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ANTIBODIES TO NEWLY RECOGNIZED MURINE 13-18 KDa PITUITARY PEPTIDES CROSSREACT WITH GROWTH HORMONE AND PROLACTIN FROM SEVERAL SPECIES, INCLUDING MAN

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SUMMARY: Recently we identified five novel peptides of M_r 13,000 to 18,000 (designated P13, P14, P16, P17, and P18 according to approximate M_r) in the anterior pituitary gland of rat and man that appeared related to GH and PRL in regulation and structure. We have now raised polyclonal antibodies in the rabbit to four of these peptides--P13, P14, P17 and P18--isolated from rat anterior pituitary; the rabbit injected with P16 did not produce antibodies. Besides reacting with their respective immunogens, antisera to all four peptides crossreacted, quite unexpectedly, with human GH and with human, porcine, and ovine PRL. Antiserum to P17, in addition, crossreacted very strongly with rat PRL, while P18 antiserum crossreacted not only with human GH but also with its 20K and cleaved variants. These results provide strong evidence for the structural relatedness of these peptides to GH and PRL, and raise the possibility that they may be related functionally as well.

The mammalian adenohypophysis elaborates a number of hormonal proteins, the major two being GH and PRL. Recently, we have identified five additional anterior pituitary peptides, ranging in M_r from 13,000 to 18,000, that appear related to GH and PRL in regulation and structure (1). Immunologically, they do not crossreact with currently available antisera to murine GH and PRL. Furthermore, P14 seems to be a glycosylated form of P13. Although found in large quantities (4-8 μg/mg) in the pituitary gland of several species, the physiological significance of these peptides has never been explored. Results of our current experiments indicate that one of these, the approximately 13,000 M_r band designated P13, is released in response to cold stress (2), and its tyrosine peptide map partially resembles

Abbreviations used are: GH, growth hormone; PRL, prolactin; G-PRL, glycosylated PRL; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IL, interleukin.

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that of human IL-1 (unpublished), suggesting that this molecule might have growth promoting and immunomodulatory activities. The actions of the other peptides are unknown. The subject of the present communication is our finding that antibodies generated against these peptides of murine origin crossreact strongly with GH and PRL of several species, including man.

MATERIALS AND METHODS

Tissues and hormones. Anterior pituitary tissues were obtained from rats of S/A strain (Simonsen Laboratories, Gilroy, CA), pigs of Duroc X Yorkshire breed (Meat Animal Research Center, Clay Center, NE), and human cadavers (National Pituitary Agency, NIH). Purified preparations of rat PRL (NIAMDD-RP-1), ovine PRL (NIH-P-S12), human PRL (NIADDK-I-7), rat GH (NIAMDD-RP-1), and human GH (NIH HS2243E) were gifts from the Hormone Distribution Program of the NIH, and porcine PRL (USDA-B1) and porcine GH (USDA-B1) were gifts from the U.S.D.A.

Production of antisera. Each peptide electroeluted from gels, as described below, was suspended in Freund's complete adjuvant. The suspension was injected into the popliteal lymph node of a rabbit by the method of Goudie et al. (3), as described earlier (4). Approximately 80-100 μg of protein in each case was injected into a 2-3 kg female New Zealand White rabbit. After four weeks, a booster injection of the same amount of antigen, suspended in Freund's incomplete adjuvant, was administered subcutaneously at multiple sites. The rabbits were bled 10 days later and the immunological reactivity of the antisera was determined by Western blot analysis.

Electroelution. Proteins for immunization were recovered from dye-stained gels by electroelution using a BIO-RAD electroeluter (model 422). Protein bands of interest were excised from the gel, minced into small pieces, and electrophoresed in SDS-containing Tris buffer at 60 mA for 3 hr. The eluted material was dialyzed against distilled water to remove some of the NaDodSO4 and other salts, and it was then lyophilized.

Protein assay. The protein content of the eluates was determined by the Pierce bicinchoninic acid method (5). Bovine serum albumin was used as the standard.

Gel electrophoresis. Anterior pituitary tissues were homogenized in Laemmli's 2-mercaptoethanol-containing sample buffer, in the ratio of 20 mg/ml. The homogenates were heated in boiling water for 3 min and then centrifuged at 1,000 x g for 15 min. A 25 μl aliquot of the supernatant, or a 5X diluted sample, was loaded on the gel. Purified hormone preparations were also boiled in sample buffer and loaded in 25 μl volumes. SDS-PAGE was performed in 1.5-mm thick and 12-cm long slab gels of 12% acrylamide, using the buffer system of Laemmli (6). The proteins in gels were stained with Coomassie brilliant blue R or transferred onto nitrocellulose paper for immunostaining.

