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PHOSPHORYLATION OF THE GLYCOPROTEIN HORMONE α-SUBUNIT SECRETED BY HUMAN TUMOR CELL LINES

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The synthesis of ectopic proteins by tumors is thought to result from derepression of normally silent genes. One approach to a better understanding of this phenomenon is to characterize the physicochemical properties of the ectopic products, comparing them to their normal counterparts. In the following communication, evidence will be presented to indicate that the glycoprotein hormone α-subunits secreted by a number of human tumor cell lines are phosphorylated. This novel covalent modification occurs in cell lines derived from both trophoblastic (JAR, JEG) and nontrophoblastic (HeLa, ChaGo) tumors. A choriocarcinoma cell line (JAR), which secretes both hCG-α and hCG-β, phosphorylates only the α-subunit.

The glycoprotein hormones (chorionic gonadotropin, CG; follicle stimulating hormone, FSH; luteinizing hormone, LH,; thyroid stimulating hormone, TSH) are a family of proteins composed of two nonidentical subunits, α and β, which are associated noncovalently. These hormones share a common α-subunit but have distinct (though similar) β-subunits, which bestow biological specificity to the hormones. Both polypeptide subunits undergo post-translational modification by glycosylation at Asn or Ser residues (1,2).

In addition to their synthesis in pituitary (LH, FSH, TSH) and placenta (CG), one or more of these hormones or the isolated α- or β-subunits are also synthesized by a variety of tumors (3-8) and tumor-derived cell lines (9-19). The free α-subunit is secreted by cell lines established from both trophoblastic and non-trophoblastic tumors. In the latter instance, the hormone subunit is considered to be an ectopic protein, i.e. one characteristic of a cell-type other than that from which the tumor originated. Generally, the ectopic product is quite similar to its normal counterpart, but differences have been reported. In this regard, the glycoprotein hormone

Abbreviations used: hCG, human chorionic gonadotropin; FSH, follicle stimulating hormone; LH, luteinizing hormone; TSH, thyroid stimulating hormone; Btr, sodium butyrate; MEM, minimum essential medium; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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α-subunit secreted by a variety of tumor cells in culture demonstrates a lower isoelectric point, a higher apparent molecular weight, and altered oligosaccharide side chains when compared to urinary human chorionic gonadotropin (hCG) α-subunit as a standard. It has been demonstrated that several tumor cell lines fucosylate the α-subunit (20,21), a modification normally found on hCG-β but not on hCG-α (1,2). Cole et al. (22) report that the free α-subunit produced by a malignant trophoblast cell line (JAR) contains, in addition to the two usual N-linked oligosaccharide side chains, an additional O-linked carbohydrate moiety that is not present on α-subunit found in association with hCG-β. Moreover, the tumor free α-subunit will not combine with isolated hCG β-subunit to form intact hCG (i.e., αβ dimer).

The ectopic α-subunit secreted by HeLa cells has recently been purified and characterized (G. S. Cox and R. A. Rimerman, manuscript submitted). Several differences were observed in direct comparisons between the HeLa protein and urinary hCG-α. Although various interpretations could be suggested to account for the observed differences, a unifying hypothesis that encompassed a number of observations was that HeLa cells might modify the ectopic subunit by phosphorylation. The results presented below describe a direct test of this hypothesis.

Materials and Methods

Materials. Powdered culture media, sera, antibiotics, and other supplements were obtained from Grand Island Biological Company. Plastic tissue culture flasks were products of Corning. Radioisotopes including [^{35}S]methionine and [^{32}P]orthophosphate were purchased from New England Nuclear. Rabbit antiserum to intact hCG was obtained from Cappel Laboratories, and materials for electrophoresis were supplied by Bio.Rad Laboratories. Purified hCG and the isolated α- and β-subunits (preparation CR-123) were generously provided by the Center for Population Research of the National Institute of Child Health and Human Development.

