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Immunoaffinity Isolation of Native Membrane Glucocorticoid Receptor from S-49^{++} Lymphoma Cells

Biochemical Characterization and Interaction with Hsp 70 and Hsp 90

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The membrane glucocorticoid receptor (mGR), previously correlated with glucocorticoid-induced lymphocytolytic competency, was purified under nondenaturing conditions from mGR-enriched mouse S-49 T lymphoma cells. Proteins were immunoaffinity batch adsorbed to BUGR-2 monoclonal antibody-coupled protein A Sepharose 4B beads, and elution by epitope competition was compared with standard denaturation procedures. Elution with BUGR-2 epitope peptides released multiple mGRs (42–150 kDa) and heat shock proteins 70 and 90, suggesting that mGR interacts with these protein chaperones under physiological conditions. The mGR-heat shock protein 90 interaction was inhibited by 1 μ*M* geldanamycin. Several other mGR binding partners were captured and most were dissociated from mGR by 0.6 *M* salt. Peptide maps of purified mGR displayed immunoreactive bands unique to mGR. Scatchard analysis estimated a k_d value of 239 n*M* and a B_{max} of 384 fmol/mg protein for mGR, compared to a k_d of 19.5 n*M* and a B_{max} of 90.3 fmol/mg protein for the intracellular GR (iGR). The rank order of affinities for mGR were RU-486 > dexamethasone > triamcinolone acetonide = aldosterone. Other steroids had no significant binding affinity. These results show that epitope-purified mGR on the plasma membrane of mouse lymphoma cells is similar but not identical to iGR.

Key Words: Membrane glucocorticoid receptor, epitope elution, heat shock proteins.

Introduction

Glucocorticoids have various biochemical, physiological, and pathological effects in virtually every organ system. The ability of glucocorticoids to inhibit growth and cause involution of immature lymphoid tissues *(1)*, and to evoke in vitro lymphocytolysis, are the basis of common treatment regimens for lymphoproliferative disorders. Although glucocorticoids have been successful in the treatment of certain kinds of leukemias and lymphomas, the mechanism of this lytic response is not clear. It is generally accepted that glucocorticoids and many other steroid hormones bind to a cytoplasmic/nuclear receptor, which triggers RNA-dependent protein synthesis. In this case, steroid receptors are ligand-activated transcriptional factors capable of coordinately inducing or repressing the expression of cell-specific target genes. However, not all effects of steroid hormones are believed to involve the cytoplasmic/nuclear steroid hormone receptor. Various groups have provided evidence for specific binding of several steroid hormones to sites on the plasma membrane [reviewed in *(2)*]. Evidence for membrane-initiated steroid hormone responses includes changes in neurosecretion or neuronal excitability that occur within seconds of steroid administration, when *de novo* protein synthesis is inhibited or when access to intracellular receptors has been blocked [reviewed in *(2)*]. These actions of steroids that occur independently of classical intracellular receptors may have important roles in the regulation of various physiological, pathological, and pharmacological effects.

Using both the murine S-49 and human CCRF-CEM cell lines, we have previously shown that there is a higher M_r glucocorticoid receptor (GR) located on the plasma membrane (mGR) of various leukemic and lymphoma cells and cell lines, and that there is a correlation between the presence of this mGR form and the ability of these cells to respond in vitro to the lymphocytolytic activity of glucocorticoids *(3–6)*. That removal of functional membrane proteins by mild trypsinization rendered these cells resistant to the apoptotic effect of glucocorticoids, suggested mGR's involvement in the cell death response *(7)*. The multiple molecular sizes of this mGR have been shown to be both similar (94–97 kDa) to that of the intracellular GR (iGR) and larger (> 125 kDa) *(8)*. In addition to binding

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glucocorticoids, this GR form is also recognized by both monoclonal and polyclonal antibodies (Abs) directed against different epitopes in the iGR molecule *(3,4,6,8)*. Because all leukemic patients do not respond positively to therapeutic glucocorticoids *(9,10)*, we have studied the quantity of mGR in malignant lymphocytes from pediatric leukemic patients, compared to lymphocytes from normal healthy individuals *(4)*. Fluorescence-activated cell sorting confirmed the variable quantities (some very high) of mGR on lymphocytes taken from individual leukemic patients, while very low amounts of mGR were always observed on lymphocytes taken from normal individuals.

The classification of the mGR protein as a modified form of the iGR has been a major point of controversy. Previous studies from our laboratory have shown that mGR exhibits both similarities to and differences from iGR; observed differences include molecular size and cellular localization *(3,4,6–8)*. On the other hand, both mGR and iGR are recognized by the three different anti-GR Abs tested, suggesting they have similar or identical epitopes *(11)*. Other similarities between mGR and iGR are that they both interact with glucocorticoid response element DNA *(11)*, exhibit several common proteolytic enzyme cleavage sites *(11)*, and can undergo phosphorylation *(11)*. Therefore, we postulate that mGR is a modified form of the iGR. Similarly, we have reported an immunological characterization of, and unique function for, a membrane form of the estrogen receptor *(12–14)*.

The identification of this mGR as a novel cell surface hormone receptor and its potential role in lymphocytic leukemia make it an interesting subject for further characterization. In this report we present the characteristics of immunoaffinity-purified native mGR, including the size, structure, and hormone-binding and specificity properties of this novel mGR isolated from mGR-enriched S-49 mouse lymphoma cells ($S\text{-}49^{++}$) compared to those of the classical iGR isolated from the same cells. We also demonstrate mGR interactions with other plasma membrane protein constituents, including the chaperonins heat shock protein (hsp) 70 and hsp 90.

