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Phosphorylation of the secreted, free α subunit of human chorionic gonadotropin

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ABSTRACT **Phosphorylation of secretory proteins is an uncommon event. In this manuscript, the phosphorylation of human chorionic gonadotropin, a glycoprotein hormone secreted by the JAR choriocarcinoma cell line, is described. Labeling of JAR cells with $^{32}PO_4$ indicates that both the intracellular and the secreted forms of the free α subunit are phosphorylated. Although the secreted αβ dimer also incorporates $^{32}PO_4$, there is little detectable phosphorylation of the intracellular precursors of αβ dimer, suggesting that dimer phosphorylation occurs as a late event in post-translational processing. In addition, phorbol 12-myristate 13-acetate markedly stimulates the phosphorylation of both intracellular and secreted forms of free α subunit and to a lesser extent of secreted αβ dimer. *In vitro* assays, using homogenates of JAR cells as a source of protein kinase activity, indicate that the uncombined α subunit is preferentially phosphorylated. The phosphorylation sites are on serine and threonine residues in the α subunit.**

Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of two dissimilar subunits, α and β, joined noncovalently. It is secreted from normal first-trimester placenta as well as from a number of malignant trophoblastic cell lines, including the JAR choriocarcinoma cell line. We have utilized this cell line to characterize the biosynthesis and post-translational processing of hCG (1–5). Two asparagine-linked oligosaccharide chains are added cotranslationally to each of the subunits prior to formation of the αβ dimer. The association of the newly synthesized subunits is rapid and is accomplished while the molecules are in the rough endoplasmic reticulum. Combination of the subunits, however, is incomplete, and 51% of the total α and 44% of the total β subunits synthesized in JAR cells remain uncombined (5). Trimming and processing of the oligosaccharide chains occur as the hCG forms are translocated through the cell, after which the dimer as well as the uncombined subunits are secreted continuously into the culture medium.

Studies in this laboratory have been designed to determine what post-translational modifications are involved in regulating the amount of αβ dimerization and in the cellular translocation events of hCG and its free subunits. Many proteins have specific recognition signals that are important in the delivery of proteins to their respective compartments in the cell. For example, the mannose-6-phosphate residue on acid hydrolases is essential for the targeting and subsequent delivery of these enzymes to the lysosomes (6, 7). Similar recognition signals, either in the amino acid sequence or in the carbohydrate moiety, have been implicated in the regulation of protein translocation between intracellular compartments, such as from the endoplasmic reticulum to the Golgi and eventually to the cell membrane (8). Various studies have shown that different secretory glycoproteins can be translocated through the same cell at different rates (9, 10). This argues against a bulk-phase, continual movement of proteins through the cell and supports the theory that specific recognition of amino acid or carbohydrate structure may mediate protein translocation.

Post-translational modifications may also regulate subunit combination of hCG in trophoblastic cells. As noted above, only about one-half of each subunit synthesized is able to combine to form the dimer; thus, the synthesis of subunits is not a rate limiting factor for combination. This suggests that some post-translational modification of either subunit may limit dimer formation. For example, we have shown (11) that there are differences in disulfide bond formation between the intracellular forms of hCG-dimer β and of free β that may alter their ability to combine with the α subunit. In addition, we have reported (12) that about 50% of the free α subunit has an additional O-linked oligosaccharide chain, not observed in combined dimer α. In the bovine pituitary, O-glycosylation of the free α subunit blocks combination with the β subunit of luteinizing hormone (13), and such a modification may also inhibit hCG αβ dimer formation.

We now report that in JAR cells, the free α subunit is phosphorylated both in intact cells and in a cell-free system obtained from JAR cells. This phosphorylation event is stimulated by phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (14). Although phosphorylation of intracellular proteins is a widely accepted mechanism for amplification of intracellular signals (15, 16), phosphorylation of secretory proteins is a rare phenomenon. Examples of these include transformation-specific proteins (17, 18), fibronectin (19), certain milk proteins (20), and lipomodulin (21). Hepatocytes, in general, synthesize a large number of phosphoproteins, yet secrete only a small percentage of these, including apolipoprotein B (22), vitellogenin-derived proteins (23), and a 63-kDa protein (24).

