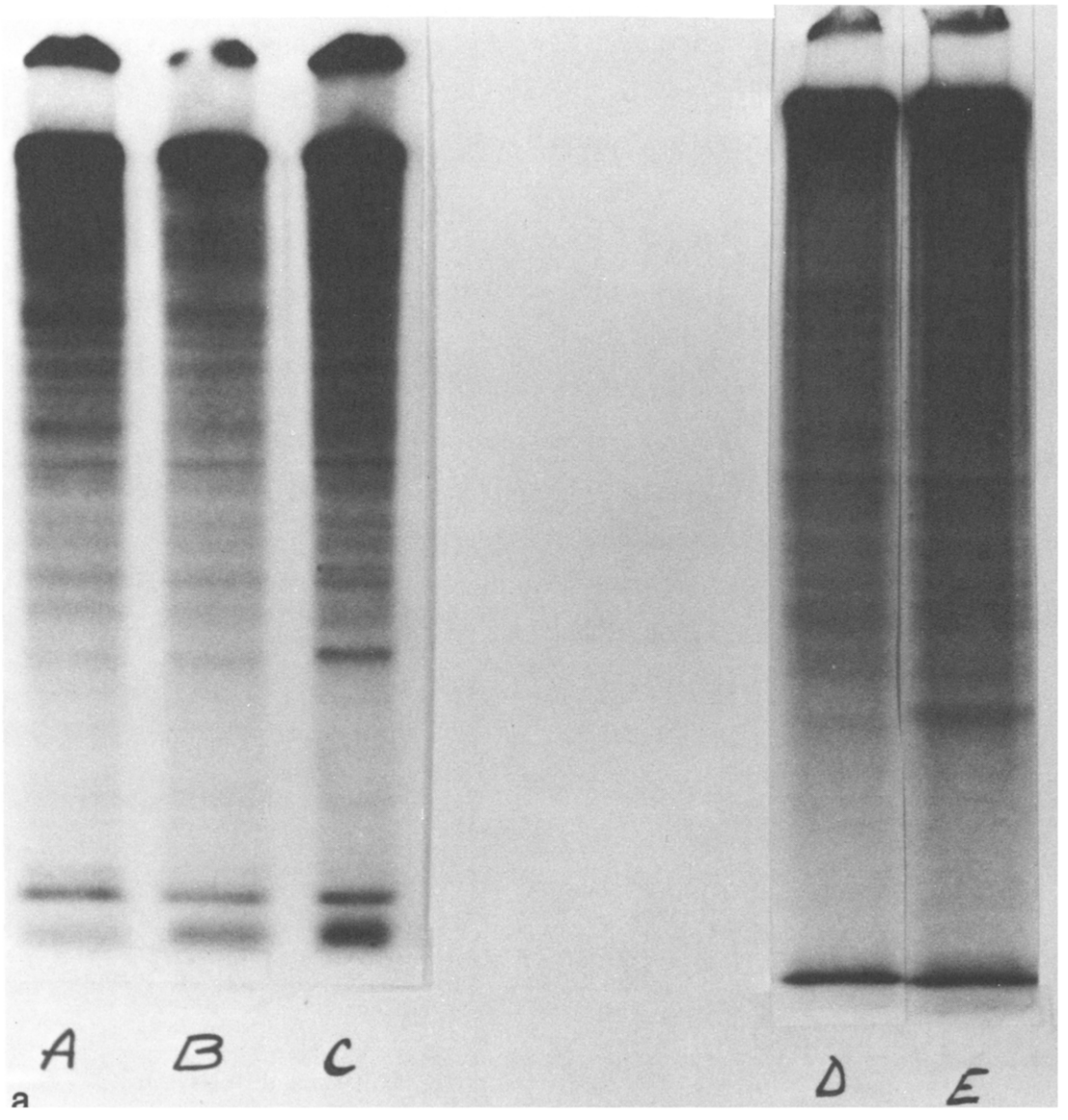


Fig. 1a. Autoradiogram of SDS slab gel electrophoresis of extracts from purified nuclei representing phosphorylated material from 8-day chick embryo back epidermis cultured *in vitro* in BGJ_b medium in the absence(A) or in the presence of (B) 2.5S NGF, 4 µg/ml or (C) EGF, 4 µg/ml for 4 hours following a 2 hour equilibration period. ^{32}P-phosphate, 1 mCi/ml of culture media, was added during the final hour. For the *in vivo* studies, (D) H_2O, 150 µl or (E) EGF, 150 µg in 150 µl of H_2O was injected into the amniotic sac of 8-day chick embryos through a window in the shell. After 2 hours, the epidermis was prepared as for the *in vitro* experiments and cultured with ^{32}P-phosphate, 1 mCi/ml, in the media for 1 hour. In all experiments, the tissue was rinsed and homogenized, the purified nuclei prepared for gel electrophoresis, and the gels dried and exposed to X-ray film for autoradiography. Each sample consisted of the entire back epidermis from a single chick embryo.