Results

mGR-Protein Interactions-hsp 70 and hsp 90

Plasma membrane particulates extensively washed to rid them of possible cytosolic GR contaminants were extracted with detergent, and the extracted proteins incubated with anti-GR Ab (BUGR-2)-coupled protein A beads to capture membrane-resident GR. After further washing, the samples were eluted from the beads by boiling, resolved by SDS-PAGE, transblotted, and probed with either BUGR, anti-hsp 70 or anti-hsp 90. Figure 1 shows the results. Western analysis with BUGR-2 (lane 1) showed multiple immunoreactive bands ranging in size from 42 to 150 kDa (including a band corresponding to the traditional size of

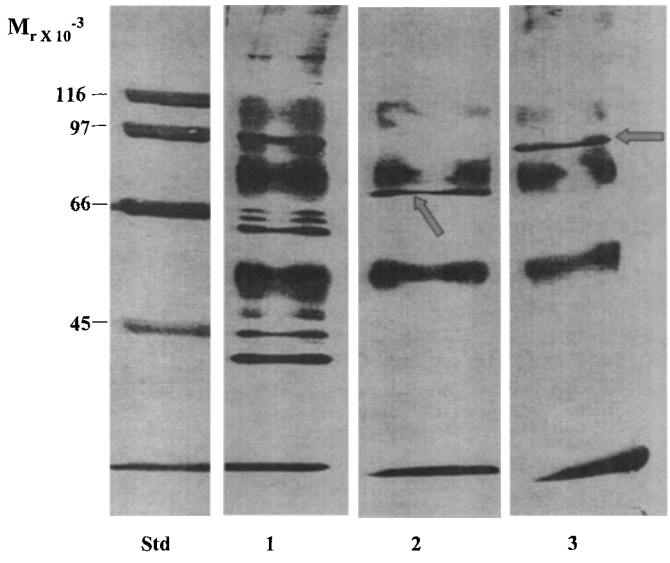

Fig. 1. Western blot analysis of immunoaffinity column-captured proteins eluted by denaturation. Captured membrane proteins were washed with buffer no. 4, boiled in buffer no. 5 for 5 min, and resolved by 7.5% SDS-PAGE before Western analysis with either BUGR-2 anti-GR (lane 1), anti-hsp 70 (lane 2) or anti-hsp 90 (lane 3) antibodies; std denotes M_r standards. Arrows in lanes 2 and 3 show hsp 70 and hsp 90, respectively.

GR [94–97 kDa]), and those matching our previous identifications of mGR (125 kDa and 150 kDa). Similar immunoidentification analysis with monoclonal antibodies (courtesy of Dr. David Toft) demonstrated hsp 70 (lane 2, 70 kDa), or hsp 90 (lane 3, 90 kDa), although other band sizes were also present. Incubation of plasma membrane particulates with protein A Sepharose 4B beads in the absence of BUGR-2 produced no immunoreactive bands when probed with any of the monoclonal Abs employed previously (data not shown). These data show that these heat shock proteins copurify with immuno-captured mGR. However, the multiplicity of bands suggested that either the harshness of the boiling treatment or stickiness of fragments to the beads were complicating the analysis of the proteins.

Therefore, to further confirm the likelihood of protein–protein interactions, to eliminate contaminating proteins that might stick to the beads and be shed during the harsh boiling conditions, and to assess more specifically the interactions between mGR and the hsp 70 and hsp 90 under more physiological conditions *(15)*, we employed epitope-based competitive elution for mGR. Western blot analyses of these preparations are shown in Fig. 2. The preparation probed with BUGR (lane 3) reveals a predominant mGR band at 94 kDa (doublet), as well as receptor bands at 42 kDa, 44 kDa, 125 kDa (doublet), and 150 kDa. Similar analyses employing monoclonal Abs to either hsp 70 or hsp 90 as probes showed much more simplified banding patterns for both hsp 70 (lane 2) and hsp 90 (lane 1). Therefore, hsp's 70 and 90 interact with and copurify with mGR.

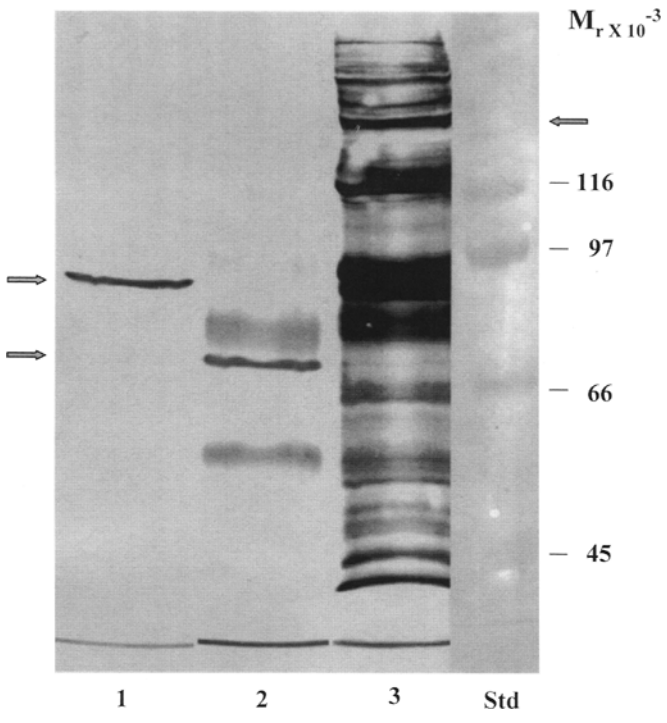

Fig. 2. Western blot analysis of immunoaffinity column-captured proteins eluted by epitope competition. Captured membrane proteins were washed with buffer no. 4, eluted with BUGR-2 epitope and resolved by 7.5% SDS-PAGE before Western analysis with either anti-hsp 90 (lane 1), anti-hsp 70 (lane 2), or BUGR-2 anti-GR (lane 3) antibodies; std denotes M_r standards. Arrows in lanes 1, 2, and 3 show hsp 90, hsp 70 and the 150 kDa mGR, respectively.

