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THE COVALENT LABELING OF PROTEINS BY 17β-ESTRADIOL, RETINOIC ACID, AND PROGESTERONE IN THE HUMAN BREAST CANCER CELL LINES MCF-7 AND MCF-7/Adr^R

NORIKO TAKAHASHI* and THEODORE R. BREITMAN

Laboratory of Biological Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

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Summary—In this study we analyzed the covalent binding to proteins of 17β-estradiol (E2), retinoic acid (RA), and progesterone in MCF-7 and MCF-7/Adr^R cells. MCF-7 cells have receptors for E2 and progesterone. MCF-7/Adr^R cells do not have these receptors. After a 1-day incubation period with either [^{3}H]E2, [^{3}H]progesterone, or [^{3}H]RA the levels of covalently bound radioactivity was between 1.4- to 2-fold greater in MCF-7 cells than in MCF-7/Adr^R cells. We analyzed the labeled proteins with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and fluorography. About 40 proteins were labeled by E2 in MCF-7 cells and about 10 of these proteins were the only proteins labeled by E2 in MCF-7/Adr^R cells. We saw that the same 8 proteins were labeled by RA in both cell lines. Progesterone labeled 2 proteins with M_r values of 37,000 and 20,000 in MCF-7 cells. These 2 proteins had mobilities that were the same as proteins that were labeled by either E2 or RA in both MCF-7 and MCF-7/Adr^R cells. Besides these 2 proteins, we saw proteins of M_r 51,000 (p51) and 55,000 that were covalently labeled by E2 in MCF-7 cells and by RA in both MCF-7 and MCF-7/Adr^R cells. The p51 had the same mobility on 2D-PAGE as an 8-azido-[^{32}P]cAMP-labeled protein. This protein is probably RII$_\alpha$, the type II cAMP-binding regulatory subunit of type II cAMP-dependent protein kinase. These results suggest that the estrogen receptor, while not obligatory, might still modulate the covalent linkage of E2 to protein. In addition, our results raise the possibility that some effects of some ligands of the thyroid/sterioid hormone receptor family may involve the covalent linking of these hormones to proteins, including RII$_\alpha$.

INTRODUCTION

Retinoic acid (RA), 17β-estradiol (E2), and progesterone are ligands for related nuclear receptors that are members of the thyroid/steroid hormone family [1]. It is generally believed that these receptors act as DNA-binding transcription factors and that the binding of the ligand to its receptor modulates this activity.

There is evidence that some effects of members of the thyroid/steroid hormone family on some cell types may be independent of a nuclear receptor [2–4]. Thus, doisynolic-type acids bind poorly to uterine estradiol receptors (ER) but have potent uterotropic and vaginal-cell cornification activity in ovariectomized mice [3]. *In vitro*, some doisynolic-type acids are more potent than E2 in promoting the growth of ER-positive MCF-7 cells [3]. Finally, estrogens and progestagens enhance growth of prostatic cells which have no ER or progesterone receptors [2].

These apparent nonreceptor effects on cell growth are unexplained. Several possibilities have been presented and evaluated [3] including a model in which estrogenic or androgenic agents act by diminishing the effect of a cell-growth inhibitor [2, 5].

A mechanism that may account for some hormone effects is a covalent modification of cellular proteins by hormones or their metabolites. In MCF-7 cells the E2 metabolite 16α-hydroxyestrone is covalently bound to the ER and other nuclear matrix proteins [6] by a pathway that may be nonenzymatic [7]. We showed that both E2 and RA are bound covalently to proteins in MCF-7 cells [8]. The covalent binding of RA to proteins (retinoylation) occurs in many cell types and on a variety of proteins [9, 10] including RI and II, the cAMP-regulatory subunits of cAMP-dependent protein

*To whom correspondence should be addressed.

kinase (PKA) type I and II [11]. Breast tumor cell proliferation is inhibited by either cAMP [12, 13] or RA [13–15]. It is believed that most, if not all, effects of cAMP involve its activation of PKA by binding to and releasing the regulatory subunit from the catalytic subunit. Thus, both RA and cAMP may affect the same regulatory protein in breast tumor cells. In contrast to RA and cAMP, E2 enhances proliferation of ER-positive breast tumor cells [16]. The possibility that the covalent labeling of protein by E2 is involved in the mechanism of this growth effect prompted us to examine the proteins labeled by E2 and RA in ER-positive MCF-7 cells and ER-negative mutant MCF-7/Adr^R cells.