MATERIALS AND METHODS

***In Vivo* Labeling, Immunoprecipitations, and NaDodSO$_4$/PAGE.** JAR human choriocarcinoma cells, obtained from Roland Pattillo (25) (Medical College of Wisconsin), were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum as described (1, 2). For biosynthetic labeling, cells were grown to 50–60% confluency in 100-mm Petri dishes. They were incubated with $^{32}PO_4$ (as $H_3{}^{32}PO_4$ carrier-free, ICN) at 0.5 mCi/ml (1 Ci = 37 GBq) in phosphate-free medium (Flow Laboratories) with 10% (vol/vol) fetal calf serum for 18 or 22 hr. Incubations were done in the presence of PMA, phorbol 13-monoacetate (PAc), or vehicle. Drug stocks were made in dimethyl sulfoxide, the final concentration of which in the medium did not exceed 0.01%. Both phorbol analogs were used at a final concentration of 100 nM. Cell lysates and media were collected as described (1, 5), except that protease inhibitors (20 mM

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Abbreviations: hCG, human chorionic gonadotropin; PMA, phorbol 12-myristate 13-acetate; PAc, phorbol 13-monoacetate.

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EDTA/2 mM phenylmethylsulfonyl fluoride/leupeptin at 1 μg/ml/antipain at 2 μg/ml/benzamidine at 10 μg/ml/Trasylol at 10 units/ml/chymostatin at 1 μg/ml/pepstatin at 1 μg/ml) and the phosphatase inhibitor sodium vanadate at 2 mM were added to the lysate buffer. The free α subunit and the intact αβ dimer were precipitated with specific antisera, and then the antibody complexes were subsequently collected with protein A-Sepharose CL-4B (Sigma) (5). The hCG forms were eluted from these pellets by boiling in $NaDodSO_4$ electrophoresis sample buffer [0.0625 M Tris·HCl, pH 6.8/10% (vol/vol) $NaDodSO_4$/0.1% bromophenol blue/10% (vol/vol) glycerol] with 5% (vol/vol) 2-mercaptoethanol for 5 min. Proteins were fractionated by $NaDodSO_4$/PAGE (5–20%), according to the method of Laemmli (26). Radioactive bands were visualized by fluorography.

***In Vitro* Assays.** JAR cells were grown to 95% confluency in four or five 150-mm^2 flasks. The cells were washed several times in homogenizing buffer (0.25 M sucrose/10 mM Tris·HCl, pH 7.2) and harvested by scraping. Whole cells were pelleted by centrifugation and resuspended in the same buffer with the addition of 0.1% Triton X-100. This cell suspension was sonicated to disrupt cell membranes. Nuclei were pelleted by centrifugation at 500 × *g* for 3 min, and the supernatant was adjusted to a final protein concentration of 1.5 mg/ml, as determined by the method of Lowry *et al.* (27) using bovine serum albumin as a standard.

cAMP-Dependent Kinase Assays. Activity was measured by the method of Gill and Walton (28) with modifications by Moore *et al.* (29). The incubation mixture contained 10 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM ATP containing 3×10^5 cpm of [γ-^{32}P]ATP, 1 μM cAMP, histone at 1 mg/ml, and 50 mM potassium phosphate (pH 6.8) in a final volume of 200 μl. In some experiments, 10 mM NaF and 2.5 mM theophylline were employed to potentiate the effects of cAMP. The reaction was initiated by the addition of 50 μg of JAR protein, and incubation was for 10 min at 30°C. Proteins were then precipitated with 5% (wt/vol) trichloroacetic acid, and results of triplicate assays were quantitated by scintillation counting.

***In Vitro* Phosphorylation of hCG.** The conditions for this assay were adapted from the cAMP-dependent kinase assay. The 200-μl reaction mixture contained 50 mM potassium phosphate (pH 6.8), 10 mM $MgCl_2$, 10 mM NaF, and 0.5 mM ATP containing 3×10^5 cpm of [γ-^{32}P]ATP. Intact urinary hCG and the purified subunits (obtained from the Center for Population Research, National Institute of Child Health and Human Development, National Institutes of Health, batch CR-123) were used at a concentration of 0.5 mg/ml. The reaction was initiated by the addition of 50 μg of JAR post-nuclear homogenate. After 24 hr at 30°C, 200 μl of $NaDodSO_4$ electrophoresis sample buffer was added. The samples were immediately boiled for 5 min, and the proteins were separated by $NaDodSO_4$/PAGE.

Phosphoamino Acid Analysis. The α bands were cut from the gel and eluted in 0.1% $NaDodSO_4$/5 mM 2-mercaptoethanol/50 mM NH_4HCO_3, pH 8, at 37°C for 24 hr in the presence of 100 μg of bovine serum albumin. The eluate was lyophilized, and the residue was hydrolyzed in 6 M HCl for 3 hr at 100°C. Phosphoamino acids were then separated by HPLC on a Altex Ultra-sphere C_{18}-IP column (Beckman) equilibrated with 5 mM tetrabutylammonium phosphate, pH 2.5, at a flow rate of 1.0 ml/min. Identification of standard phosphoamino acids was achieved after post-column derivatization with *o*-phthalaldehyde on a Kratos FS950 fluorometer (Kratos Analytical Instruments, Ramsey, NJ). Of the total recovered counts, 70% were detected in the phosphoamino acids serine and threonine.

