Cross-linking of a Growth Hormone Releasing Factor-binding Protein in Anterior Pituitary Cells*

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Growth hormone-releasing factor (GRF) stimulates the release of growth hormone from the anterior pituitary and is related to the peptides of the glucagon/secretin family. Although the mechanism of action of this hormone has been studied in considerable detail, little is known concerning the GRF receptor itself. We have attempted to label the GRF receptor by chemically coupling the [125I]-GRF analog [His², Nle¹⁵]-hGRF(1-32)-NH₂ (GRFa) (where Nle is norleucine) to plated rat anterior pituitary cells with the protein cross-linker disuccinimidyl suberate (DSS) (0.1 mM). Verification of biological activity of the [125I]-GRFa was confirmed prior to the cross-linking experiments using the reverse hemolytic plaque assay. Whole cell extracts prepared from the cross-linked cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography of the dried gels. Four bands of 72, 50, 30, and 26 kDa were detected in autoradiograms from cells exposed to the labeled analog for 20 min (22 °C) followed by exposure to DSS for 2 min. The 72-kDa band was interpreted to be bovine serum albumin, which was used as a carrier in initial studies. The 50- and 30-kDa bands were very faint and probably represent nonspecific binding sites since they were unchanged in the presence of excess unlabeled GRFa. The 26-kDa band was diminished in a concentration-dependent manner by unlabeled rat GRF, GRFa, and to a lesser extent by vasoactive intestinal peptide (VIP). It is unlikely, however, that GRFa was acting as a VIP receptor since the labeled analog did not induce prolactin secretion (VIP is a prolactin secretagogue). GRFa also increased cellular cAMP to levels similar to GRF and greater than VIP. Autoradiographs from gels run under nonreducing conditions revealed the 26-kDa band as the major species, indicating that, if a polymeric form of this binding protein exists, it does not involve disulfide linkages. Thus, the best candidate for the putative GRF receptor is the 26-kDa band. We have further demonstrated that the higher concentrations of DSS used previously (5 mM) result in diffuse autoradiograms with multiple bands, suggesting that caution should be exercised when interpreting cross-linking data under these conditions.

The hypothalamic neuropeptide GRF is a member of the glucagon/secretin/VIP family and is the primary stimulus for GH release from the somatotroph of the anterior pituitary (AP) (1-3). The human-derived peptide exists primarily in a 44- and 40-residue form and both possess similar biological activity (3-5). Mechanistically, GRF induces GH secretion in a cAMP- and calcium-dependent manner (1, 6, 7) and the regulation of GRF receptor-adenylate cyclase coupling is thought to involve GTP-binding regulatory proteins (8, 9). However, the chemical nature of the GRF receptor remains unclear. Initial characterization of GRF binding to the cell surface has shown that binding sites exist in bovine (10) and rat (11) AP cells with Kᵦ values of 3 nM and 41 pM, respectively. Furthermore, GRF binding to rat AP cells is influenced by glucocorticoids (11) and can undergo down regulation (12). Other studies indicate that GRF can act at VIP receptors in intestinal epithelium (13, 14) and pancreas (15), while VIP is known to be a weak GRF agonist in the AP (16). The concentrations of each hormone required for these cross-receptor interactions, however, exceed physiologic levels and are undoubtedly related to the homology of these peptides (14).

Recently, the molecular mass of the putative GRF receptor was reported to be 75 KDa based on chemical cross-linking of [125I]-hGRF(1-40)-hydroxide (hGRF) to rat AP cells (17). In the present study, we have cross-linked a biologically active, radioiodinated GRF analog (GRFa) to cultured rat AP cells. The results of our work indicate a lower molecular weight binding site and suggest that the 75-kDa protein is BSA. This discrepancy and the difficulties involved in interpreting cross-linking data are considered.

EXPERIMENTAL PROCEDURES

Preparation of Iodinated Peptides—GRFa, hGRF (kindly provided by Drs. J. Rivier and W. Vale, The Salk Institute, La Jolla, CA) and VIP (Peninsula Laboratories) were radioiodinated by the chloramine-T method (18) and purified by reverse phase HPLC on an Ultraphase ODS column. Labeled compounds were diluted in phosphate-buffered saline and used in cross-linking experiments at a concentration of approximately 1 nM (100 Ci/mg).