EXPERIMENTAL

Cells

The human breast cancer cell lines MCF-7 and MCF-7/Adr^R [17] were obtained from Dr R. I. Glazer. Cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Gaithersburg, MD). Cell cultures were grown at 37°C in a humidified atmosphere of 5% CO_2 in air and subcultured every week.

Cells were removed from the surface of the tissue culture flask with trypsin–EDTA (0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}, GIBCO). Cells (3×10^5) were suspended in 5 ml of IMDM containing 10% fetal bovine serum and plated in 25 cm^2 tissue culture flasks.

We estimated cell number on an electric particle counter (Coulter Electronics, Hialeah, FL) and viability by Trypan blue dye exclusion.

Labeling of cellular proteins by E2, progesterone, and RA

The medium was removed from cultures that were near confluency. The cells were washed once with a serum-free medium composed of RPMI 1640 (GIBCO) supplemented with 10 mM 4-(2-hydroxyethyl)-l-piperazinethane sulfonic acid, 5 μg of insulin (Collaborative Research, Bedford, MA)/ml and 5 μg of transferrin (Collaborative Research)/ml. Serum-free medium (5 ml) was added to each flask and then we added 5 μl of a stock solution containing either E2 ([2,4,6,7,16,17,-^{3}H(N)], 150 Ci/mmol), progesterone ([1,2,6,7,21-^{3}H(N)], 193 Ci/mmol), RA ([11,12-^{3}H(N)], 40–60 Ci/mmol), or non-radioactive RA. The final concentrations of E2 and progesterone were 50 nM. The final concentration of RA was 100 nM. All radioactive compounds were purchased from Du Pont-New England Nuclear (Boston, MA). The radioactive compounds and RA were dissolved in absolute ethanol and diluted into the growth medium such that the final concentration of ethanol was no higher than 0.1%.

After a 24-h incubation, the medium was removed and the cells were detached from the surface either with 1 ml of trypsin–EDTA or with a cell scraper. RPMI 1640 containing 10% fetal bovine serum (1 ml) was then added. The cells were harvested by centrifugation and washed extensively with phosphate-buffered-saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2) containing 600 μg of bovine serum albumin (essentially fatty acid free, Sigma, St Louis, MO)/ml.

Photoaffinity labeling with 8-azido-[^{32}P]cAMP

Total cell extracts, prepared from cells grown as described above, were labeled with 8-azido-[^{32}P]cAMP (ICN Biomedicals, Irvine, CA) in the absence and presence of unlabeled 100 μM cAMP essentially by the procedure of Walter and Greengard [18] with slight modification [19]. These conditions result in a quantitative binding of 1 mol of 8-azido-[^{32}P]cAMP to 1 mol of the RI and II regulatory subunits [18]. Binding of 8-azido-[^{32}P]cAMP was specific since no radiolabeling was seen in samples incubated in the presence of 100 μM unlabeled cAMP before addition of 8-azido-[^{32}P]cAMP.

Preparation of samples for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cell pellets prepared as described above were extracted five times by the Bligh-Dyer [20] procedure with $CHCl_3$–CH_3OH–H_2O (1:2:0.8). The delipidated residue was collected by centrifugation at 10,000 g for 5 min in a microcentrifuge and then dried in a centrifugal vacuum device (Savant, Farmingdale, NY). The dried residue was dissolved in isoelectric focusing buffer containing 9.5 M urea, 2% NP-40, and 2% ampholytes (pH 3.5–10, Pharmacia LKB, Uppsala, Sweden).