RESULTS

Our studies have been designed to identify post-translational modifications that are important in the regulation of intracellular translocation and subunit combination of hCG. Because protein phosphorylation is a common modulator of protein function, we examined JAR cells for their ability to phosphorylate hCG. JAR cells were incubated for 18 hr with $^{32}PO_4$ in the presence of vehicle (0.01% dimethyl sulfoxide) or 100 mM PMA. The labeling media and cell lysates were immunoprecipitated, and the hCG forms were fractionated by $NaDodSO_4$/PAGE. The results, depicted in Fig. 1*A*, show that both secreted free α and αβ dimers were phosphorylated under basal conditions; however, PMA stimulated phosphorylation of free α more than of αβ dimer. The phosphorylation of the intracellular hCG precursors is shown in Fig. 1*B*. Only the free α subunit was phosphorylated intracellularly under control conditions, and PMA treatment enhanced $^{32}PO_4$ incorporation into free α. We have observed in this and other experiments (data not shown) that the intracellular phosphorylated form of α migrates slightly faster than the ^{35}S-labeled JAR α standard. The intracellular forms of the αβ dimer did

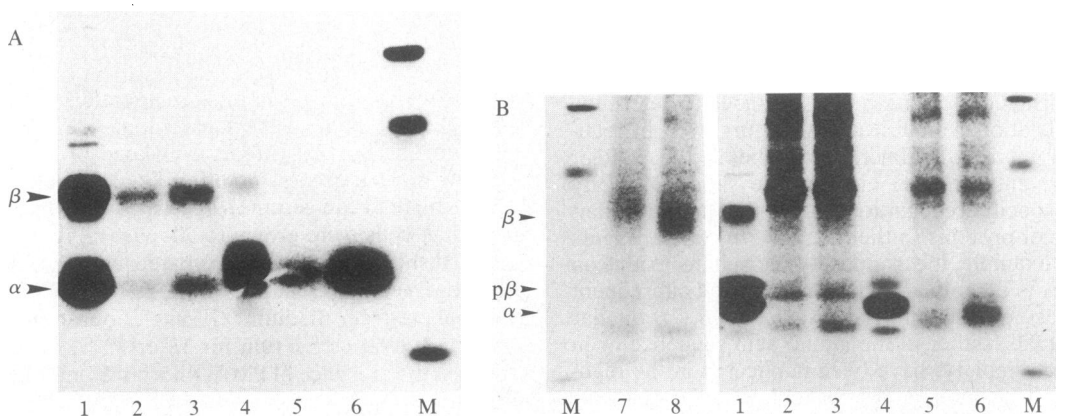

FIG. 1. Phosphorylation of hCG and its subunits by JAR cells. JAR choriocarcinoma cells were incubated with $H_3{}^{32}PO_4$ at 0.5 mCi/ml for 18 hr in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 100 nM PMA. Both the media (*A*) and cell lysates (*B*) were immunoprecipitated with anti-αβ (lanes 1, 2, and 3) and anti-α (lanes 4, 5, and 6) antisera. [^{35}S]Methionine-labeled (100 μCi/ml) (lanes 1 and 4) JAR intracellular and secreted hCG forms were collected in the same experiment as markers. The immunoprecipitated samples were analyzed by $NaDodSO_4$/PAGE. Arrows indicate the migration of the α and β subunits of hCG as well as the intracellular β precursor (pβ). Cell lysates incubated in the presence (lane 8) and absence (lane 7) of PMA were clarified by immunoprecipitation with normal rabbit serum (lanes 7 and 8) before addition of anti-αβ and anti-α antisera. Molecular size markers were (from the top) bovine serum albumin (69 kDa), ovalbumin (46 kDa), cytochrome *c* (12.3 kDa).

not appear to be phosphorylated in control or in PMA-treated cells. However, a band that comigrates with intracellular β precursor in the lanes with the anti-$\alpha\beta$ antibody precipitated material was slightly phosphorylated. This band could represent free β since in these experiments it would have coimmunoprecipitated with $\alpha\beta$.