Preparation of Cultured Anterior Pituitary Cells—Cells were obtained from freshly dissected AP of male Sprague-Dawley rats (180-220 g, body weight, Hilltop Lab Animals, Inc.) and dispersed according to our established procedure (1, 8, 19). Dispersed cells were seeded into 24-well plates (Primaria, Falcon) and maintained with RPMI 1640 medium supplemented with 7.5% horse serum, 2.5% fetal bovine serum (all from Gibco), and antibiotics.

GRFa-Cell Cross-linking—Three days after plating, cells were washed 3 times (1 h each) with bicarbonate- and serum-free RPMI 1640 medium containing 25 mM Hepes (Sigma) and antibiotics. After the third wash, radioiodinated peptide with or without unlabeled hormone was added to each well. Binding was allowed to occur for 20 min, at which time the cross-linker DSS (Pierce Chemical Co.) was added. The cross-linking reaction was run for 2-20 min at 22 °C and was stopped by the addition of 40 mM glycine. After removing the

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1 The abbreviations used are: GRF, growth hormone releasing factor; rGRF and hGRF, rat and human growth hormone releasing factor, respectively. GRFa, [His⁴, Nle¹⁵]-rGRF(1-32)-NH₂; Nle, norleucine; BSA, bovine serum albumin; AP, anterior pituitary; DSS, disuccinimidyl suberate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography.
medium from each well, the cells were solubilized in Nonidet P-40 (Sigma) and Laemmli sample buffer as described by Wood and O’Dorisio (20).

Electrophoresis and Autoradiography—Solubilized cell extracts were applied to 10% Laemmli gels (21) and run at 20 mA/gel. Gels were then fixed-stained and dried, followed by autoradiography for 4–7 days with Kodak XAR-5 film in the presence of a Du Pont Cronex intensifier screen. Developed autoradiograms were analyzed on a Quickscan Jr. densitometer (Helena Laboratories) at 525 nm.

Reverse Hemolytic Plaque Assay—Plated cells were prepared for the hemolytic plaque assay essentially as described by Anderson et al. (22) using the method of Frawley and Neill (23) with modifications (24). Briefly, normal rat AP cells were dispersed with trypsin (0.1%, Cooper Biomedical) and adjusted to a concentration of 300,000 cells/ml. These cells were mixed with 1 ml of protein-A-coupled ox erythrocytes (Colorado Serum) in Dulbecco’s minimal essential medium (GIBCO) containing 0.1% BSA and 20 μM HEPEs and loaded directly into a Cunningham chamber. After a 1-h attachment period, the chamber was washed with RPMI 1640 containing 10% heat-inactivated horse serum and antibiotics and then the cells were incubated for 20 h at 37 °C (95% O2, 5% CO2). Labeled or unlabeled hormone was then added with a 1:150 dilution of GH antibody (courtesy of Dr. J. D. Neill, University of Alabama, Birmingham, AL) or 1:60 dilution of prolactin antibody (AFP C238, National Hormone and Pituitary Program, Baltimore, MD) followed by incubation for 1 h at 37 °C. The cells were then incubated with Dulbecco’s minimal essential medium containing 0.1% BSA and guinea pig complement (1:50, Colorado Serum) for 15 min at 37 °C. Cells were fixed in 1% glutaraldehyde for 5 min at 4 °C, rinsed, stained, and air-dried. The circumference of a plaque was defined as the first line formed by a complete string of intact erythrocytes, taken back to a common background density (22). Fifty or more randomly chosen plaque areas were determined for each slide using a Zeiss Videoplan system (22).

Cellular Cyclic AMP and Protein Levels—Cyclic AMP was extracted from the plated AP cells with 0.1 N HCl and measured by radioimmunoassay (25). Proteins were measured by the method of Lowry et al. (26) using BSA (Sigma) as a standard.

RESULTS AND DISCUSSION

Chemical cross-linking has been used with considerable success to identify several peptide hormone receptors, including insulin (27), glucagon (29, 29), VIP (20, 30), and prolactin (31). Conditions for cross-linking vary according to the system under study, the nature of the cross-linker, and the specificity and biological activity of the probe.

