Disruption of Raf-1/Heat Shock Protein 90 Complex and Raf Signaling by Dexamethasone in Mast Cells*

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Antigen stimulation of mast cells via the IgE receptor, FceRI, results in the recruitment of the cytosolic tyrosine kinase, Syk, and the activation of various signaling cascades. One of these, the extracellular signal-regulated kinase (ERK2) cascade, is inhibited by low concentrations of the immunosuppressant drug, dexamethasone, probably at a step prior to the activation of Raf-1 (Rider, L. G., Hirasawa, N., Santini, F., and Beaven, M. A. (1996) J. Immunol. 157, 2374–2380). We now show that treatment of cultured RBL-2H3 mast cells with nanomolar concentrations of dexamethasone causes dissociation of the Raf-1/heat shock protein 90 (Hsp90) complex. Raf-1 bereft of this protein fails to associate with the membrane or Ras in antigen-stimulated cells. Upstream events such as the Syk-dependent phosphorylation of Shc, the engagement of Shc with the adapter protein, Grb2, and the activation of Ras itself are unaffected. Interestingly, the counterpart of Raf-1 in the c-Jun N-terminal kinase (JNK) cascade, MEKK-1 (mitogen-activated protein kinase/ERK kinase), is similarly associated with Hsp90, and this association as well as the activation of MEKK-1 are disrupted by dexamethasone treatment. Disruption of the ERK and JNK cascades at the level of Raf-1 and MEKK-1 could account for the inhibitory action of dexamethasone on the generation of inflammatory mediators in stimulated mast cells.

The exposure of mast cells to anti-inflammatory glucocorticoids such as dexamethasone suppresses antigen-induced secretion of granules and de novo production of arachidonic acid-derived metabolites and inflammatory cytokines (see citations in Refs. 1 and 2). Phospholipase C and MAP1 kinase-related signaling events are also inhibited (2, 3). These responses, however, are not equally affected by glucocorticoids. Nanomolar concentrations of dexamethasone, for example, suppress activation of the ERK2 MAP kinase as well as the production of arachidonic acid and the cytokine, TNFα, but only partially inhibit phospholipase C-mediated events and degranulation (2, 4).

In investigating the mechanisms of actions of dexamethasone, we have focused on the ERK2 activation cascade (i.e. Ras → Raf-1 → MEK → ERK2) because production of both arachidonic acid via phospholipase A2 and TNFα in the RBL-2H3 mast cell line is dependent on the activation of this cascade (5). The phosphorylations of Raf-1, MEK, ERK2, and cytosolic phospholipase A2 are blocked in dexamethasone-treated cells whereas early antigen-induced events, such as the tyrosine phosphorylation of the IgE receptor (FceRI) and its associated tyrosine kinases, Lyn and Syk, are unaffected (2). Thus, dexamethasone appears to act at the level of, or at a step proximal to, the activation of Raf-1.

The events that link the antigen-induced activation of Syk to activation of Raf-1 include Syk-dependent tyrosine phosphorylation of the receptor docking protein, Shc, and the association of phosphorylated Shc with the adapter protein Grb2, which is constitutively associated with the Ras guanine nucleotide exchange factor, Sos (6). These associations lead to conversion of Ras to its active GTP-bound state (7) as well as the activation of Raf-1, MEK, and ERK2 (8, 9). As reported here, the phosphorylation of Shc, its association with Grb2, and the activation of Ras are not impaired in dexamethasone-treated cells even though activation of Raf-1, MEK, and ERK2 is blocked. Furthermore, Raf-1 fails to translocate from cytosol to membrane and associate with Ras possibly because dexamethasone disrupts binding of Hsp90 to the Raf-1 multimeric complex without affecting other proteins in this complex, namely 14-3-3, p50 (Key), and FKB65 (9, 10). Similar preliminary findings are reported for MEKK-1, the counterpart of Raf-1 in the JNK cascade. The findings identify a new site of action of dexamethasone and highlight the critical role of Hsp90 in MAP kinase signaling.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: dexamethasone from Sigma; 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) from Pierce; polyclonal antibodies against Raf-1, Src, MEKK-1, and SHIP and monoclonal antibodies against 14-3-3 were from Santa Cruz Biotechnology Inc.; monoclonal antibodies against Raf-1, Grb2, and Hsp90 were from Transduction Laboratories; other antibodies, kinase substrates, and Ras activation kit were from Upstate Biotechnology; enhanced chemiluminescence kit was from Amersham Pharmacia Biotech; radiolabeled products were from NEN Life Science Products. The antigen, DNP-BSA, 125I-IgE, and DNP-specific monoclonal IgE were gifts from Drs. Henry Metzger and Juan Rivera (National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH). Rabbit polyclonal antibodies against p50 (Key) and FKB65 were gifts of Dr. Gary Perdew (Department of Veterinary Science, Pennsylvania State University, University Park, PA) and Dr. Stephanie Simsek (National Cancer Institute, Frederick Cancer Research and Development Center, respectively).