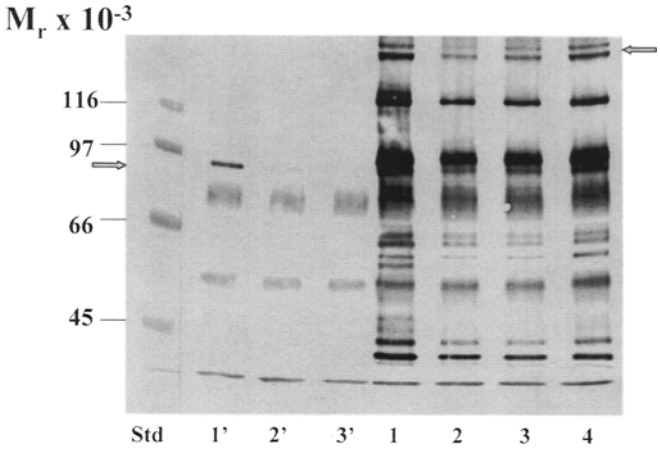

Fig. 3. Western blot analysis of proteins immuno-captured by BUGR-2. S-49^{++} cells were treated with either 1 μ*M* geldanamycin (lanes 2 and 2'), 5 μ*M* geldanamycin (lanes 3 and 3'), 10 μ*M* geldanamycin (lane 4) or vehicle (lanes 1 and 1') for 1 h at 37°C in roller bottles. Immuno-captured membrane proteins were washed with buffer no. 4, boiled in buffer no. 5 for 5 min, and resolved by 7.5% SDS-PAGE before Western analysis with either anti-hsp 90 (lanes 1'–3') or BUGR-2 anti-GR (lanes 1–4) antibodies; std denotes M_r standards. Arrow in lane 1' and 4 indicate hsp 90, and the 150 kDa mGR, respectively.

Inhibition of hsp 90-mGR Interaction by Geldanamycin

To examine the specificity of the mGR-hsp 90 interaction, cells were pretreated with either 1 μ*M*, 5 μ*M*, or 10 μ*M* geldanamycin for 1 h at 37°C in roller bottles and Western blot analysis was performed as described above, employing both BUGR-2 and anti-hsp 90 Abs as probes. Figure 3 shows the results. Immunoprecipitation of control plasma membrane particulates by BUGR-2-coupled protein A Sepharose 4B beads revealed both mGR and hsp 90 by Western blot (lanes 1 and 1', respectively). However, immunoprecipitation of geldanamycin-treated plasma membrane extracts revealed only mGR (lanes 2, 3, and 4); no hsp 90 was cross-immunoprecipitated (lanes 2' and 3'). These results show that the mGR-hsp 90 interaction can be inhibited by geldanamycin.

Epitope Specificity of Immuno-Capture and Salt Resistance of Accessory Protein Binding to mGR

To confirm the specificity of BUGR-2 based competitive elution of mGR from Ab-coupled protein A Sepharose beads, elution with a peptide not related to the BUGR-2 epitope was used as a negative control. Plasma membrane extracts from S-49^{++} cells were mixed with either BUGR-2 epitope or a nonspecific epitope (part of the human GR hinge region). These preparations were separately mixed with BUGR-2-coupled protein A beads, washed, eluted by boiling, and then resolved by SDS-PAGE. Further analysis by both Coomassie staining (right panel) and Western blot (left panel) is shown in Fig. 4. The BUGR-2 epitope successfully competed with mGR binding to BUGR-2, unlike the nonspecific epitope (compare lanes 1 and 2). Under these experimental conditions, the results also show that multiple high and low M_r peptides were co-captured with mGR (lane 2'); only some of these proteins were immunoreactive with BUGR (lane 2). To investigate the relative affinities of mGR for these co-immunoprecipitating proteins, we analyzed samples eluted after a more stringent buffer wash (0.6 *M* NaCl). Several of the nonimmunoreactive peptides disappeared after the high stringency buffer wash (lane 3'), compared to immunoreactive bands which resisted this wash (lane 3). These data suggest that mGR binds with higher affinity to BUGR than to its protein partners.

Affinity and Number of Receptor Binding Sites of mGR vs iGR

Cytosolic and plasma membrane preparations obtained from S-49^{++} cells were used to estimate receptor sites and dissociation constants according to the method of Scatchard *(16)*. Representative results for iGR (A & B) and mGR (C & D) are shown in Fig. 5 and are summarized in Table 1. Binding of labeled dexamethasone to mGR was successfully competed with 1–10 m*M* unlabeled dexamethasone at values averaging 60%, and this binding was saturable (Fig. 5D). The affinity of dexamethasone for this iGR is similar to that reported by others; the affinity of

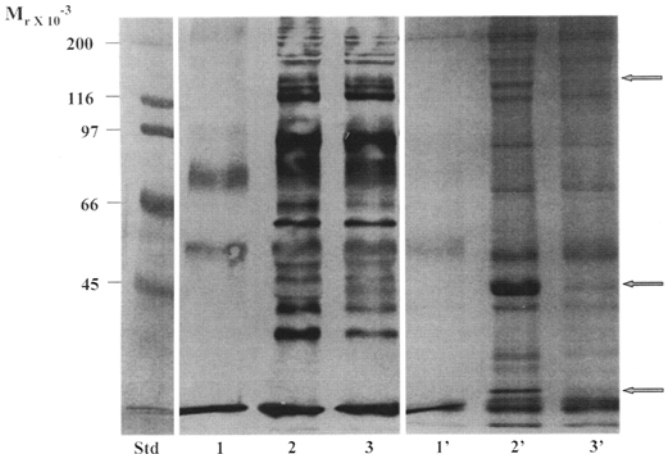

Fig. 4. Western blot analysis and Coomassie staining of proteins immuno-captured by BUGR-2 in the presence of either BUGR-2 epitope (lanes 1 and 1') or nonspecific (hinge region of human GR) epitope (lanes 2, 2', 3, and 3'). The beads were washed with buffer no. 4 containing either 0.3 *M* NaCl (lanes 1, 1', 2, and 2') or 0.6 *M* NaCl (lanes 3 and 3'), boiled in buffer no. 5 for 5 min, and resolved by 9% SDS-PAGE before Western analysis with BUGR-2 (lanes 1–3) or Coomassie staining (lanes 1'–3') of the gel; std denotes M_r standards. Arrows show nonimmunoreactive accessory proteins which are dissociated from mGR by 0.6 *M* NaCl wash.

dexamethasone for mGR was significantly less than that for the classical iGR isolated from the same cells (~16-fold). Therefore, purified mGR exhibits lower affinity for dexamethasone than does its intracellular counterpart in S-49^{++} cells, although the mGR density (per protein in the sample) is fourfold higher.

Steroid Specificity of mGR

The ability of various steroid hormones to displace [^{3}H]dexamethasone from mGR was used to assess the specificity of glucocorticoid binding to these plasma membrane binding sites. The steroid hormone receptor antagonist, RU-486, competed most efficiently for these binding sites, followed by dexamethasone, triamcinolone acetonide, and aldosterone (Fig. 6). Progesterone, 17β-estradiol, testosterone, hydrocortisone and retinoic acid were ineffective at displacing [^{3}H]dexamethasone from mGR.