Western blotting. Proteins from the gels were electrophoretically transferred onto nitrocellulose paper by the method described by Burnett (7). For determining immunological crossreactivity, antisera from the immunized rabbits were reacted, at a dilution of 1:1,000, with the

nitrocellulose paper. ^{125}I-labeled Protein A (130,000 CPM/ml) was used to visualize the immunoreactive bands.

RESULTS

Fig. 1 shows the migration patterns in SDS-PAGE of the murine pituitary proteins under investigation and the electrophoretic purity of the gel eluates that we used for immunization. Eluates of bands P18, P17, and P16 were quite homogeneous; P14 and P13 eluates were slightly contaminated with each other.

Each of the five eluates was injected into a rabbit. Immunoblotting revealed the presence of antibodies to pituitary proteins in 4 of 5 animals; the rabbit injected with P16 did not produce antibodies either to P16 or to any other pituitary protein tested.

Results of the immunological crossreactivities of the four antisera with GH and PRL from rat, man, pig and sheep are presented in Figs. 2-4.

The anti-P13 serum reacted very weakly with the P13 band in rat pituitary extract (Fig. 2, Panel B), but the anti-P14 serum reacted quite strongly with the rat P14 band (Fig. 2, Panel C). Neither of the two antisera

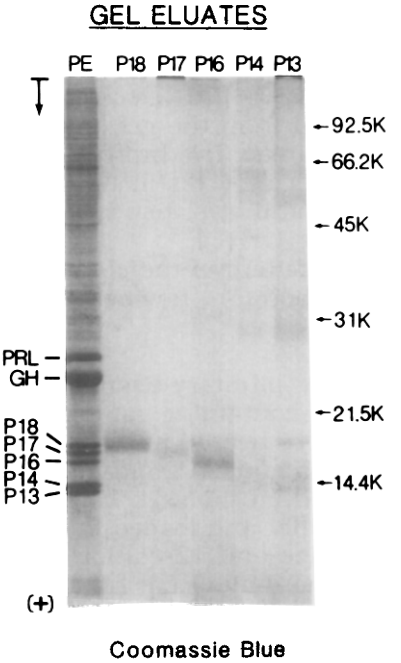

Fig. 1. Separation of the 13,000 to 18,000 M_r murine pituitary peptides by SDS-PAGE under reducing conditions, and demonstration of electrophoretic purity of the electroeluted peptides used for immunization. Note: a band with the R_f of P18 seen in the P13 eluate is not a contaminant but an artifact of staining. PE = pituitary extract.

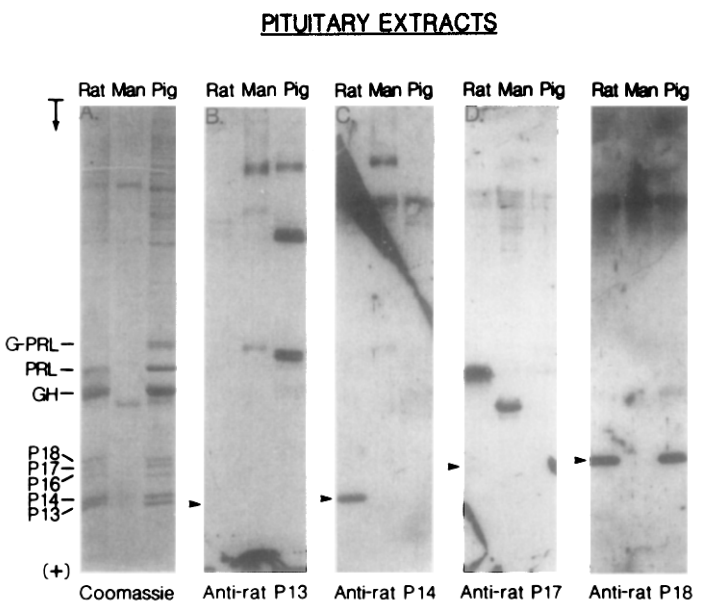

Fig. 2. Western blot analysis showing the immunological crossreactivity of the murine P13, P14, P17, and P18 antisera with proteins in the pituitary extracts of rat, man, and pig. Extract from 0.1 mg equivalent of anterior pituitary tissue was used in each case. Arrowheads indicate the positions of the immunoreactive P13, P14, P17 and P18 bands in the murine pituitary extract.

crossreacted with the P13 and P14 bands in human and porcine pituitary extracts. Both antisera elicited an immunoreactive band having the R_f of G-PRL in human pituitary extract. The anti-P13 serum, in addition, reacted with the GH band and a doublet just ahead of G-PRL in the porcine pituitary extract.