Cell Culture and Experimental Procedures—Experiments were performed with RBL-2H3(m1) cells, a genetically engineered subline of RBL-2H3 cells made to express muscarinic m1 receptors (11). Cells were plated in 60-mm2 (5 × 105 cells/10 ml) or 145-mm2 (1 × 106 cells/30
ml) dishes in modified Eagle’s medium with Earle’s salts, supplemented with 15% fetal bovine serum. For examination of Shc phosphorylation and Ras activation, fetal bovine serum was omitted. Cultures were incubated (37°C) for 18 h with 0.5 μg/ml DNP-IgE to achieve 100% occupancy of FcεRI by IgE in the presence of dexamethasone or vehicle (dimethyl sulfoxide < 0.1%), which did not impair cell viability (>95% viable by trypan blue exclusion). The cultures were then washed with a glucose saline/PIPES buffer (5) and stimulated in the same buffer for 5 min or as indicated. Cultures were placed on ice, and all subsequent manipulations were performed with ice-cold reagents. Cells were washed with phosphate-buffered saline and then lysed (10 min) in 1.0 ml of the following buffers: A (25 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 12.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin), B (20 mM HEPES, pH 7.3, 1% Triton X-100, 10% glycerol, 12.5 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 30 μg/ml leupeptin, 30 μg/ml aprotinin, and 25 mM p-nitrophenyl phosphate), C (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 2 mM EDTA, and the protease/phosphatase inhibitors listed for buffer B), or D (25 mM HEPES, pH 8.0, 5 mM KCl, 119.4 mM NaCl, 1 mM MgCl2, 0.5 mM CaCl2, 5.6 mM glucose, 1% Nonidet P-40, and the protease/phosphatase inhibitors listed for buffer A). For Western blot analysis, in vitro kinase assays, and cell fractionation, cells were lysed in buffer A, B, or C, respectively. For chemical cross-linking experiments, cells were lysed in buffer D before addition of 2 mM DTSSP and, 20 min later, 40 mM KCl to terminate the reaction. Proteins were immunoprecipitated from this mixture as described below.

Cell Fractionation—Cell lysates in buffer C were homogenized in a Dounce homogenizer (~20 strokes) and centrifuged at 500 × g for 5 min to remove nuclei and unbroken cells. Samples of equivalent protein content (Bio-Rad protein assay kit) were centrifuged at 100,000 × g for 45 min to separate the soluble (cytosolic fraction) and insoluble fractions. The latter fraction was resuspended in 1 ml of buffer C, supplemented with 1% Triton X-100, rehomogenized (~5 strokes), and centrifuged at 100,000 × g for 45 min to obtain the solubilized membrane fraction.

Immunoblotting—Whole cell lysates in buffer A were clarified by centrifugation at 500 × g (5 min) for immunoprecipitation. Samples of equivalent protein content of the clarified lysates as well as the cytosolic and membrane fractions described above were incubated briefly with agarose beads before overnight incubation with the appropriate polyclonal antibody. Samples were then incubated for a further 2 h with Protein A-agarose beads. The beads were washed 4 times with buffer A and dissolved in 35 μl of Laemmli buffer (12). Proteins were separated by SDS-PAGE. Blots were probed with the indicated primary antibodies and peroxidase-labeled secondary antibodies and visualized by chemiluminescence.