Peptide Mapping of mGR vs iGR

Affinity-labeled cytosolic and plasma membrane preparations from S-49^{++} cells were first immunocaptured with BUGR-2-coupled protein A beads and then digested with α-chymotrypsin, trypsin, or V8 protease while still on the beads. Eluted peptides or fragments were resolved by SDS-PAGE and analyzed by Western blotting. Analysis of α-chymotrypsin (30 μg/mL)–treated mGR extracts revealed no significant differences between iGR and mGR

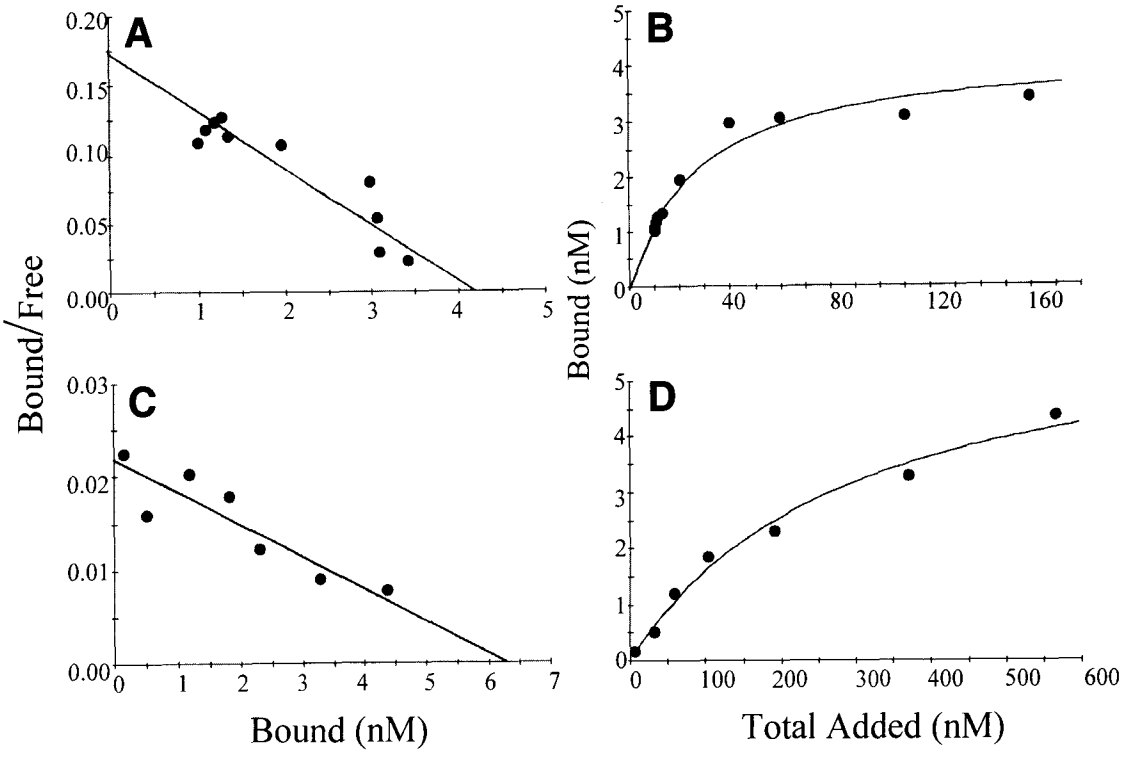

Fig. 5. Saturation binding (B and D) and Scatchard (A and C) analysis of dexamethasone binding to iGR (cytosolic, A and B) and mGR (plasma membrane, C and D) from S-49^{++} cells. Either cytosolic or plasma membrane extracts were incubated with increasing concentrations of [^{3}H]dexamethasone (50.0 Ci/mmol) at 4°C for 2 h in triplicate. Nonspecific binding was determined in the presence of either 1 μ*M* or 10 μ*M* unlabeled dexamethasone, respectively, and subtracted from total binding to give specific binding. Bound and free hormone were separated using 5% dextran-coated charcoal. Scatchard analysis was performed using the KELL computer program.

Table 1
Scatchard Analysis of Glucocorticoid Binding to mGR and iGR from S-49^{++} Cells[a]

GR	k_d (n*M*)	B_{max} (n*M*)	fmol/mg protein
mGR	239 ± 36*	5.03 ± 0.42	384 ± 34*
iGR	19.5 ± 4.4	4.90 ± 0.57	90.3 ± 12

[a]Saturation binding assays were performed by incubating either iGR or mGR preparations with increasing concentrations of [^{3}H]dexamethasone (50.0 Ci/mmol) at 4°C for 2 h in triplicate. Nonspecific binding was determined in the presence of either 1 μ*M* or 10 μ*M* unlabeled dexamethasone, respectively, and subtracted from total binding to give specific binding. Bound and free hormone were separated using 5% dextran-coated charcoal. K_d and B_{max} values were obtained by Scatchard analysis using the KELL computer program. Data presented are the means of three experiments ± SEM. *Significantly ($p < 0.05$) different from iGR.

as major immunoreactive peptide fragments of 42 kDa, 19 kDa, and 16 kDa were observed in both preparations (not shown). However, both trypsin (30 μg/mL) and V8 protease (40 μg/mL) produced unique immunoreactive mGR cleavage products of 32 kDa and 16kDa, respectively (Fig. 7, see arrows). In each case where a new smaller fragment appears, the absence of larger fragments in that digestion, compared to the undigested sample, indicates that a new cleavage site was used in the mGR sample. Note however, that the major immunoreactive fragments generated by these enzymes were identical, confirming their similarity over large portions of the sequence.