Cell culture. HeLa cells were obtained from the American Type Culture Collection (CCL 2.2) and maintained on plastic in minimum essential medium (MEM) supplemented with 6% L-glutamine, 5% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. ChaGo cells were obtained through the courtesy of Dr. Bruce Weintraub at the National Institutes of Health, and BeWo and JAR cultures were kindly provided by Dr. R. A. Pattillo at the Medical College of Wisconsin. ChaGo and the choriocarcinoma cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics as noted above. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 and were routinely subcultured by trypsinization when cultures neared confluence.

Radiolabeling. To label cultures, the medium was removed, the cell sheet was washed twice in MEM formulated without phosphate and supplemented with 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) plus 2% dialyzed calf serum, and then incubated for 16-24 hr in the same medium with or without 3 mM sodium butyrate (Btr) and containing 50-100 μCi/ml of $^{32}P_i$. The medium was cleared of detached cells by centrifugation, and the secreted proteins were precipitated with $(NH_4)_2SO_4$. Insoluble material was collected by centrifugation, dissolved in distilled water, and chromatographed

on Sephadex G-25 [equilibrated and eluted with phosphate-saline buffer (50 mM potassium phosphate, 0.15M NaCl, pH 7.4)] to remove $(NH_4)_2SO_4$ and unincorporated orthophosphate.

Immunoprecipitation. Secreted proteins were concentrated and separated from $^{32}P_i$ as described aobve. Appropriate column fractions (Sephadex G-25) were adjusted to contain 50 mM Tris. Cl (pH 7.4), 1% Triton X-100, 0.5 M NaCl, and 0.02% NaN_3. Formalin-fixed, heat inactivated Staphylococcus aureus (Cowan I strain, ATCC 12598) cells were added to 1 ml of the above mixture in order to deplete the sample of radiolabeled material that would bind non-specifically to either IgG in the serum or the S. aureus cells. After incubation for 30 min at 37°C, the solutions were clarified by centrifugation. The supernatants were then incubated for 24 hr at room temperature following the addition of 10 µl of either preimmune, anti-α, or anti-hCG serum. The specificity of the antiserum raised against purified hCG α-subunit has been described previously (28). Antibody against intact hCG was purchased from Cappel Laboratories. These preparations demonstrated comparable reactivity toward both α- and β-subunits. The solutions were again supplemented with 200 µl of a 10% (w/v) suspension of formalin-fixed, heat-inactivated S. aureus cells and incubated at 37°C for 30 min. The immune complexes were colleted by centrifugation, then washed successively with 50 mM Tris.Cl (pH 7.4), 0.15M NaCl; 50 mM Tris.Cl (pH 7.4), 0.15M NaCl, 1% Triton X-100, 2M urea; and 50 mM Tris.Cl (pH 6.8).

Polyacrylamide gel electrophoresis (PAGE). After washing the immune precipitates, the final pellets were resuspended in SDS-PAGE sample buffer [25 mM Tris.Cl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol], heated at 60°C for 20 min to release bound material, and clarified by centrifugation. The supernatants were boiled for 5 min in a water bath, then subjected to electrophoresis at approximately 25 mA in slab gels containing 12.5% polyacrylamide using the Tris-glycine electrode buffer described by Laemmli (34). In some experiments, culture media were chromatographed on Sephadex G-25 to remove unincorporated $^{32}P_i$ and analyzed directly on SDS-PAGE as described above without prior immunoprecipitation. Following electrophoresis, gels were stained with Coomassie blue, destained, dried under vacuum, and subjected to autoradiography at -60°C on Kodak XAR film using intensifying screens.

Results and Discussion

The data presented in Fig.1 indicate that HeLa cells secrete a variety of phosphorylated proteins as determined by electrophoresis of conditioned medium in polyacrylamide gels containing sodium dodecylsulfate (SDS-PAGE). Sodium butyrate, which increases α-subunit production in HeLa cells (14, 17, 23-25), had minimal effect on the overall pattern of secreted phosphoproteins (compare lanes 1,3, and 5 with lanes 2,4, and 6, respectively). A notable exception was the dramatic increase in autoradiographic signal corresponding to a protein with apparent M_r 24,600. This protein migrated slightly behind a purified hCG α-subunit standard run in parallel (M_r 22,400; arrow), a property characteristic of the tumor free α-subunit (20,21,25,26).