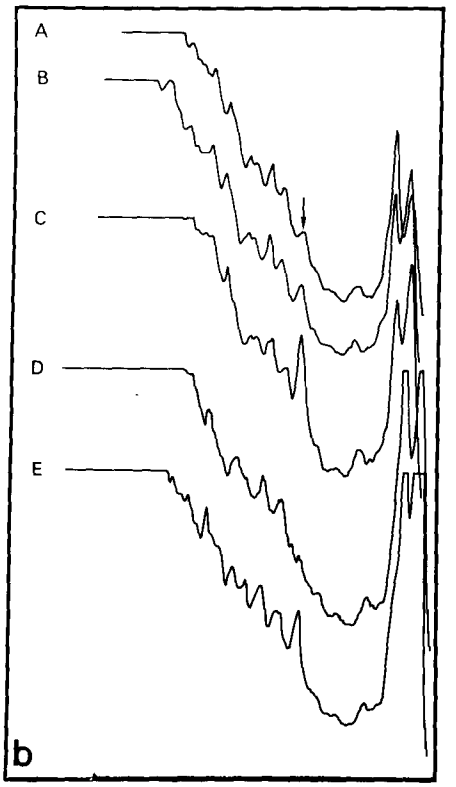
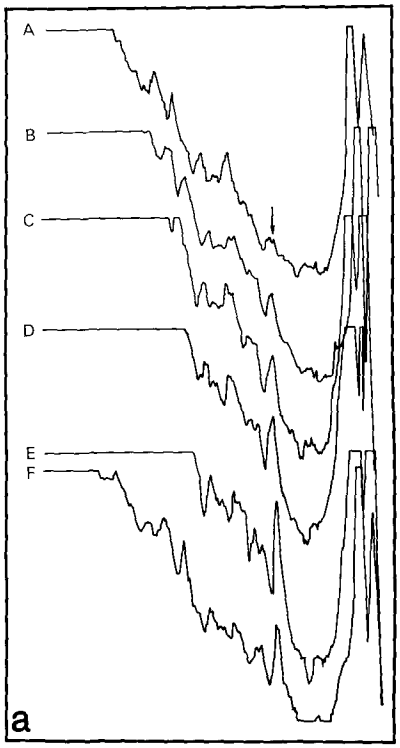


Fig. 1b. Optical density tracings of the autoradiograms shown in (1a). The peak designated with the arrow corresponds to the area on the autoradiogram demonstrating increased phosphorylation of a specific material with a molecular weight of 30,000. The tracings labelled (C) and (E) correspond to the samples treated with EGF in vitro and in vivo respectively.

treatment of superior cervical ganglia in vivo or in vitro (32). However, as seen both in the autoradiogram and the optical density tracing (Fig. 1A and B), NGF did not increase the phosphorylation of this particular protein in this system, demonstrating the specificity of EGF. Concomitant with the in vitro studies of phosphorylation, ODC activity was measured 4 hours after addition of EGF and found to be 5 times higher than either control or NGF-treated tissue activity (data not shown).

For the in vivo studies, EGF was injected into the amniotic sac of 8-day old embryos through a window in the shell of the egg 2 hours before the epidermis was removed and incubated with ^{32}P in the culture medium. This



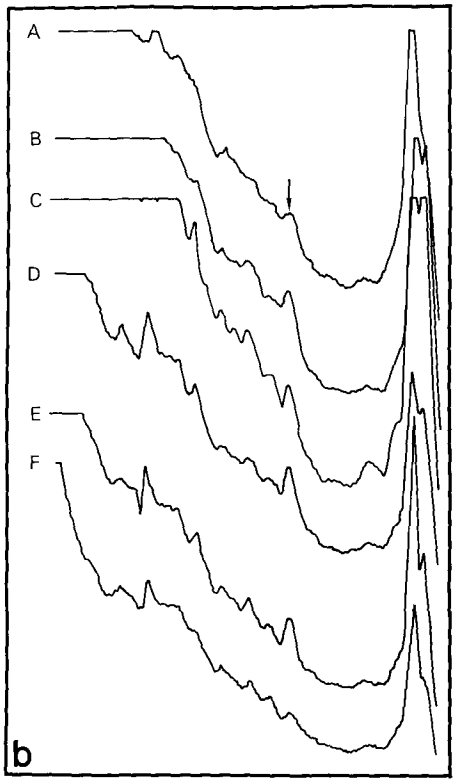


Fig. 2a. Optical density tracings of autoradiograms of samples of chick embryo epidermis treated for 4 hours in vitro with various concentrations of EGF: (A) control (B) 0.01 µg/ml (C) 0.1 µg/ml (D) 1.0 µg/ml (E) 4.0 µg/ml (F) 7.0 µg/ml. Same methodology of preparation of tissues as described in Fig. 1.