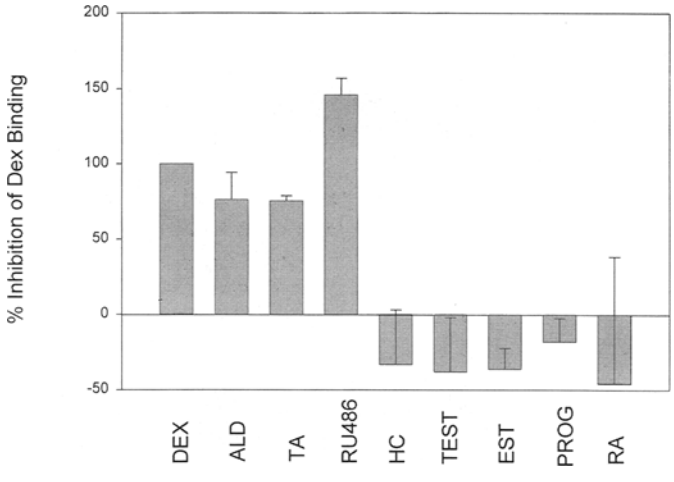

Fig. 6. Steroid binding specificity of mGR as revealed by competition with 100-fold excess concentrations of competing hormones. Plasma membrane preparations were labeled for 2 h at 4°C with 100 n*M* [^{3}H]dexamethasone ± 10 μ*M* unlabeled steroid hormones: DEX: dexamethasone; ALD: aldosterone; TA: triamcinolone acetonide; RU-486; HC: hydrocortisone; TEST: testosterone; EST: 17β-estradiol; PROG: progesterone; and RA: retinoic acid. Bound and free hormone were separated using 5% dextran-coated charcoal. The ability of a 100-fold excess of unlabeled dexamethasone to compete for [^{3}H]dexamethasone binding to mGR was taken as 100%, and the relative ability of the other steroids to compete was normalized to this value. Values are means of three experiments ± SEM.

Discussion

We have developed a novel immunoaffinity purification scheme in which mGR-containing extracts can be further purified by adsorption to BUGR-2-coupled protein A Sepharose beads, then competitively eluted with the BUGR epitope under nondenaturing conditions. The competitive epitope elution approach is not only an effective method of breaking antigen-Ab interactions, but also is preferable for studying protein–protein interactions, owing to the absence of harsh experimental conditions (high salt, sulphydryl reducing agents, heat, detergents) usually employed to remove proteins from their noncovalent attachment to Abs. Western blot analysis reveals eluted mGR at 94 kDa (doublet), 125 kDa (doublet), and 150 kDa, as well as probable fragments at 42 kDa and 44 kDa. This molecular size heteroge-

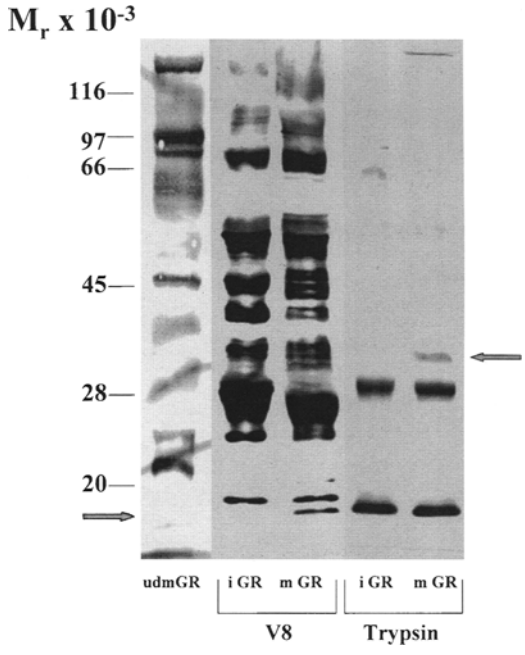

Fig. 7. Peptide maps of V8 protease and trypsin digestion products from intracellular glucocorticoid receptors (iGR) vs membrane glucocorticoid receptors (mGR). Cytosolic and plasma membrane preparations were immuno-captured on BUGR-2-coupled protein A beads, washed with buffer no. 4 and treated with either 40 μg/mL V8 protease for 90 min at 25°C or 30 μg/mL trypsin for 20 min at 10°C. The reactions were stopped by immediately freezing in dry ice or by the addition of a 10X concentration of soybean trypsin inhibitor, respectively. The beads were pelleted at 1000*g* and boiled in buffer no. 5 for 5 min. The denatured samples were then resolved on a 10% SDS-PAGE before Western analysis with BUGR-2 antibody. UdmGR represents undigested preparation. Arrows indicate the unique 16 kDa V8 protease-generated and 32 kDa trypsin-generated mGR fragments.

neity shows that mGR exists in these cells in various high molecular weight forms, and is highly susceptible to proteolytic enzymes (shown here experimentally) like its intracellular counterpart. Consistent with previous observations *(3,8)*, mGR is significantly larger in M_r than the iGR (150 kDa vs 94 kDa); these size differences have been suggested to be due to posttranslational modifications of mGR *(8)*. Peptide mapping with trypsin, α-chymotrypsin, and V8 protease was employed to study structural differences between mGR and iGR. Unique V8 protease and tryptic digestion products of mGR were observed, which suggests that either peptide sequence or a modification at the V8 protease and tryptic sites have been altered in mGR. It is possible that posttranslational modifications change the conformation of the modified protein and therefore expose or obscure proteolytic cleavage sites. Several examples have been reported where glycosylation and introduction of bulky carbohydrates impart protection against proteolysis via steric hindrance *(17–19)*. Glycosylation is a very likely candidate form of posttranslational modification, as many membrane proteins are glycoproteins. This hypothesis is strengthened by our previous observations of characteristic glycoprotein streaking in two-dimensional gels *(8)*. Although phosphorylation of mGR has also been observed *(11)*, such a M_r variation between mGR and iGR cannot be explained by this form of modification.

Is mGR a contamination of our membrane preparations with iGR? We think not for the following reasons. First, our membrane preparations are extensively washed and a similar technique used to prepare membranes for analysis of membrane estrogen receptor has shown membrane ER to be a completely unique size compared to intracellular ER *(13)*. Second, the commonly used mouse pituitary tumor cell line ATt-20 contains a high concentration of the intracellular (cytosolic/nuclear) GR, but we have not found any mGR in these cells using a variety of assays like immunocytochemistry *(3)*, FACS *(20)* and Western (unpublished observation from our lab) analyses, nor are they sensitive to glucocorticoid-induced lysis. However, if we stably transfect ATt-20 cells with the 1A splice variant of the GR cDNA, these cells express mGR by FACS analysis and also become sensitive to glucocorticoid-induced lymphocytolysis *(21,20)*. Based upon these data, we do not think that the high M_r mGR is the result of contamination of the membrane with cytoplasmic/nuclear receptor or nonspecific adsorption.