To further identify this protein, culture media from cells labeled with $^{32}P_i$ in the absence or presence of 3 mM Btr were immunoprecipitated with antiserum raised against hCG α-subunit (28). As seen in Fig.2, there was a single autoradiographic band in samples receiving anti-α serum (lanes 2 and 4)

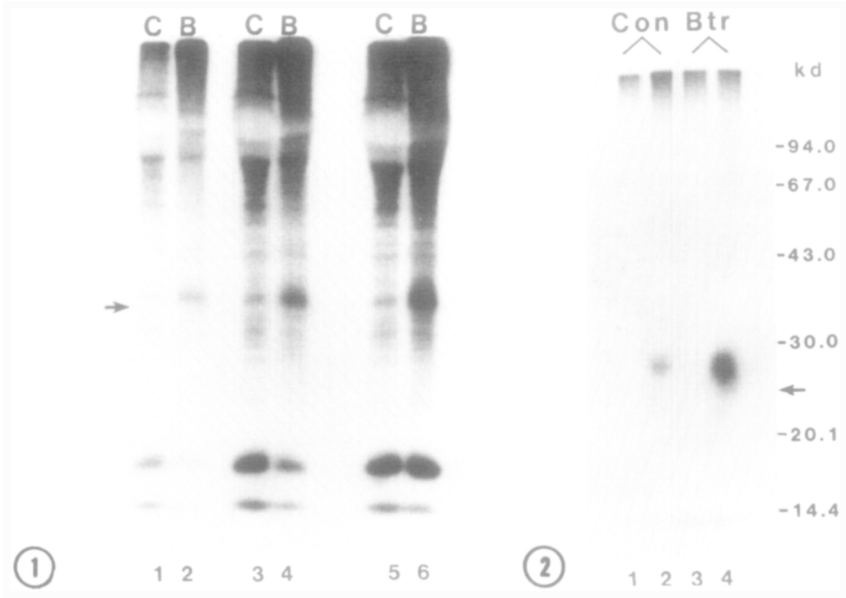

Figure 1. Analysis of HeLa secreted phosphoproteins by SDS polyacrylamide gel electrophoresis. To induce α-subunit, cultures were preincubated in medium supplemented with 3 mM Na butyrate (B) for 24-36 hr prior to labeling with $^{32}P_i$. Control flasks (C) received no supplements. After cells were incubated with $^{32}P_i$ as described in the Materials and Methods, the medium was desalted by chromatography on Sephadex G-25. Aliquots of material eluting in the void volume [corresponding to either 5,000 cpm (lanes 1,2), 10,000 cpm (lanes 3,4), or 20,000 cpm (lanes 5,6) of ^{32}P] were adjusted to contain 25 mM Tris•Cl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol (SDS-PAGE sample buffer) in a final volume of 40μl. These samples were heated at 100° C for 5 min and then electrophoresed at 25 mA in 12.5% polyacrylamide slab gels in the buffer system described by Laemmli (34). Purified hCG α-subunit (arrow) was run on a parallel lane. After electrophoresis, the gel was stained, destained, dried, and subjected to autoradiography as described in the Materials and Methods.

Figure 2. Immunoprecipitation of α-subunit from HeLa cultures labeled with [^{32}P]orthophosphate. Cells were cultured and labeled with $^{32}P_i$ in the absence (Con) or presence (Btr) of 3 mM sodium butyrate as described in the Materials and Methods and Fig. 1. Media samples were incubated with either preimmune (lanes 1 and 3) or anti-α serum (lanes 2 and 4). The immune complexes were washed as described in the Materials and Methods and analyzed by SDS-PAGE. The arrow denotes the position of urinary hCG-α (CR-123) run on a parallel lane, and migration of molecular weight standards is denoted by their apparent molecular weights (M_r) in kilodaltons (kd).

that migrated slightly slower than the purified α-subunit standard (arrow). This band was not evident in samples incubated with preimmune serum (lanes 1 and 3), and the signal was enhanced in the immunoprecipitated medium from Btr-treated cells (lane 4) compared to that from untreated cells (lane 2). All samples contained nonspecifically adsorbed material of high molecular weight which was present in the medium at relatively high concentrations (see Fig.1). Similar patterns (except for variability due to the extent of non-specific adsorption) were obtained when cells were labeled with [^{32}S]methionine (data not presented).