2D-PAGE

2D-PAGE was according to O'Farrell [21]. First dimension isoelectric focusing gels contained 2% ampholytes (pH 3.5–10). Second

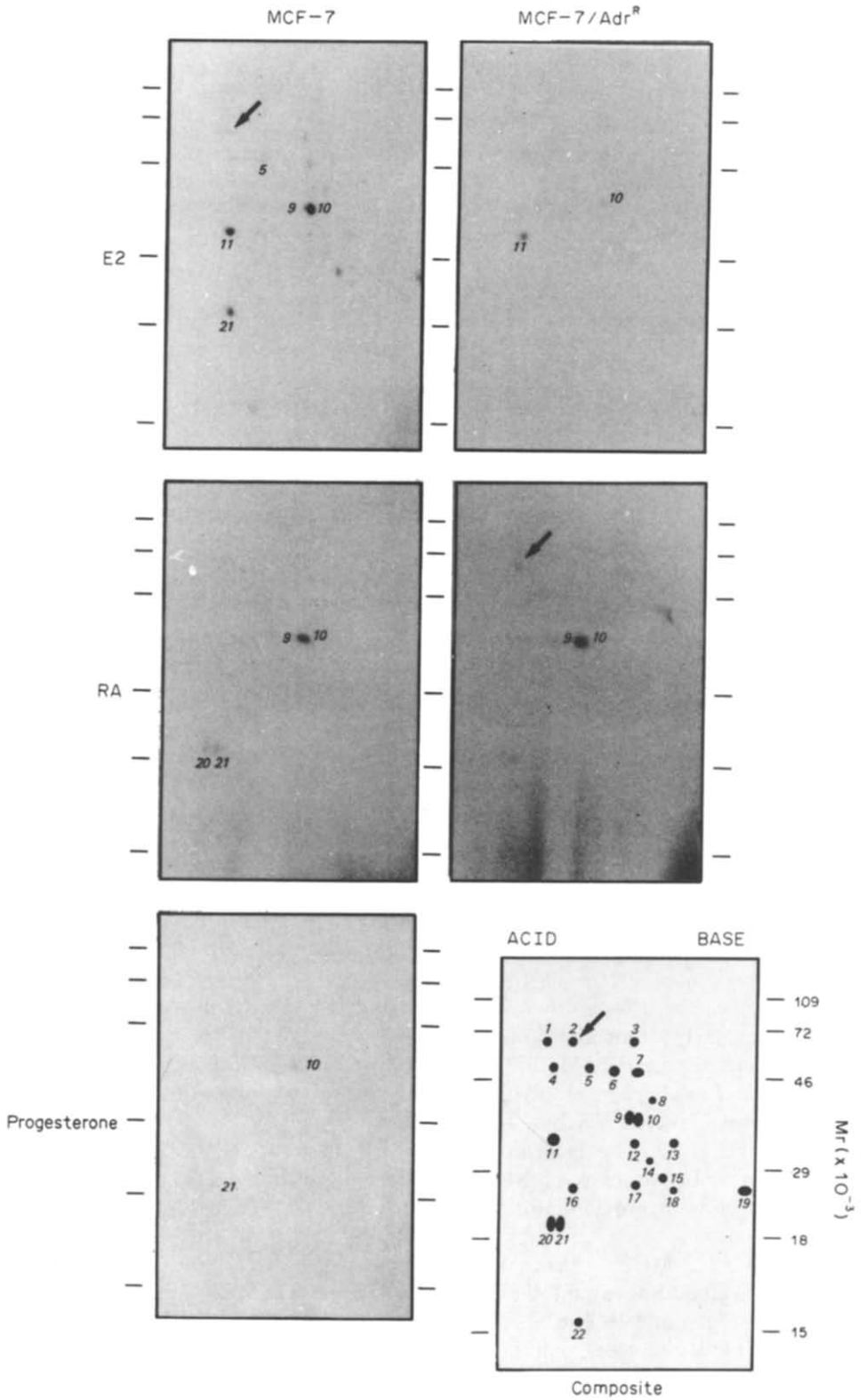

Fig. 1. 2D-PAGE pattern of proteins labeled by E2, RA, and progesterone in MCF-7 and MCF-7/Adr^R cells. MCF-7 and MCF-7/Adr^R cells were grown for 24 h with either 50 nM [^{3}H]E2, 50 nM [^{3}H]progesterone, or 100 nM [^{3}H]RA. The number of MCF-7 cells harvested and extracted by the Bligh-Dyer procedure for each condition was 2.25×10^6 cells for E2, 2.55×10^6 cells for progesterone, and 0.9×10^6 cells for RA. The comparable values for MCF-7/Adr^R cells were 0.96×10^6 for E2, 1×10^6 for progesterone or 0.86×10^6 for RA. The residues were dissolved in isoelectric focusing buffer and analyzed by 2D-PAGE and fluorography. Gels were exposed to the film for 60 days. The *composite* was drawn manually and the position of each *numbered* [^{3}H]protein was assigned based on its mobility compared with proteins that were stained by Coomassie Blue R-250. *Arrows* show the predicted position of the major retinoylated protein of HL60 cells [42] and correspond to *protein 2* of MCF-7 and MCF-7/Adr^R cells.