Phorbol esters have been shown to exert their effects predominantly by activation of protein kinase C (14). To further elucidate the specificity of the action of PMA in JAR cells, we tested the effect of a phorbol analog, PAc, that does not activate protein kinase C. JAR cells were incubated for 22 hr with $^{32}PO_4$ in the presence of vehicle, 100 nM PAc, or 100 nM PMA (Fig. 2). PMA stimulated $^{32}PO_4$ incorporation into the secreted free α subunit compared to control levels. The inactive analog PAc did not significantly alter the phosphorylation of free α from control levels. In contrast, phosphorylation of secreted hCG dimer was not significantly affected by treatment with either the active or the inactive analog. To discount the possibility that the phosphorylation was a serum-mediated event, the experiments were repeated in serum-free medium (data not shown). The profiles of subunit phosphorylation were unchanged, indicating that the observed stimulatory effect of PMA was indeed mediated by the cells and not by the serum. These data suggest that PMA may be acting through cellular protein kinase C activation to stimulate the phosphorylation of the free α subunit.

In vitro assays were utilized to further characterize the kinase activity in JAR cells. Keutmann and co-workers (30, 31) had demonstrated selective phosphorylation of the free β subunit *in vitro*, using the catalytic subunit of cAMP-dependent kinase as an enzyme source. We assayed JAR cells for cAMP-dependent kinase activity based on $^{32}PO_4$ incorporation into histone H1. Addition of cAMP at concentrations ranging from 1 pM to 1 μM was unable to stimulate phosphorylation. For example, $^{32}PO_4$ incorporation was 340 and 290 nmol phosphate/min per μg of protein, respectively, in membranes from control and from 1 μm cAMP-treated membrane preparations. Neither the addition of 10 mM NaF nor of 2.5 mM theophylline had a potentiating effect. This suggests that cAMP-dependent kinase does not have a major role in hCG phosphorylation in JAR cells.

Conditions of the assay were modified to maximize the hCG kinase activity. Preliminary characterization of the enzyme activity under these conditions was done using histone H1 as a substrate. The substrate was saturating at a concentration of 0.5 mg/ml. In addition, the reaction was linear for 2 hr, and $^{32}PO_4$ incorporation continued throughout a 24-hr period. Purified urinary hCG $\alpha\beta$, α, and β were

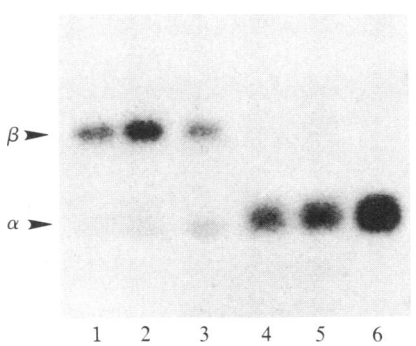

FIG. 2. Preferential stimulation of phosphorylation of secreted free α subunit by PMA. JAR cells were incubated alone (lanes 1 and 4), with 100 nM PMA (lanes 3 and 6), or with PAc (lanes 2 and 5) in the presence of $^{32}PO_4$ for 22 hr. Media samples were immunoprecipitated with anti-$\alpha\beta$ (lanes 1–3) and anti-α (lanes 4–6) antisera and analyzed by $NaDodSO_4$/PAGE. Arrows indicate the migration of secreted JAR α and β standards.

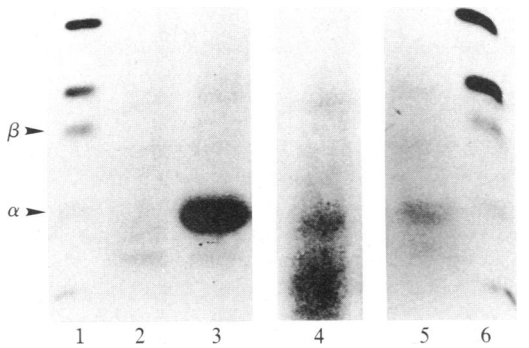

FIG. 3. *In vitro* phosphorylation of hCG and its subunits. Purified urinary hCG dimer (lane 5) and dissociated α (lane 3) and β (lane 4) subunits were incubated with a post-nuclear homogenate fraction from JAR cells in the presence of [γ-^{32}P]ATP for 24 hr and analyzed by $NaDodSO_4$/PAGE. Arrows indicate the migration of JAR α and β standards. Lane 2, JAR homogenate alone. Lanes 1 and 6, molecular size standards as in Fig. 1.

incubated with a post-nuclear homogenate of JAR for 24 hr. The reaction was stopped by boiling in $NaDodSO_4$ electrophoresis sample buffer, and the proteins were fractionated by $NaDodSO_4$/PAGE. The fluorograph is shown in Fig. 3. Only the uncombined α subunit was significantly phosphorylated, and the uncombined β and the intact $\alpha\beta$ dimer incorporated negligible amounts of $^{32}PO_4$.