It was of interest to examine other tumor cell lines that produce the glycoprotein hormone α-subunit in order to determine whether subunit phosphorylation was unique to HeLa. The results presented in Fig.3 and Fig.4 indicate that a variety of tumor cell lines carry out this modification. Media from HeLa (cervical carcinoma) and ChaGo (bronchogenic carcinoma) cells labeled with $^{32}P_i$ were immunoprecipitated with anti-α serum and analyzed by SDS-PAGE. As seen in Fig.3, both cell lines secreted an immunoreactive phosphoprotein that migrated with M_r slightly greater than the urinary hCG-α standard (arrow). Similarly, the data presented in Fig.4 indicate that two choriocarcinoma cell lines phosphorylate a protein that can be immunoprecipitated from JAR medium with antisera directed against the free α-subunit (lane 3) or intact hCG (lane 4), and from JEG medium with anti-α serum (lane 2) but not with preimmune serum (lane 1). Since the anti-hCG serum employed will precipitate the free α- and β-subunits as well as the heterodimer hCG (data not presented), the results depicted in Fig.4 suggest that β-subunit is not phosphorylated by the JAR choriocarcinoma cell line.

In this regard, Ryan and coworkers (29,30) have demonstrated that hCG-β can be phosphorylated *in vitro* on Thr 97 by the catalytic subunit of skeletal muscle cAMP-dependent protein kinase; no phosphorylation of free hCG-α or of either subunit in intact hCG could be detected. Compared to unmodified hCG, the hormone formed by recombining phosphorylated β-subunit with hCG α-subunit exhibited reduced capacity for receptor binding (29%) and adenylate cyclase activation (69%). The significance of these observations with regard to a physiologic role for hCG phosphorylation is uncertain since phosphate could not be detected in the hormone purified from human pregnancy urine.

A number of other polypeptide hormones seem also to be phosphoproteins. It has recently been reported that naturally-occurring pituitary human growth hormone contains organically-bound phosphorous (31), and that it can be phosphorylated *in vitro* by the catalytic subunit of protein kinase (31) and by the epidermal growth factor-stimulated tyrosine kinase (32). Phosphorylated forms of ACTH have also been demonstrated (33). The functions of these phosphorylated hormones have not yet been defined.

The data presented above demonstrate that the glycoprotein hormone α-subunit secreted by continuous cell lines established from both nontrophoblastic (HeLa and ChaGo) and trophoblastic (JAR and JEG) tumors is phosphorylated. Thus, subunit phosphorylation is not unique to its ectopic expression since the gonadotropin is considered a eutopic product of choriocarcinoma. The site(s) of phosphorylation remains to be elucidated, as does the biological significance of this heretofore unrecognized covalent modification of hCG-α. Whether its phosphorylation is characteristic of a transformed phenotype is currently under investigation. One can speculate that such