In Vitro Kinase Assays—Raf-1, MEK, and ERK were immunoprecipitated from whole cell lysates (5 × 106 cells) or cytosolic/membrane fractions (from 15 × 106 cells) as described above except that immunoprecipitates were collected after 2 h of incubation with the required antibody. They were then washed four times with buffer B (diluted with an equal volume of phosphate-buffered saline) and once in a MOPS buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). The assay mixture consisted of immunoprecipitate, [γ-32P]ATP (10 μCi in 150 μl cold ATP), 25 mM MgCl2, substrate, and the MOPS buffer (final volume 35 μl). The substrates used were 15 μM inactive mouse MAP kinase kinase-GST (ERK-2-GST; for MEK activity), 45 μM inactive mouse p24 MAP kinase-GST (ERK-2-GST; for MEK activity), or 70 μM myelin basic protein peptide (for ERK activity). The mixtures were incubated at 30°C for 30 min (for Raf-1 and MEK activities) or 12 min (for ERK activity) and then solubilized in 35 μl of Laemmli buffer. The 32P-labeled proteins were separated by SDS-PAGE for autoradiographic detection.

RESULTS AND DISCUSSION

Dexamethasone Inhibits Activation of Raf-1, MEK, and ERK-2 but Not Upstream Events—Exposure of RBL-2H3(m1) cells to 100 nM dexamethasone for 18 h resulted in almost complete suppression of the antigen-induced activation of Raf-1, MEK, and ERK as determined by in vitro assay of the immunoprecipitated kinases from cell extracts (Fig. 1A). Initial experiments had indicated that suppression of Raf activation was time-dependent (data not shown but see later figure) and was prevented by co-treatment with the glucocorticoid receptor antagonist, RU-486 (1 μM) to indicate the probable involvement of this receptor (13). The activation of Raf-1 was blocked whether cells were stimulated with antigen, the calcium-mobilizing agent, thapsigargin, the protein kinase C stimulant, phorbol 12-myristate 13-acetate, or the muscarinic m1 agonist, carbachol (14, 15) (Fig. 1B). These results indicated a common point of convergence for these various stimulants, inhibitable by dexamethasone, for activation of Raf-1.

Upstream events of Raf-1 activation were unaffected by dexamethasone treatment. In addition to the previously noted lack of effect on antigen-induced tyrosine phosphorylation of FcεRI, Lyn, Syk, and the guanine nucleotide exchange factor, Vav (2), the tyrosine phosphorylation of Shc isoforms (p46hsc and p52hsc) and the increased association of Shc with Grb2 (6) were still apparent in dexamethasone-treated cells (Fig. 1C). Also, the tyrosine phosphorylation of SHIP that coimmunoprecipitates with Shc (16) was also unaffected (Fig. 1C). Thus, dexamethasone appeared to target a step proximal to Raf-1 activation.

Dexamethasone Disrupts the Hsp90-Raf-1 Complex and Raf-1 Signaling; Comparisons with Other Kinase-Hsp90 Complexes—Chemical cross-linking and immunoprecipitation studies revealed that the Raf-1-14-3-3-p50cdc37-FKBPD59/62Hsp90 complex remained intact in the dexamethasone-treated cells except for a loss of Hsp90. As shown in Fig. 2A, antigen stimulation by itself caused a consistent increase (~50%, mean of five experiments) in the amount of Hsp90 and a decrease (~45%, mean of three experiments) in the amount of 14-3-3 associated with the Raf-1 complex. In dexamethasone-treated cells, Raf-1 was partially bereft of Hsp90 and failed to show an increased association with Hsp90 following antigen stimulation. Dexamethasone, however, appeared not to affect the association of 14-3-3 with Raf-1 and its decrease upon antigen stimulation. The immunophilin, FKBPD59/62, and p50cdc37 (blots for p50cdc37 are shown in Fig. 2A) were unaffected by either antigen stimulation or dexamethasone treatment.

Dexamethasone acts through the glucocorticoid receptor in a time-dependent fashion (see Fig. 2D). However, no detectable change in expression of Raf-1 (see also Ref. 2), Hsp90, or any other Raf-1-associated proteins was evident by immunoblotting of extracts of dexamethasone-treated cells in three series of experiments (data not shown). Other Hsp90 complexes, namely complexes of Hsp90 with MEKK-1, Lyn, Src, and MEK were also examined (Fig. 2B). Hsp90 association with MEKK-1 has not been previously reported, but this enzyme is the hierarchical counterpart of Raf-1 in the JNK activation cascade, and its activation is suppressed in dexamethasone-treated RBL-2H3 cells (3). MEKK-1 was also associated with Hsp90, and this association was markedly inhibited by 100 nM dexamethasone. The other kinase complexes remained intact with the possible exception of the Src-Hsp90 complex, which showed a small (~25%) loss of Hsp90. Although such experiments provided no simple explanation for the loss of Hsp90 from Raf-1 and MEKK-1, dexamethasone acts presumably through induction or suppression of synthesis of a protein that regulates association of Hsp90 with Raf-1 and possibly MEKK-1.