The epitope elution technique also represents a way of identifying protein partners for the mGR. Western blot analysis of BUGR epitope-eluted mGR with anti-hsp 70 and anti-hsp 90 monoclonal Abs show that hsp 70 and hsp 90 were cross-immuno-captured with mGR by BUGR-2-coupled protein A beads. Because neither hsp 70 nor hsp 90 is recognized by BUGR-2, this observation suggests that a protein–protein interaction between mGR and heat shock proteins may occur under physiological conditions. It is well known that hsp 70 and hsp 90 are chaperones for intracellular steroid hormone receptors, including iGR, and they have been proposed to function as regulators of receptor function *(22–25)*. However, this is the first evidence showing heat shock protein interactions with cell surface steroid receptors. While hsp 90 participates in multiple signal transduction pathways *(26)* and is vital for maintaining iGR in a conformation favorable for hormone binding *(22)*, hsp 70 has been shown to be a prerequisite for hsp 90 binding to iGR and activation of steroid-binding activity *(23,24)*. The role of hsp 70 and hsp 90 in relation to mGR is unclear at the present time, and we can only conjecture it to be similar to that observed with iGR *(22,25)*. Perhaps these chaperones are assisting mGR interactions with membrane constituents or with other proteins required in its signaling cascades. Several examples of heat shock protein heterocomplexes assisting in the assembly of supramolecular receptor structures containing protein kinases have been reported *(25,27,28)*. Such membrane-associated tyrosine kinases are involved in the signal transduction elicited following activation of cell surface receptors. Another possible role for heat shock proteins here is in transport of mGR to the plasma membrane. Hsp's protect lipophilic domains of proteins while conveying them to their membrane destinations, thus the name chaperonin. An example of this is the Rous sarcoma virus using hsp 90 to transport its tyrosine kinase to plasma membranes *(29)*.

Geldanamycin is a benzoquinone ansamycin, which binds specifically to hsp 90 and inhibits hsp 90 binding to steroid receptors *(30)*. This stable and pharmacologically specific interaction has been shown to disrupt iGR function in intact cells *(31)*. Geldanamycin was used here in an attempt to inhibit the hsp 90–mGR interaction. Geldanamycin at both 1 μ*M* and 5 μ*M* inhibited the cross-immunoprecipitation of hsp 90 by BUGR, suggesting the interaction between hsp 90 and mGR is similar to that observed between hsp 90 and iGR.

Nearly all of the captured proteins observed by Coomassie staining were successfully eluted by BUGR epitope inclusion with the partially purified mGR plasma membrane extract. Inclusion of a peptide representing a different epitope on the same protein was ineffective in releasing these proteins, suggesting that the eluting peptide did not act by steric hindrance or some other nonspecific biochemical activity, but that it acted by specifically interrupting a molecular recognition. Many other proteins were co-captured with mGR that were not recognized by our available Abs. Sorting out the identities of these protein bands as mGR cleavage products, or as actively associated proteins, needs careful analysis. For instance, several of the proteins observed by Coomassie staining were not observed on the Western blot. Only two of these nonimmunoreactive proteins were identified as the protein chaperones, hsp 70 and hsp 90; a more stringent wash with 0.6 *M* NaCl of the adsorbed protein A Sepharose 4B beads dissociated several

other lower molecular weight nonimmunoreactive proteins from mGR, suggesting they may also be accessory proteins to mGR. The specific role of these proteins and nature of interaction with mGR is unclear at the present; they may also be involved in the function of mGR.

Glucocorticoid binding affinity for mGR obtained from S-49^{++} cells was estimated to be 239 n*M*. The extracted mGR exhibited significantly less affinity for glucocorticoids than did the iGR from S-49^{++} cells. However, the mGR density was significantly greater than iGR density. The meaning and importance of the differences in glucocorticoid binding affinity and receptor density between mGR and iGR is unclear at the present time. These sample preparations are very different, and these differences may not reflect the relative concentration of GR in the cell membrane. It is also possible that the ligand most specific for the membrane version of GR has as yet not been tested. Additionally, one may not be certain whether the observed affinity and receptor number reflect the true value for mGR because of the possible influence of experimental manipulations on the protein. Detergents were employed to extract mGR and the actual separation of the receptor from its lipid environment in the membrane could alter its conformation and thus its steroid binding pocket. We currently know too little about the three-dimensional structure of membrane proteins and how much the membrane itself contributes to their conformation to conjecture further. However, the glucocorticoid binding affinity (k_d) for the mGRs here is in agreement with k_d values reported from the few other groups examining cell surface glucocorticoid receptors in the rat liver and brain (100 n*M* and 480 n*M*, respectively) *(32,33)*. An exception to this class of relatively lower affinity mGRs has been reported for amphibian neuronal membranes *(34)*. The relatively high affinity (k_d = 0.51 n*M*) of this mGR for glucocorticoids may be associated with it mediating rapid, stress-induced neuronal changes; this function may require more efficacy for glucocorticoids than the other reported mGRs.

Glucocorticoids are effective in treating certain leukemic patients, and this favorable clinical response is believed to be mediated through the GR *(1,10)*. The presence of mGR has been correlated with the apoptotic effect of glucocorticoids on both rodent and human leukemic cell models. The lower affinity of glucocorticoids for mGR could mean that pharmacological concentrations only obtainable with clinical treatment strategies are necessary for the therapeutic lymphocytolytic actions of glucocorticoids, consistent with the concentrations of glucocorticoids that are typically used to achieve apoptosis in in vitro systems.

Steroid binding specificity studies for mGR showed an order of displacement as follows: RU-486 > dexamethasone > triamcinolone acetonide = aldosterone. The steroid hormone receptor antagonist, RU-486 exhibited the highest affinity for mGR; synthetic receptor antagonists are well-known to exhibit higher binding affinities than their agonist counterparts. Triamcinolone acetonide, a synthetic glucocorticoid similar to dexamethasone, and aldosterone, a mineralocorticoid, exhibited similar affinity for this mGR, although they do not bind as well as dexamethasone. Surprisingly, hydrocortisone, a naturally occurring glucocorticoid with high affinity for iGR, had no affinity for mGR. The chemical structure of hydrocortisone is very similar to that of dexamethasone, lacking only the 1, 2-double bond, the 9α-fluoro substituent, and the 16α-methyl group. Therefore, one or more of these functional moieties may be required for binding of mGR to glucocorticoids. As expected, other steroid hormones, such as progesterone, 17β-estradiol, testosterone, and retinoic acid showed no significant displacement of glucocorticoids from mGR. The inability of nonadrenocorticoid hormones to displace [^{3}H]dexamethasone from mGR suggests a specificity in binding of this group of hormones to mGR and so implies that such binding is not the result of nonspecific partitioning of the steroid into the lipophilic plasma membrane.