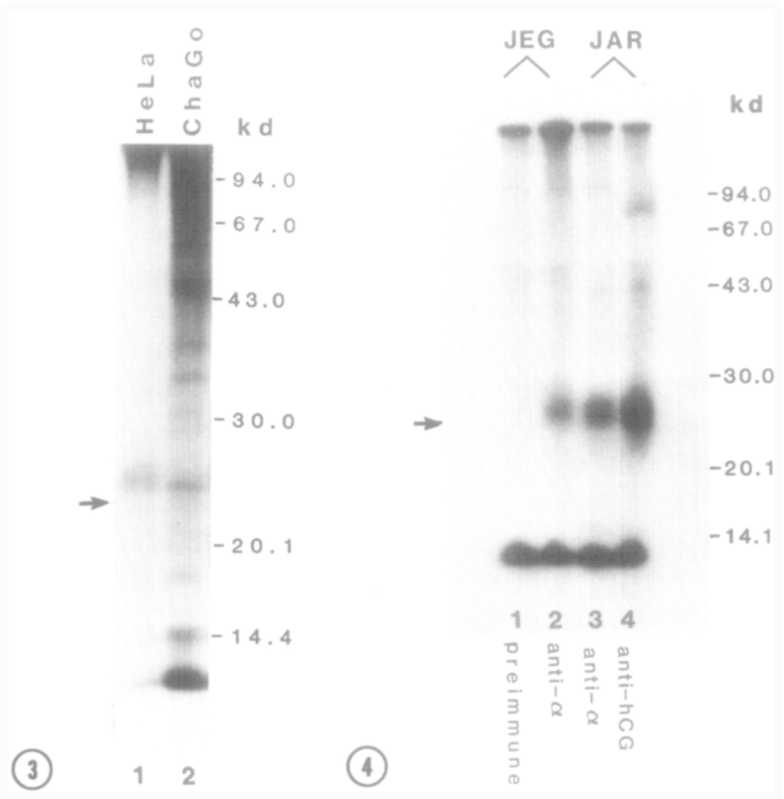

Figure 3. Phosphorylation of the glycoprotein hormone α-subunit by various nontrophoblastic tumor cell lines. Cells were maintained in RPMI-1640 medium containing 10% fetal calf serum (ChaGo) or in MEM containing 5% calf serum (HeLa) and other supplements as described in Materials and Methods. Cell sheets were washed and then incubated overnight in MEM formulated without phosphate and supplemented with 50 mM HEPES buffer (pH 7.2), 2% dialyzed calf serum, and 50 μCi/ml of $^{32}P_i$. Media were collected from HeLa (lane 1) and ChaGo (lane 2) cultures and immunoprecipitated with anti-α serum as described in Materials and Methods, except the pre-precipitation with S. aureus cells was omitted, and the immune complexes were washed in 50 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Electrophoresis and autoradiography were performed as described in Fig 2.

Figure 4. Phosphorylation of hCG-α by two trophoblastic tumor cell lines. The JAR and JEG choriocarcinoma cell lines were maintained in RPMI-1640 medium containing 10% fetal calf serum and the other supplements as listed in Materials and Methods. Cell sheets were washed twice and incubated 24 hr in phosphate-deficient MEM containing 50 mM HEPES buffer (pH 7.2), 2% dialyzed calf serum, and 50 μCi/ml of [^{32}P]orthophosphate. Media were collected from JEG (lanes 1 and 2) and JAR (lanes 3 and 4) cultures and incubated with preimmune (lane 1), anti-α (lanes 2 and 3), or anti-hCG (lane 4) sera. The anti-α serum was generated in this laboratory as described previously (28), and the anti-hCG serum was purchased from Cappel Laboratories. The latter antiserum demonstrates excellent cross reactivity with both free α- and β-subunits in standard RIA and immunoprecipitation protocols (data not presented). Immune complexes were washed and analyzed by SDS-PAGE as described above. Urinary hCG α-subunit (arrow) and molecular weight markers were run on parallel lanes and stained with Coomassie blue.

modification may produce an α-subunit with biological functions independent of hormone formation; or that the phosphorylated α-subunit may be incapable of

associating with β-subunits; or that if combination can occur, the phosphorylated hormone may have altered properties (e.g., receptor binding).