In this study, we have used GRFa as a probe for the identification of GRF-binding sites. This radioiodinated analog has been reported to bind GRF-specific sites and induce GH secretion from the AP (11). Using the reverse hemolytic plaque assay, we confirmed the biological activity of both unlabeled and radioiodinated GRFa, and determined that this analog did not induce prolactin secretion (Table I). This was an important control because GRF has been reported to interact with VIP receptors which can stimulate prolactin release (13–16). Accordingly, as a positive control, we validated that VIP stimulates prolactin release in the hemolytic plaque assay (data not shown). In contrast, radioiodinated hGRF (at a comparable specific activity to GRFa) was virtually inactive as a GH secretagogue (Table I). GRFa also stimulates cellular cyclic AMP levels to an extent similar to that for hGRF and to a much higher degree than comparable concentrations of VIP (Table II). These data indicate that 125I-GRFa is a more suitable probe than 125I-hGRF for the study of the GRF receptor.

When AP cells were incubated with 125I-GRFa followed by cross-linking with low (0.1 mM) concentrations of DSS for 2 min, four different molecular weight species were apparent in autoradiograms from sodium dodecyl sulfate-polyacrylamide gels (Fig. 1, lane 4). A 75-kDa band was present only if BSA was included in the cell medium (Fig. 1, lanes 4–6). Indeed, incubation of 125I-GRFas with BSA in the absence of AP cells also yielded an identical 72- to 75-kDa band. Two other bands (50 and 30 kDa) were also detected in the presence of cells but were very faint. These bands probably represent nonspecific binding sites because they were preserved even when cells were co-incubated with excess, unlabeled hormone (Fig. 2A).

The fourth band (26 kDa) was the most prominent and decreased in intensity when excess (1 μM) unlabeled GRFas or human GRF was co-incubated with the AP cells (Fig. 1, lanes 1–6). The 26-kDa species also decreased in a concentration-dependent manner in response to unlabeled rat GRF (or to a significantly lesser extent) VIP (Fig. 2, A and H). It is not surprising that higher concentrations of VIP decreased the intensity of the GRFas band since VIP and rat GRF share considerable homology (12 of 32 amino acids) (32). Although VIP stimulates prolactin secretion from the AP, VIP can also act as a weak GRF agonist at concentrations greater than 10 nM (16). This correlates well with the results in Fig. 2B in which a significant decrease in the intensity of the 26-kDa band is only apparent if unlabeled VIP is co-incubated with 125I-GRFa at concentrations above 10 nM. Thus, in addition to the data from the reverse hemolytic plaque assay for prolactin, these results indicate that it is unlikely that the

### Table I

| Condition | Hormone measured | Mean plaque area (mm²) |
|-----------|------------------|------------------------|
| Control   | GH               | 5,400 ± 600            |
| 1 nM GRFa | GH               | 21,600 ± 1,600         |
| 10 nM GRFa| GH               | 30,650 ± 2,900         |
| 100 nM GRFAs | GH       | 36,500 ± 2,900        |
| Control   | PRL              | 5,070 ± 350            |
| 1 nM GRFa | PRL              | 5,280 ± 250            |
| 10 nM GRFa| PRL              | 4,940 ± 150            |
| 100 nM GRFAs | PRL       | 5,330 ± 300           |
| Control   | hGRF             | 6,190 ± 180 (n = 2)    |
| 1 nM hGRFas | GH         | 3,500 ± 700 (n = 2)   |
| ~1 nM hGRFas | GH      | 9,030 ± 580 (n = 2)  |
| Control   | PRL              | 5,670 ± 100 (n = 2)    |
| 125I-GRFa | PRL              | 4,580 ± 10 (n = 2)     |

### Table II

| Condition | Cyclic AMP (pmol/mg protein) |
|-----------|-----------------------------|
| Control   | 23.3 ± 1.0                  |
| 1 nM GRFas | 141 ± 6                    |
| 10 nM GRFas | 655 ± 51                   |
| Experiment 2 | Control | 17.2 ± 1.4                 |
| 10 nM hGRF(1–40) | 814 ± 66                  |
| 10 nM VIP | 75.8 ± 14.7                |
predominant GFRα binding is to VIP receptors. Somatostatin, which does not act at the GFR receptor (33, 34), had no effect on the 26-kDa band (Fig. 2, A and B).