dimension gels were 10–20% gradient polyacrylamide gels (Geltech, Hudson, OH). Gels were fixed, stained with Coomassie Blue R-250, and prepared for fluorography with ENTENSIFY (Du Pont-New England Nuclear) according to the manufacturers instructions. The dried gels were exposed to Kodak XAR-5 film at $< -80°C$. Molecular weight markers were from GIBCO.

RESULTS

Labeling of MCF-7 and MCF-7/Adr^R proteins by [^{3}H]E2, [^{3}H]progesterone, and [^{3}H]RA

We incubated MCF-7 or MCF-7/Adr^R cells for 24 h with radiolabeled E2, progesterone, and RA. Radioactivity was in the residues after the Bligh-Dyer extraction to remove free lipid. Based on this radioactivity we estimated that in MCF-7 cells there were 150 fmol of E2, 71 fmol of progesterone, and 600 fmol of RA covalently bound per 10^6 cells. The corresponding values for MCF-7/Adr^R cells were 75 fmol of E2, 48 fmol of progesterone, and 440 fmol of RA. The labeled proteins were analyzed by 2D-PAGE and fluorography (Figs 1 and 2). In MCF-7 cells we saw about 40 proteins labeled by [^{3}H]E2 (Fig. 2). Three of the most highly labeled proteins were *proteins 10*, *11*, and *21* (Figs 1 and 2). In contrast, in MCF-7/Adr^R cells, *proteins 10* and *11* were the only proteins labeled by [^{3}H]E2 after a 2-month exposure to the film (Fig. 1). About 10 proteins were seen after the gel was exposed to the film for 13 months (Fig. 2) *Protein 10* was the highest labeled protein in MCF-7 cells and *protein 11* was the highest labeled protein in MCF-7/Adr^R cells. As shown in Fig. 1, *proteins 10* and *21* of MCF-7 cells were labeled less by [^{3}H]progesterone than by [^{3}H]E2. The incorporation of [^{3}H]progesterone into proteins of MCF-7/Adr^R cells was too low to provide informative 2D-PAGE fluorograms.

The proteins of either MCF-7 cells or MCF-7/Adr^R cells were labeled to about the same extent by [^{3}H]RA (Figs 1 and 2). The labeling of *proteins 9* and *10* was greater than that of *proteins 20* and *21* (Figs 1 and 2). After exposure of the gel to the film for 13 months we saw retinoylated *proteins 2* (arrows), *3*, *5*, *7*, and *11* (Fig. 2).

Labeling of proteins in MCF-7 and MCF-7/Adr^R with 8-azido-[^{32}P]cAMP

In examining the patterns for proteins that were labeled with E2, RA, and progesterone we noted that *proteins 10* and *21* were labeled by each of the three radioactive hormones (Figs 1 and 2). In addition, *protein 5* appeared to have the same mobility on 2D-PAGE as a retinoylated cAMP-binding protein identified in HL60 cells as an isoform of RII [11]. To investigate further this possibility, we prepared MCF-7 total cellular proteins photoaffinity labeled with 8-azido-[^{32}P]cAMP. Specific immunoprecipitation of RII was carried out using an anti-RII$_\alpha$ antisera (described in Ref. [22]) (kindly provided by Drs D. Ogreid and S. O. Doskeland, Institute of Anatomy, University of Bergen, Bergen, Norway). These immunoprecipitates were electrophoresed on SDS–PAGE gels. In the adjoining lanes, we ran immunoprecipitates obtained with the anti-RII$_\alpha$ antisera of total cellular proteins from HL60 cells photoaffinity labeled with 8-azido-[^{32}P]cAMP. The labeled immunoprecipitate from MCF-7 cells had the same M_r value of 51,000 as the labeled immunoprecipitates from HL60 cells shown in Fig. 6 of Ref. [11].