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References

1. Kessler, M. J., Reddy, M. S., Shah, R. H., and Bahl, O. P. (1979) J. Biol. Chem. 254, 7901-7908.
2. Kessler, M. J., Mise, T., Ghai, R. D., and Bahl, O. P. (1979) J. Biol. Chem. 254, 7909-7914.
3. Blackman, M. R., Rosen, S. W., and Weintraub, B. D. (1978) Adv. Int. Med. 23, 85-113.
4. Sheth, N. A., Saruiya, J. N., Ranadine, K. J., and Sheth, A. R. (1974) Br. J. Cancer 30, 566-570.
5. Rees, L. H. (1975) J. Endocrinol. 67, 143-175.
6. Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. P., and Ross, G. T. (1973) Ann. Intern. Med. 78, 39-45.
7. Weintraub, B. D. and Rosen, S. W. (1973) J. Clin. Invest. 52, 3135-3142.
8. Rosen, S. W. and Weintraub, B. D. (1974) N. Engl. J. Med. 290, 1441-1447.
9. Rabson, A. S., Rosen, S. W. Tashjian, A. H., and Weintraub, B. D. (1973) J. Natl. Cancer Inst. 50, 669-674.
10. Story, M. T. and Hussa, R. O. (1980) J. Clin. Endocrinol. Metab. 50, 1057-1061.
11. Pattillo, R. A., Hussa, R. O., Story, M. T., Ruckert, A. C. F., Shalaby, M. R., and Mattingly, R. F. (1977) Science 196, 1456-1458.
12. Pattillo, R. A. and Gey, G. O. (1968) Cancer Res. 28, 1231-1236.
13. Lieblich, J. M., Weintraub, B. D., Rosen, S. W., Chou, J. Y., and Robinson, J. C. (1976). Nature 260, 530-532.
14. Ghosh, N. K. and Cox, R. P. (1976) Nature 259, 416-417.
15. Chou, J. Y. (1978) In Vitro 14, 775-778.
16. Benveniste, R., Conway, M. C., Puett, D. and Rabinowitz, D. (1975) Endocrinology 105, 581-587.
17. Lieblich, J. M., Weintraub, B. D., Rosen, S. W., Ghosh, N. K., and Cox, R. P. (1977) Nature 265, 746.
18. Ruddon, R. W., Anderson, C., Meade, K. S., Aldenderfer, P. H., and Neuwald, P. D. (1979) Cancer Res. 39, 3885-3892.
19. Cole, L. A., Hussa, R. O., and Rao, C. V. (1981) Cancer Res. 41, 1615-1619.
20. Ruddon, R. W., Bryan, A. H., Hanson, C. A., Perini, F., Ceccorulli, L. M., and Peters, B. P. (1981) J. Biol. Chem. 256, 5189-5196.
21. Morrow, J. S., Weintraub, B. D., and Rosen, S. W. (1983) Biochem. Biophys. Res. Commun. 112, 115-125.
22. Cole, L. A., Perini, F., Birken, S., and Ruddon R. (1984) Biochem. Biophys. Res. Commun. 122, 1260-1267.
23. Ghosh, N. K., Rukenstein, A., and Cox, R. P. (1977) Biochem. J. 166, 265-274.
24. Ruddon, R. W., Anderson, C., Meade, K. S., Aldenderfer, P. H., and Neuwald, P. D. (1979) Cancer Res. 39, 3885-3892.

25. Chou, J. Y., Robinson, J. C., and Wang, S. S. (1977) Nature 268, 543-544.
26. McClure, D. S. and Cox, G. S. (1984) Arch. Biochem. Biophys. 233, 93-105.
27. Dean, D. J., Weintraub, B. D., and Rosen, S. W. (1980) Endocrinology 106, 849-858.
28. Cox, G. S. (1981) Biochemistry 20, 4893-4900.
29. Keutmann, H. T., Ratanabanangkoon, K., Pierce, M. W., Kitzmann, K., and Ryan, R. I. (1983) J. Biol. Chem. 258, 14521-14526.
30. Ratanabanangkoon, K., Keutmann, H. T., Kitzmann, K., and Ryan, R. J. (1983) J. Biol. Chem. 258, 14527-14531.
31. Liberti, J. P., Antoni, B. A., and Chlebowski, J. F. (1985) Biochem. Biophys. Res. Commun. 128 , 713-720.
32. Baldwin, G. S., Grego, B., Hearn, M. T. N., Knesel, J A., Morgan, F. J., and Simpson, R. J. (1983) Proc. Natl. Acad. Sci. USA 80, 5276-5280.
33. Bennett, H. P. J., Brubaker, P. L., Seger, M. A., and Solomon, S. (1983) J. Biol. Chem. 258, 8108-8112.
34. Laemmmli, U. K. (1970) Nature 227, 680-685.