When reacted with purified hormone preparations (Fig. 3), both antisera crossreacted weakly with glycosylated (8) and unglycosylated forms of ovine, porcine and human PRL, and with human GH. In the human PRL preparation used, a G-PRL band was not visible by dye-staining (Fig. 3, Panel A), yet both antisera elicited an immunoreactive band in that region. It is likely that a protein other than human G-PRL migrating in the area strongly crossreacted with P13 and P14 antisera. Possibility for the existence of such a 25 KDa protein co-migrating with human G-PRL was suggested from the results of an earlier study as well (9).

The anti-P17 serum crossreacted very strongly with the rat PRL and human GH bands in pituitary extracts (Fig. 2, Panel D) as well as in purified hormone preparations (Fig. 4, Panel B). In addition, it weakly crossreacted with the unglycosylated and glycosylated forms of porcine PRL. Its crossreaction with human GH included the 20K variant and the large amino

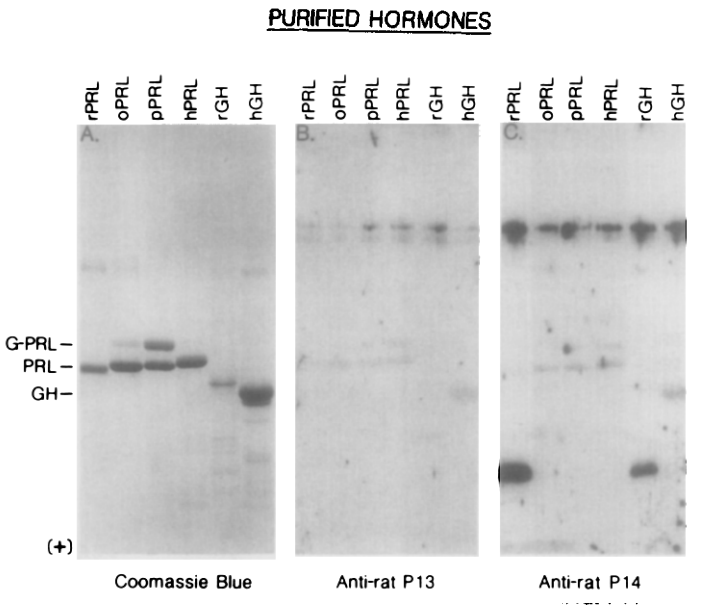

Fig. 3. Western blot analysis showing the immunological crossreactivity of murine P13 and P14 antisera with rat, ovine, porcine, and human PRL, and rat and human GH. Approximately 15 µg of protein was used for dye-staining (Panel A) and 5 µg for immunostaining (Panels B and C). The small letters r, o, h, and p stand for rat, ovine, human and porcine species.

terminal fragment (F_1) of cleaved human GH (Fig. 4, Panel B). It did not crossreact with rat or porcine GH, or human PRL.

The antiserum to P18, on the other hand, crossreacted with GHs of all three species tested, both in the pituitary extracts (Fig. 2, Panel E) and in purified preparations (Fig. 4, Panel C). Unlike anti-P17 serum, however, its crossreaction with human GH did not include the 20K and cleaved variants. It weakly crossreacted with human and porcine PRLs also, but not with rat PRL. Furthermore, it crossreacted strongly with the P18 band in rat and porcine pituitary extracts, but not in human pituitary extract (Fig. 2, Panel E).

DISCUSSION

PRLs and GHs of different species have various degrees of sequence homology (10). The immunological crossreactivities observed in these experiments between antisera to the new pituitary polypeptides and GH and PRL give strong indications that the pituitary peptides designated P13, P14, P17 and P18 share antigenic epitopes with GHs and PRLs of different species, and are thus structurally related to them to some extent. The crossreactivities further suggest that the peptides are related to one another also, since antibodies raised individually against them crossreacted with a