We also prepared 2D-PAGE autoradiograms of total cellular proteins of MCF-7 and MCF-7/Adr^R cells photoaffinity labeled with 8-azido-[^{32}P]cAMP. We saw at least 11 cAMP-binding proteins (Fig. 3). Based on our studies in HL60 cells [11] it was possible that *proteins b* and *c* (M_r of 51,000) were isoforms of RII and that *proteins d–i* (M_r of 47,000) were isoforms of RI. An 8-azido-[^{32}P]cAMP-binding protein (*protein c*, Fig. 3) and the proteins covalently labeled with [^{3}H]E2 or [^{3}H]RA (*protein 5*, Fig. 2) had the same mobilities. *Proteins j*, *k*, and *l* (Fig. 3) may be proteolytic fragments of either RI or II or both.

The pattern of cAMP-binding proteins in MCF-7 cells differed from that of MCF-7/Adr^R cells (Fig. 3). We saw more isoforms of RI in MCF-7/Adr^R cells than in MCF-7 cells. Thus, *proteins d*, *e*, *g*, *h*, and *i* were found at much higher levels in MCF-7/Adr^R cells. MCF-7 cells had a higher level of the RII isoform *protein c* but did not have detectable levels of *proteins j*, *k*, and *l*.

In HL60 cells the levels of RI and II increase about 2-fold after growth for 24 h with 100 nM RA [11]. We did not see a similar increase either with MCF-7 cells or with MCF-7/Adr^R cells (Fig. 3). However, after growth of MCF-7/Adr^R cells with 100 nM RA we saw an apparent decrease in the level of *protein g* and a marked increase in the level of *protein f*.