In summary, the similarities between mGR and iGR include recognition by the anti-GR monoclonal Ab, BUGR-2, protease-generated immunoreactive fragments, binding specificity for adrenocorticoids, and interaction with the receptor chaperones, hsp 70 and hsp 90. Observed differences in mGR compared to iGR from S-49^{++} cells include cellular localization, a lesser affinity for glucocorticoids, a larger molecular size, lack of hydrocortisone binding, and generation of unique immunoreactive proteolytic fragments by trypsin and V8 protease. The inability to dissociate hsp 90 from mGR under high salt washing conditions is different from the phenomenon with iGR, where hsp 90-GR complexes dissociate at > 0.3 *M* salt *(25)*. The observed relatively high affinity interaction between mGR and the heat shock proteins may help to elucidate the role of mGR in the signal transduction pathways of glucocorticoid-evoked lymphocytolysis. These characteristics may help to explain some of the many unanswered questions about cell surface steroid hormone receptors.

Materials and Methods

Materials

The labeled steroids [^{3}H]dexamethasone and [^{3}H]dexamethasone 21-mesylate (50.0 Ci/mmol) were obtained from Dupont NEN (Boston, MA, USA). Unlabeled dexamethasone, triamcinolone acetonide, aldosterone, progesterone, hydrocortisone, β-estradiol, testosterone, retinoic acid, Nonidet P-40 (NP-40), RNase, trypsin, α-chymotrypsin, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DNase I, V8 protease (Endoproteinase Glu-C from *Staphylococcus aureus* V8) and protein A immobilized on Sepharose CL-4B beads were obtained from Boehringer Mannheim Corp. (Indianapolis, IN, USA). RU-38486 was a generous gift from Roussel-Uclaf (Romainville, France). Anti-hsp 70 *(35)* and anti-hsp

90 *(36)* monoclonal Abs were a kind gift from Dr. David Toft. Geldanamycin was provided by the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD). All other reagents were obtained from commercial sources as indicated. The BUGR-2 anti-mouse GR monoclonal Ab *(37)* was produced by culturing the hybridoma-producing cells in RPMI 1640 media and purifying the Ab from the medium supernatant on a protein A-Sepharose 4B affinity column *(37)*.

Cell Culture

S-49 cells were obtained from the ATCC (Bethesda, MD, USA) and grown in RPMI 1640 (Fisher Scientific, Pittsburgh, PA, USA) in the presence of 10% bovine calf serum (JRH Biosciences, Lenexa, KS, USA) at 37°C and 5% CO_2 in air. Using sequential cell-separation techniques previously described [immunopanning *(3)*, fluorescent cell sorting *(8,38)*, and finally, soft agar cloning *(39)*], stable mGR-enriched cell lines ($S\text{-}49^{++}$) were produced from the S-49 T lymphoma cells.

Buffers

The following buffers are referred to in the text by number: (no. 1) 20 m*M* (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]) sodium salt, 10 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, disodium salt, and 1 m*M* dithiothreitol, containing protease inhibitors (83 μg/mL aprotinin, 100 μg/mL bacitracin, 125 μg/mL trypsin inhibitor, 2 μg/mL pepstatin, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* diisopropylfluorophosphate), pH 7.6; (no. 2) 20 m*M* Tris-HCl, 140 m*M* NaCl, and 10 m*M* sodium molybdate, containing protease inhibitors (83 μg/mL aprotinin, 100 μg/mL bacitracin, 125 μg/mL trypsin inhibitor, 2 μg/mL pepstatin, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* diisopropylfluorophosphate), pH 8.2; (no. 3) buffer no. 2 containing 9.4% 10X DNase buffer (4 m*M* Tris-HCl, 10 m*M* NaCl, and 60 m*M* $MgCl_2$, pH 7.9), 0.85% NP-40, 1 μg/mL RNase, and 33 U/mL DNase I (grade I); (no. 4) 10 m*M* phosphate buffer, 300 m*M* NaCl, 2.7 m*M* KCl, and 0.025% NP-40, pH 7.4; (no.5) 62.5 m*M* Tris-HCl, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue, pH 6.8; (no. 6) 25 m*M* Tris base, 192 m*M* glycine, and 20% methanol (v/v); (no. 7) 20 m*M* Tris-HCl, 500 m*M* NaCl, pH 7.5; (no. 8) 20 m*M* Tris-HCl, 500 m*M* NaCl, 0.05% Tween-20, 1% gelatin, pH 7.5.

Preparation and Labeling of iGR

From 1 to 2×10^9 mGR-enriched cells ($S\text{-}49^{++}$) cells were harvested and washed with phosphate-buffered saline (PBS). The cells were resuspended in buffer no. 1 at a 1:1 packed cell volume to buffer ratio and disrupted at 4°C using a 50-W model ultrasonic processor (three cycles, 10 s each time; Sonics and Materials, Danbury, CT) with a 2-mm probe, with the output control set to 60-Hz voltage. The homogenate was centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant containing iGR was recovered and the protein concentration determined by the Bradford protein assay *(40)*. The cytosolic preparation was labeled with [^{3}H]dexamethasone 21-mesylate for 2 h at 4°C. Specific binding of extracted iGR was estimated by charcoal assay *(8,41)*.