The low molecular weight of the GFR-binding protein prompted us to ask if the 26-kDa species is a proteolytic fragment of a larger subunit and if a larger disulfide-linked binding complex exists. No such higher molecular weight bands were detected in autoradiograms when cell extracts were prepared in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin/ml) or if gels were run in the absence of sulfhydryl reducing agents, β-mercaptoethanol or dithiothreitol (data not shown). If a polymeric form of the GFR receptor exists, it is probably through noncovalent interactions.

The difference between the mass of the GFR-binding protein in our study (26 kDa) and the higher mass species (75 kDa) recently reported (17) led us to expose AP cells to the high concentrations of DSS and the long cross-linking time described in this earlier report. Under those conditions (5 mM DSS, 20-min cross-linker incubation), we obtained autoradiograms with a diffuse appearance (Fig. 3) reminiscent of the earlier study. Although the authors attributed the diffuse appearance of these patterns to the presence of glycoproteins, we think that excess cross-linker and longer incubation are responsible because much more distinct autoradiograms are evident at 0.1 mM DSS for 2 min (compare Figs. 1 and 2A to Fig. 3). Furthermore, with 5 mM DSS we resolved multiple bands both for 125I-GFRα and 125I-VIP (Fig. 3). As with GFRα, 125I-VIP also formed a 72- to 75-kDa complex only in the presence of BSA (Fig. 3, lane 7), confirming the earlier work of Wood and O’Dorisio (20). The multiple bands obtained at these higher concentrations of DSS are most likely due to nonspecific cross-linking of cellular proteins to the labeled hormone, although it is possible that smaller GFR-binding subunits may have cross-linked to form higher molecular weight complexes. In this regard it is important to note that,
in time course studies for cross-linking at 0.1 mM DSS, we observed an increase in the intensity of the 26-kDa GRFa band up to 30 min; no higher mass species was evident (data not shown). We have also observed that excess unlabeled GRFs can diminish the 75-kDa band under conditions of high cross-linker concentrations (Fig. 3, lane 4) and occasionally at 0.1 mM DSS as well (Fig. 1, lane 6). Because albumin is known to bind several circulating hormones, this interaction with GRF is not surprising.

Another possible explanation for the discrepancy in GRF-binding protein size is the use of different GRF probes. The data from the hemolytic plaque assay indicate that 125I-GRF (which exposes the 75-kDa binding site (17)) has very little biological activity at a specific activity comparable to 125I-GRFa (Table I). This may be due to the iodination in the human GRF peptide of Tyr-1 and the possible oxidation of Met-27 which could have compromised the binding and activity of this hormone (11). In contrast, the biological activity of 125I-GRFa was comparable to that of the unlabeled analog, confirming and extending the results of Seifert et al. (11).

Finally, it is always possible that the differences in these results are due to conditions which we have not recognized. However, acceptable current criteria for the identification of a specific GRF-binding site in AP cells have been fulfilled. These include the graded decrease in the labeling of the 26-kDa band with unlabeled peptides, and the appropriate rank order of potency (rGRF > GRFa > VIP). No effect by somatostatin-14) and biological activity. These results also illustrate the importance of proper conditions and controls for cross-linking, as well as the caution needed to interpret cross-linking data.

In conclusion, we provide evidence for a specific GRF-binding protein in AP cells. The bioactivity and specificity of the GRF analog and the conditions with which we have conducted the cross-linking experiments strongly suggest that this protein is the putative GRF receptor or a subunit thereof. Although the smaller size of this receptor is intriguing, it is not unique since the prolactin receptor is reported to have a mass of only 30 kDa (31). The isolation of this binding protein and subsequent reconstitution into an adenylate cyclase system will ultimately establish whether or not the 26-kDa species is capable itself of activating this second messenger system, thus fulfilling a requirement for a hormone receptor.

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FIG. 3. The effect of high cross-linker concentration on 125I-GRF and 125I-VIP binding to AP cells. Conditions for cross-linking are as described in Fig. 1 except that 5 mM DSS was added for 20 min after ligand binding. The concentrations of unlabeled ligand and BSA were the same as those in Fig. 1. The arrow indicates the position of the 26-kDa band.