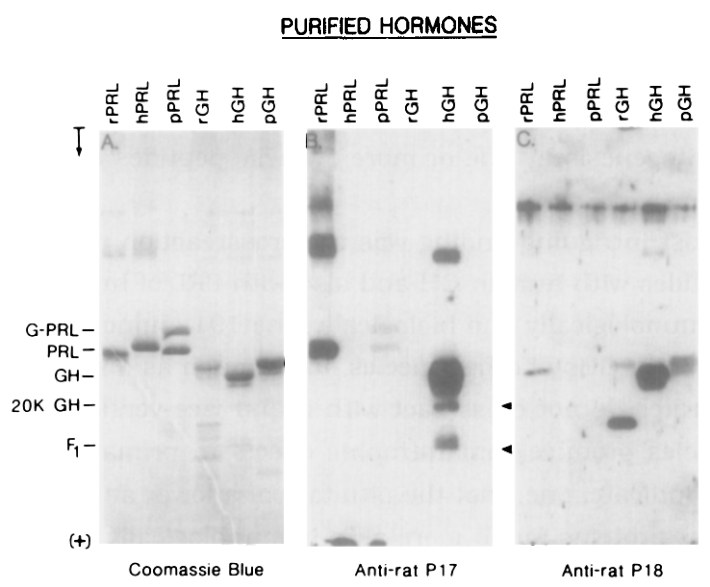

Fig. 4. Western blot analysis showing the immunological crossreactivities of murine P17 and P18 antisera with purified preparations of GH and PRL of rat, man and pig. Approximately 15 μg of protein was used for dye-staining and 5 μg for immunostaining. The small letters r, h, and p stand for rat, human and porcine species. F_1 refers to the large amino terminal fragment of cleaved human GH. Arrowheads indicate the positions of the 20K human GH and F_1 fragment of cleaved human GH. Note: in the rPRL lane of panel C, a slanted band-like line in the area of GH is not GH, but a scratch in the nitrocellulose paper.

common protein such as human GH. Tyrosine peptide maps of the peptides (1) and those of GH and PRL are in accord with the immunologic data: murine P17 showed partial resemblance to human GH, and murine P13 and P14 to human PRL. However, these peptides most likely are not fragments of GH and PRL, since P17 and P18 were found in human prolactinoma tissue completely devoid of GH (1). We have recently found that the expression of these peptides in the fetal porcine pituitary precedes that of GH and PRL (unpublished), further suggesting the genetic distinctiveness of the peptides. Therefore, these peptides must represent a new class of pituitary secretory products with immunogenic and structural features similar to those of GH and PRL.

That these peptides may indeed possess biological properties similar to those of GH and PRL is also suggested by the results of recent experiments. We (2) found a marked release of P13 and P14 from rat pituitary glands after exposure of the animal to cold, a stress stimulus that is well known to cause release of bioactive but not immunoreactive GH (11, 12, 13). Chomczynski and Brar (14) reported presence of significant mammary epithelial cell proliferating activity in a partially purified preparation of culture media from rat pituitary cells that was devoid of GH and PRL but rich

in the content of these or similar small M_r peptides. In view of rat PRL's strong crossreaction with the P17 antiserum, and those of other PRLs with antisera to the other peptides, an expression of a biological activity such as mammary mitogenesis by one or more of these peptides is not inconceivable.

The most intriguing finding was the crossreaction of antibodies to all the four peptides with human GH and also with PRL of human and other species. Immunologically and biologically, the 191 amino acid human GH is very distinct from GHs of other species, in as much as antibodies to GHs of lower vertebrates do not crossreact with it and vice-versa (15), nor do GHs of lower species produce somatotrophic effects in primates (16). Therefore, our findings indicate, one, that the pituitary of a lower animal such as the rat secretes other proteins much more akin immunologically to human GH than is the traditional murine GH; and, two, that these smaller proteins might be the progenitor of GH and PRL. Because there is significant sequence homology between GH and PRL (10), it has been postulated that the ancestor of these two proteins may have developed from a small protein by a process of gene duplication (17). However, such a small protein with sequences common to both GH and PRL has never been identified. In light of our results, it is tempting to speculate whether one or more of these peptides represents that putative ancestral protein.

The rabbit injected with P16 produced no antibodies. Although this could be due to biological variation, since only one animal was immunized, a more likely explanation could be the structure of P16. Its tyrosine peptide map (1) greatly resembles that of rat GH, and rat GH is not antigenic in rabbits (15). Recently, gene for a 17.5 KDa form of human GH, a result of alternative RNA splicing, has been demonstrated in the human pituitary (18). Because all of P16's tyrosine-containing tryptic peptides are accounted for in GH (1), P16 could represent the murine counterpart of the predicted 17.5 KDa human GH.

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