Membrane Receptor Purification and Labeling

From 1 to 2×10^9 $S\text{-}49^{++}$ cells were harvested and washed with PBS. The cells were resuspended in buffer no. 2 at a 1:1 packed cell volume to buffer ratio and disrupted at 4°C using a 50-W model ultrasonic processor (three cycles, 10 s each time; Sonics and Materials, Danbury, CT) with a 2-mm probe, with the output control set to 60-Hz voltage. The nuclear component was removed by low-speed centrifugation (600 *g*, 15 min), and the supernatant was further centrifuged at 15,000*g* for 20 min at 4°C to pellet mitochondria and lysosomes. The remaining supernatant was centrifuged at 120,000 *g* for 2 h at 4°C, yielding a plasma membrane-containing pellet *(42)*. When necessary, the plasma membrane-containing pellet was washed with buffer no. 2 and centrifuged at 120,000 *g* for 90 min at 4°C to rid it of any cytosolic contaminants. The membrane protein was resuspended in approx 1 mL of buffer no. 3 and extracted by gently mixing the pellet (with a magnetic stirring bar) for 3 h at 4°C. Care was taken in resuspending the pellet so that most of the sticky, yellow particulate was left behind. The solution was ultracentrifuged at 120,000*g* for 30 min at 4°C, and the supernatant (containing extracted mGR) was recovered. The concentration of protein extracted was determined by the Bradford protein assay *(40)*. Specific glucocorticoid binding of extracted receptor was estimated by labeling the preparation with [^{3}H]dexamethasone 21-mesylate for 2 h at 4°C, followed by the charcoal assay *(8,41)*.

Immunoaffinity Chromatography/SDS-PAGE and Western Blot Analyses

BUGR-2 monoclonal anti-GR Ab was coupled to protein A immobilized on Sepharose CL-4B beads as previously described *(43,44)*. Labeled mGR and iGR preparations (obtained from an equal number of cells) were incubated with 1:5 BUGR-2-coupled protein A beads overnight at 4°C. For competition between the mGR preparations and epitope, a 10X solution of either BUGR-2 epitope (NH_2-SVFSNGYSSPGMRPDVS-COOH) or hinge region epitope (NH_2-CNLEARKTKKKIKGIQQAE-COOH) was prepared in buffer no. 2, pH adjusted to 8.2 with 1 *N* NaOH and added to the mGR preparations to give a final epitope concentration of 4 mg/mL immediately before immunocapture with 1:5 BUGR-2-coupled protein A beads. The following morning, the beads were washed four times with wash buffer (buffer no. 4, containing 0.6 *M* NaCl where indicated) by centrifugation at 1000*g* for 10 min at 4°C. For epitope elution, samples were incubated with 12 mg/mL

BUGR-2 epitope in buffer no. 2 (pH 8.2) for 4 h, then centrifuged at 1000*g* for 10 min at 4°C. The eluted samples or beads (when epitope elution was not performed) were boiled for 5 min in buffer no. 5 and centrifuged at 12,000*g* for 10 min in a microfuge (Beckman, Palo Alto, CA, USA). The preparations were subsequently resolved by electrophoresis on either 7.5%, 9%, or 10% SDS-PAGE where indicated, as described by Laemmli *(45)*. After electrophoresis, the protein was transferred to a nitrocellulose filter by applying 400 V h (in buffer no. 6) in an electrophoretic transfer apparatus *(46)*. The filters were blocked by soaking in 3% gelatin in buffer no. 7 for 30 min at room temperature, then incubated with either 1:50 of BUGR-2, 1:100 of anti-hsp 70 (BB70) or 1:300 of anti-hsp 90 (H90-10) in buffer no. 8 for 2 h. The filters were then processed for Western blot analysis as previously described *(3,46)*.

Inhibition of mGR-hsp 90 Interaction by Geldanamycin

S-49^{++} cells were pretreated with either 1 μ*M*, 5 μ*M* or 10 μ*M*, geldanamycin (dissolved in DMSO) or vehicle for 1 h at 37°C in roller bottles. After 1 h pretreatment, cells were harvested and washed with PBS. Plasma membrane particulates were isolated and Western blot analyses of mGR and hsp 90 performed as described above.

Scatchard Analysis

Binding kinetics for iGR and mGR were estimated by Scatchard analysis *(16)* with modifications by Rosenthal *(47)*. Cytosolic or plasma membrane preparations were prepared from S-49^{++} cells as described above and incubated with increasing concentrations of [^{3}H]dexamethasone (0.1–160 n*M* and 5–600 n*M*, respectively) at 4°C for 2 h in triplicate. Nonspecific binding was determined in the presence of either 1 μ*M* or 10 μ*M* unlabeled dexamethasone, respectively, and subtracted from total binding to give specific binding. Bound and free dexamethasone were separated using 5% dextran-coated charcoal as previously described *(8,41)*. [Bound] and [Free] were determined and binding affinity and receptor numbers were estimated employing the KELL computer software (BIOSOFT, Ferguson, MO).

Specificity of Binding for Different Steroid Hormones to mGR

Plasma membranes were prepared from S-49^{++} cells as described above and labeled for 2 h at 4°C with 100 n*M* [^{3}H]dexamethasone in the presence and absence of 100-fold excess concentrations of the following unlabeled steroid hormones: dexamethasone, aldosterone, triamcinolone acetonide, 17β-estradiol, progesterone, testosterone, hydrocortisone, and retinoic acid. The progestin antagonist, RU-486 (10 μ*M*) was also used to compete for [^{3}H]dexamethasone binding. After labeling, the preparations were treated with charcoal *(8,41)*, and specific binding of the post-charcoal supernatant was estimated by subtracting the values of radioactivity in competed samples from those of uncompeted preparations. The ability of each steroid to inhibit the binding of [^{3}H]dexamethasone to mGR is proportional to its binding affinity, and was calculated by taking the inhibition ability of unlabeled dexamethasone as 100%.

Peptide Maps of mGR vs iGR

Peptide maps were generated on immuno-captured receptor preparations (described above); the washed beads were treated with the protease. The preparations were digested with either α-chymotrypsin (30 μg/mL) or trypsin (30 μg/mL) at 10°C for 20 min or V8 protease (40 μg/mL) at 25°C for 90 min. The enzymatic reaction with trypsin was arrested by addition of a 10-fold excess concentration of soybean trypsin inhibitor; the reactions with α-chymotrypsin and V8 protease were arrested by immediately freezing on dry ice. The beads were then pelleted at 1000*g* before boiling in buffer no. 5. in preparation for electrophoresis.

Statistical Analyses

Statistical analyses were performed using a one-way ANOVA. A p value of < 0.05 was accepted as indicating a significant difference between groups.

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