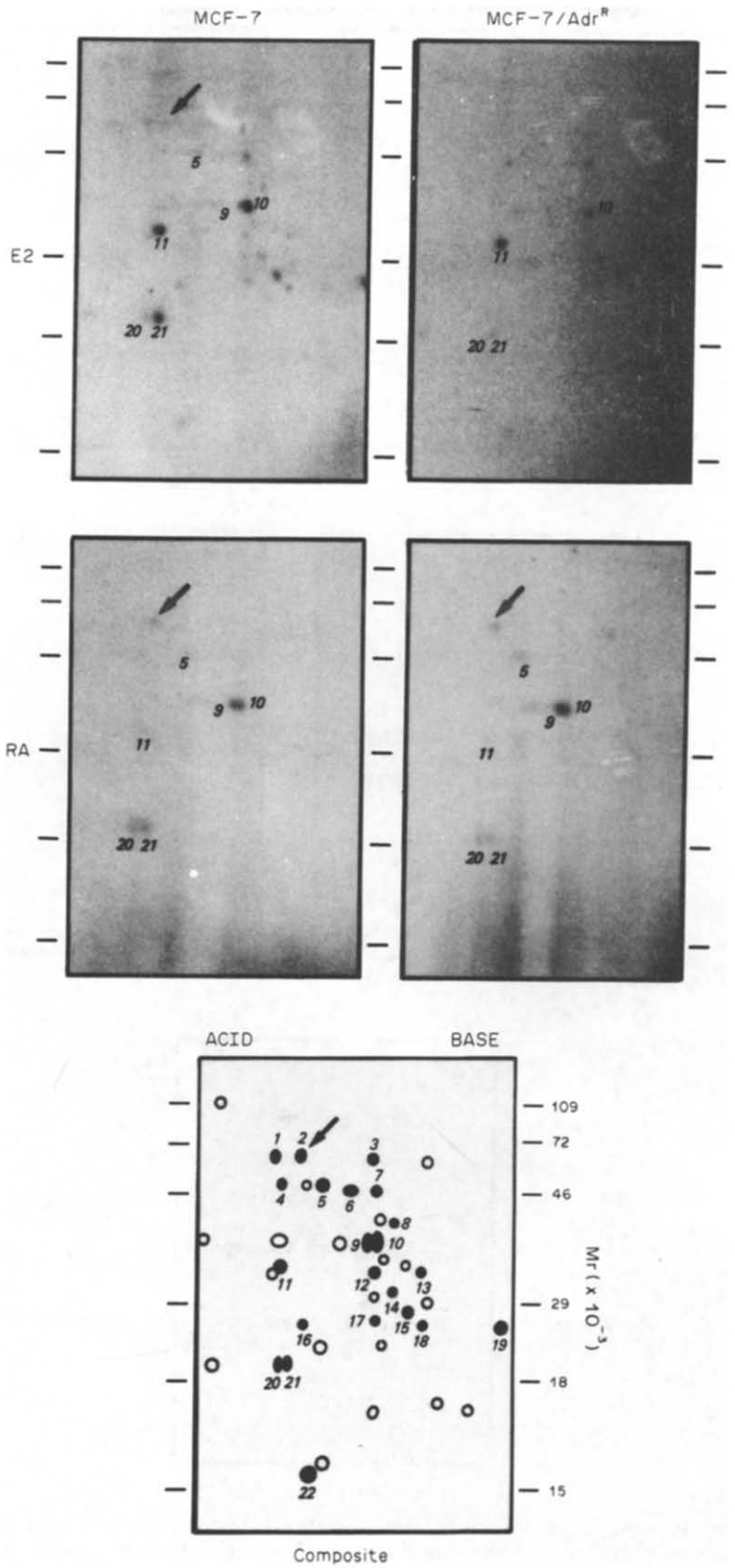

Fig. 2. Extended exposure of 2D-PAGE pattern of proteins labeled by E2 and RA in MCF-7 and MCF-7/Adr^R cells. The gels shown in Fig. 1 were exposed to the film for 13 months. The *open* symbols in the *composite* show the position of proteins that could be seen as labeled by either RA or E2 after exposure to the film for 13 months but could not be seen at 60 days.