The band corresponding to the α subunit was cut from the gel and analyzed for phosphoamino acids (Fig. 4). Total radioactivity contained in the peaks was 271 and 280 cpm, respectively, for phosphothreonine and phosphoserine. Thus, serine and threonine residues were phosphorylated in equivalent amounts in the free α subunit.

DISCUSSION

Post-translational modifications of the hCG subunits would provide an attractive regulatory mechanism for subunit combination. We have identified (11, 12) several differences between the free and combined subunits that may limit dimer

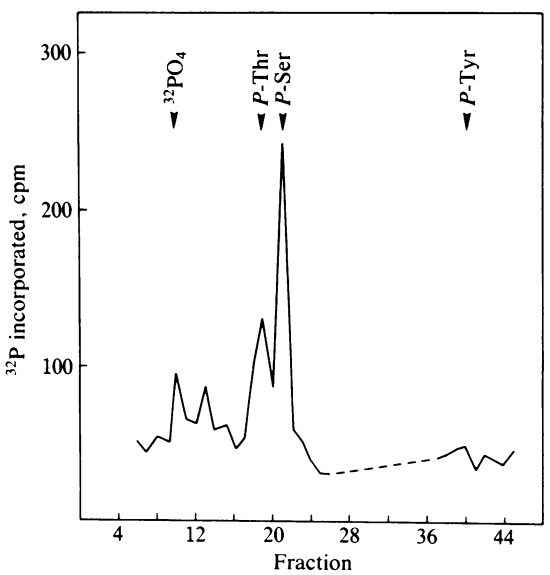

FIG. 4. Phosphorylation of serine and threonine residues in the α subunit. Phosphorylated urinary α subunit was obtained as described in Fig. 3. The α band was eluted from the $NaDodSO_4$/PAGE gel, hydrolyzed, and the phosphoamino acids separated. Unincorporated $^{32}PO_4$ eluted with the void volume. The elution times of phosphothreonine (*P*-Thr), phosphoserine (*P*-Ser), and phosphotyrosine (*P*-Tyr) are indicated.

formation, including differences in disulfide bond formation between the free and combined β subunits, and the additional O-glycosylation site in the free α subunit. We have also probed for a possible role of sulfation as a regulator of subunit combination in JAR cells, but we were unable to demonstrate sulfation of any forms of hCG. It should be noted that desialylated hCG is a substrate for sulfation by placental membranes *in vitro* (32). hCG or its subunits, however, do not appear to be sulfated in intact malignant trophoblastic cells.

Here we describe the preferential phosphorylation of the intracellular and the secreted forms of the free α subunit of hCG. Some $^{32}PO_4$ was incorporated into the secreted $\alpha\beta$ dimer, but not into the intracellular dimer subunits. This suggests that the phosphorylation of dimer occurs as a late event in post-translational processing. Possible locations for this kinase activity include both the internal and external surfaces of the plasma membrane.

We have utilized PMA, the well-known tumor promoter and activator of protein kinase C (14), to amplify the phosphorylation signal in hCG. Treatment with PMA stimulated the phosphorylation of the free α subunit. The inactive phorbol analog PAc was unable to stimulate $^{32}PO_4$ incorporation, suggesting that PMA is acting by stimulation of protein kinase C.

To better characterize the enzyme activity in JAR cells, a cell-free assay was developed. Using a post-nuclear homogenate of JAR as an enzyme source, we demonstrated preferential phosphorylation of the uncombined α subunit, confirming the *in vivo* results. In addition, phosphoamino acid analysis of the uncombined α subunit phosphorylated *in vitro* demonstrated equivalent phosphorylation of serine and threonine residues. The dissociated α and β subunits obtained from urinary hCG were used as "model" substrates for the JAR cell kinase and may not be identical to the "free" subunits synthesized in these cells. However, based on partial amino acid sequence analysis and on immunological crossreactivity (3), the specificity of the JAR kinase for the urinary and JAR-derived substrates is likely to be similar.

The α subunit of hCG contains eight residues each of serine and threonine. If the phosphorylation of free α occurs as an early event in post-translational processing, it may have an important regulatory influence on $\alpha\beta$ dimer formation. The threonine at residue 39 is the site at which O-glycosylation occurs on the free α subunit of hCG (12). In a homologous region of the free α subunit obtained from bovine pituitary, Parsons and Pierce (13) have shown that this glycosylation, as well as alkylation of a tyrosine residue two amino acids toward the amino terminus (33), block luteinizing hormone dimer formation. Thus, phosphorylation of an amino acid in this region of the α subunit may also inhibit hCG subunit combination and be a mechanism that regulates the amount of biologically active hormone formed in trophoblastic cells.

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