Fig. 2b. Optical density tracings of autoradiograms of samples of chick embryo epidermis treated in vitro with EGF, 4 µg/ml, or control buffer for various times: (A) control, 1/2 hour (B) EGF, 1/2 hour (C) EGF, 1 hour (D) EGF, 2 hours (E) EGF, 4 hours (F) control, 4 hours. ^{32}P-phosphate was added for the entire culture period in samples (A), (B), and (C) and only during the last hour of culture period in samples (D), (E), and (F).

treatment resulted in preferential phosphorylation of the same nuclear protein as did the in vitro treatment (Fig. 1A and B).

An in vitro effect of EGF on the phosphorylation of this nuclear protein could be detected at concentrations as low as 0.01 µg/ml although the greatest effects appeared at concentrations of 4 and 7 µg/ml (Fig. 2A). Also an effect was observed as early as 30 minutes after the addition of EGF

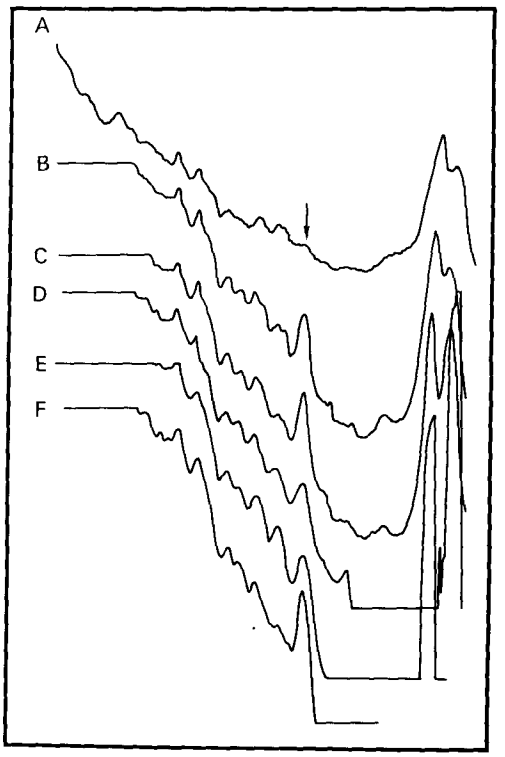


Fig. 3. Optical density tracings of autoradiograms of samples of chick embryo epidermis treated in vitro for 4 hours with: (A) control buffer, (B) EGF, 4 μg/ml, (C) EGF, 7 μg/ml, (D) dBcAMP, 10 mM, (E) IBMX, 0.5 mM, (F) EGF, 7 μg/ml and IBMX, 0.5 mM. Treatments are final concentrations in culture media. Same methodology of preparation of tissues as described in Fig. 1.

although the greatest increase was seen after 2 and 4 hours of incubation (Fig. 2B).

The degree of phosphorylation of the nuclear protein by EGF was compared with that produced by a cAMP analog and a phosphodiesterase inhibitor (Fig. 3). Although the addition of either dibutyryl cyclic AMP (dBcAMP) or 3-isobutyl-1-methylxanthine (IBMX) resulted in slightly increased phosphorylation of the protein migrating as f_1, neither produced an increase as great as did EGF at concentrations of 4 and 7 μg/ml. Phosphorylation seen after combined treatment by IBMX and EGF (7 μg/ml) was not markedly greater than that seen after EGF treatment alone.

Discussion

These results implicate, at least by temporal association, nuclear processes in the action of EGF in inducing some of its trophic responses in chick embryo epidermis *in vivo* and in organ culture. We have assumed, based on some similarities of origin, biochemical nature, active concentration levels, and time course of action of NGF and EGF, that they are producing similar effects in phosphorylating a material which has proven in the case of NGF to be the f_1 histone (unpublished observation). It is possible to speculate that there is a direct relationship of this response in this system to the regulation of gene transcriptional effects known to occur later and which result in RNA synthesis and the biochemical and morphological differences in thickening and keratinization of the epidermis. Comparable nuclear protein phosphorylations have been shown to result from hormone-target interactions or mitogenic stimulation in other systems (35-44). Though it may be regulatory in other systems (45), the change in polyamines following induction of ornithine decarboxylase in this time frame, however, may be just a facilitating factor for nuclear protein phosphorylation and transcription as it has been shown to be dependent on protein synthesis in the epidermal culture system whereas transcription is not (26).

The data strengthen the suggestion for a role of cyclic nucleotide in response to EGF. In this sytem a cAMP analog, dBcAMP, and a phosphodiesterase inhibitor, IBMX, seemed to partially mimic the effect of EGF in phosphorylating this specific nuclear protein. This finding is in support of the previous finding of a rise in cAMP and adenylate cyclase activity within one minute after EGF treatment *in vivo* (27,28). However, others have concluded that some effects, especially the mitogenic effects, of EGF may be mediated through lowered levels of cAMP (46-48). In addition, there is evidence for internalization of the growth factor following its binding to its receptor (49,50), which may represent an alternative route for producing some of its responses, other than solely through "second messenger" cyclic nucleotides. Regardless of

the initial events, our evidence supports the hypothesis that phosphorylation of a specific protein in the nucleus is involved ultimately in the response of epidermal tissue to EGF, as it appears to be in the response to NGF of its targets.

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