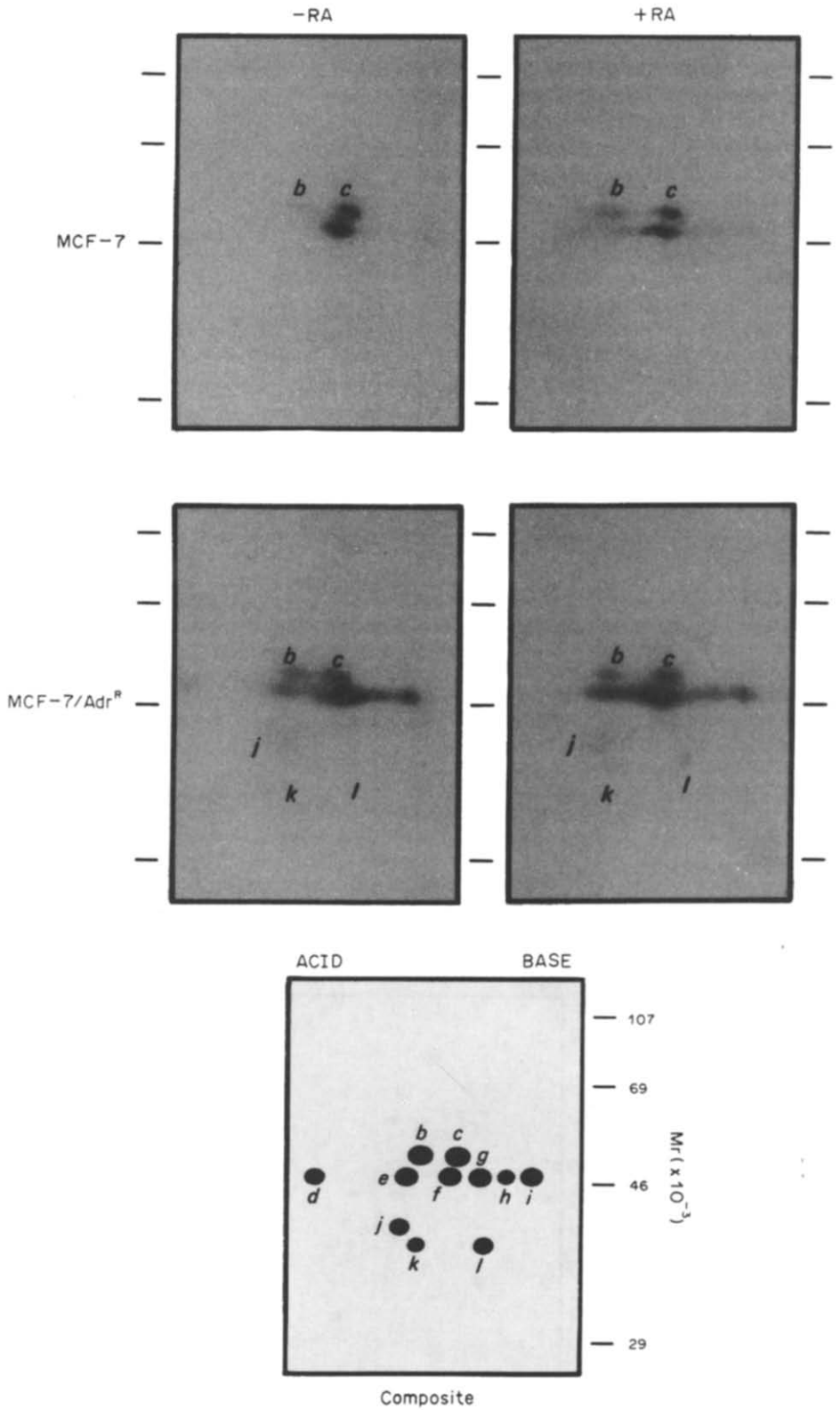

Fig. 3. cAMP-binding proteins in MCF-7 cells and MCF-7/Adr^R cells grown without and with RA. MCF-7 cells were grown for 24 h without and with 100 nM RA. Cells were removed from the surface by scraping and harvested by centrifugation. Total cellular proteins were labeled with 8-azido-[^{32}P]cAMP and separated by 2D-PAGE. The proteins from 2.8×10^6 MCF-7 cells and 3.7×10^6 MCF-7/Adr^R cells were analyzed. The dried gels were exposed to the film for 35 days. The *composite* shows the proteins from either cell line that were labeled with 8-azido-[^{32}P]cAMP. The position of each *lettered* [^{32}P]protein was based on its mobility compared with proteins that were stained by Coomassie Blue R-250.

DISCUSSION

In this study we have extended our previous findings [8] that E2 and RA are covalently bound to proteins in MCF-7 cells. The effects of these hormones on cell division in MCF-7 cells and other breast tumor cells lines have been studied extensively *in vitro* and *in vivo* [5, 13–16, 23–33]. E2 and RA bind specifically and reversibly to receptors that are members of the nuclear thyroid/steroid hormone receptor multigene family which function as regulators of gene expression [1]. MCF-7 cells contain receptors for E2, progesterone [16], and RA [34].

The explanation for the effects of E2 on the growth of MCF-7 cells is controversial. The positive correlation between ER levels and the growth response to E2 of breast tumor cell lines is the basis for the idea that these receptors play a direct and positive role in mediating growth. In contrast, results of other studies, both *in vivo* and *in vitro*, conflict with ER-dependent positive control of growth [3, 5, 35]. It is possible that some controversy from results *in vitro* is because culture conditions, which may vary widely from laboratory to laboratory, markedly influence the E2 growth effects [27, 30].

Our results in Figs 1 and 2 suggest that a mechanism of action of E2 may involve the covalent binding of E2 to protein. This reaction may be independent of the ER because proteins were modified by E2 in both receptor-positive (MCF-7) and receptor-negative cells (MCF-7/Adr^R). However, the total covalent binding of E2 was 2-fold greater in MCF-7 cells and the labeling of individual proteins was also probably greater (Figs 1 and 2). Therefore, it is possible that the ER could play a role in the covalent binding of E2 to proteins by sequestering E2 at the intracellular location where the reaction occurs. If this is a function of the ER then the concentration of E2 required for half-maximal labeling of protein may be lower in ER-positive cells than in ER-negative cells. It will be of interest to determine the intracellular localization of the labeled proteins and compare the dose-dependence of E2 covalent binding to proteins in other ER-positive and -negative cells.

We do not know the nature of the E2 moiety that is covalently bound to protein in MCF-7 and MCF-7/Adr^R cells. In MCF-7 cells the ER and other proteins localized in the nuclear matrix are covalently labeled after exposure for 28 days to [^{3}H]16α-hydroxyestrone, a metabolite of E2 [6]. This modification may occur in a nonenzymatic reaction involving the formation of a reversible Schiff base intermediate [7, 36, 37]. In the presence of 5 nM E2 the rate of 16α-hydroxyestrone formation in MCF-7 cells is about 75 fmol/10^6 cells/day [38, 39]. Thus, it is possible that 16α-hydroxyestrone is an intermediate in the covalent binding of E2 to protein that we observed. However, our results show that after only a 1-day exposure to [^{3}H]E2 the major labeled proteins in MCF-7 and MCF-7/Adr^R cells had M_r values of 37,000 and 20,000 (Figs 1 and 2). We did not see labeled proteins corresponding to the ER (M_r value of about 66,000) or the more highly labeled proteins of M_r 27,000 and >200,000 seen when MCF-7 cells are exposed to [^{3}H]16α-hydroxyestrone for 28 days [6]. Differences in incubation times, radioactive substrates, and cellular subfractions analyzed may contribute to the variations noted between our results and those in the referenced study.

The proportions of the covalent binding of E2 to proteins of MCF-7 cells that is enzymatic, nonenzymatic, or a combination of both is not known. Identification of the labeled moiety covalently bound to protein should aid in clarifying the pathway for this modification. It is of interest that tamoxifen and chlorotriansene may be proestrogens/proantiestrogens that are activated by cytochrome *P*-450 to reactive intermediates that form covalent adducts with proteins, possibly including the ER and cytochrome *P*-450 [40, 41]. Thus, the covalent attachment to specific proteins may be a mechanism for the action of many biologically-active molecules which also bind to specific receptors.

In contrast to the uncertain nature of the covalent binding of estrogens and antiestrogens to protein, the mechanisms for the covalent binding of RA to some cellular proteins are clearer. Most of the intact RA molecules bound to protein in HL60 cells is *via* a S-ester bond [42]. This reaction can occur enzymatically [43]. Retinoylation is not restricted to HL60 cells and occurs in other cell lines that respond to RA [8, 9]. Several retinoylated proteins have been identified. RI and II, the regulatory subunits of PKA, are retinoylated in HL60 cells [11] and the cytokeratins [10] and thioredoxin reductase [44] are retinoylated in keratinocytes. The chemical bond between RA and these proteins is not known and, at least for the cytokeratins, probably is not a S-ester [10].

In this study we saw that both MCF-7 and MCF-7/Adr^R cells have a retinoylated protein with a mobility on 2D-PAGE that is very similar to that seen for RII in HL60 cells (Fig. 2, *protein 5*). A protein in MCF-7 cells with the same mobility also was labeled by E2 and RA (Fig. 2, *protein 5*) and by 8-azido-[^{32}P]cAMP (Fig. 3, *protein c*). These results raise the possibility that the same protein may be retinoylated, labeled by E2, and is a cAMP-binding protein. The high resolution of the 2D-PAGE separation technique coupled with the low number of proteins that are labeled by either E2, RA, or 8-azido-cAMP strongly support this possibility. Based on an apparent M_r of 51,000 and its specific immunoprecipitation by an anti-RII$_\alpha$ serum this protein is most likely RII$_\alpha$, the most prevalent form of RII in human cells [45]. It will be of interest to learn if this protein can be colabeled by E2 and RA and if these modifications affect its intracellular distribution and function.

The possibility that RA and E2 may act by modifying a regulatory protein of PKA may aid in explaining the effects of combinations of these and other hormones on various cell types. It is well-established that there is a close interrelationship between hormones, including steroids, and PKAs to many biological responses, including cell growth and differentiation [12, 13, 46–48]. RA and agents that increase cAMP inhibit the growth of breast tumor cell lines [12–15]. While a mechanism for growth inhibition by cAMP is not known it is probable that it involves activation of PKA activity [49]. In MCF-7 cells an increase in intracellular cAMP is associated with an elevated level of PKA type II and a decrease in PKA type I [12]. Based on our evidence that RII is modified by either E2 or RA it will be of interest to examine these covalent modifications under conditions in which intracellular cAMP concentrations are varied. This information should aid in the elucidation of the physiological function of the covalent modification of proteins by E2 and RA.

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