The effects of dexamethasone were apparent at therapeutically relevant levels. Furthermore, the loss of Hsp90 from the Raf-1 complex correlated with the inhibition of antigen-stimulated Raf-1 activity whether dose (Fig. 2C) or time of exposure to dexamethasone (Fig. 2D) was varied. A 50% reduction of Hsp90 binding and Raf-1 activation was observed at, respectively, 6 and 7 nM dexamethasone (average IC50 values from three experiments). These values fall within the range of

D. S. Cissel and M. A. Beaver, unpublished data.
plasma levels (i.e. 10–20 nM) following therapeutic doses of dexamethasone in man (17). Maximal effects of dexamethasone occurred at about 15 h. These data suggested a possible link between loss of Hsp90 and impairment of Raf-1 activation. Although the role of Hsp90 and other Raf-1-interacting proteins in Raf-1 signaling require clarification (18), Hsp90 (19) is known to promote structural stability of a variety of proteins and to facilitate signal transduction (20).
role, Hsp90 binding to steroid hormone receptors permits ligand activation of the receptors, and its subsequent dissociation from the receptors permits them to become transcriptionally active (13). Activation of endothelial nitric-oxide synthase is also dependent on recruitment of Hsp90 to the enzyme complex in response to receptor stimulation (21). With respect to Raf-1, genetic studies in yeast (22) and Drosophila (23) as well as studies in mammalian cells with the Hsp90-binding agent, geldanamycin (24), suggest that Hsp90 is essential for Raf-1 stability and signaling. Geldanamycin blockade of Hsp90 binding to Raf-1 leads to degradation of Raf-1 and, as a consequence, disruption of Raf-1 signaling (24, 25). The actions of dexamethasone reported here differ in that Raf-1 signaling is still disrupted even though the levels of Raf-1 remain unchanged.

With respect to other Raf-1-associated proteins, 14-3-3 is thought to facilitate the unfolding and the interaction of Raf-1 with Ras with retention of 14-3-3 by Raf-1 in one model (26) and loss of 14-3-3 in another (27), the latter model being consistent with the present data. The effects of antigen and dexamethasone noted above raise the possibility that Hsp90, possibly in concert with 14-3-3, is also essential for successful transduction of signals by Raf-1. The role of the antigen/dexamethasone-insensitive proteins, p50cdc37 and FKBP65, in Raf-1 signaling are poorly understood.

Inhibition of Translocation of Raf-1 from Cytosolic to Membrane Fraction and Its Association with Ras—Studies with geldanamycin have suggested that Hsp90 is also essential for translocation of Raf-1 to the cell membrane (28). Assays of the cytosolic and membrane fractions of whole cell extracts (Fig. 3A) showed that Raf-1 activity was located largely in the cytosolic fraction in unstimulated cells. In antigen-stimulated cells, Raf-1 activity was increased substantially in both the cytosolic and membrane fractions although the major portion of the activity still remained in the cytosolic fraction. These increases were not apparent in dexamethasone-treated cells. Raf-1 immunoblots (Fig. 3A) showed that, like Raf-1 activity, Raf-1 protein was predominantly cytosolic, but following antigen stimulation, the amount of membrane-associated Raf-1 increased substantially, and this increase was blocked in dexamethasone-treated cells. Calculation of data from five experiments (values are noted in Fig. 3A) indicated a 2–3-fold increase in the specific activity of cytosolic Raf-1 following antigen stimulation even after correction for the contamination (≈15%) of cytosolic fractions by the plasma membrane marker, 125I-IgE-tagged FcεRI (29) (data not shown). In contrast to cytosolic Raf-1, the specific activity of membrane Raf-1 remained virtually unchanged and similar to that of cytosolic Raf-1 in stimulated cells, regardless of the state of stimulation or treatment with dexamethasone. The antigen-induced association of Raf-1 with Ras (Fig. 3B) was also impaired in dexamethasone-treated cells although Ras function, as indicated by its association with phosphatidylinositol 3'-kinase (data not shown) and a Raf-1 peptide that binds to activated Ras (Upstate Biotechnology, see legend to Fig. 3) appeared intact in both unstimulated and stimulated cells (Fig. 3C). In three experiments, the binding of Ras to Raf-1 peptide increased upon antigen stimulation and showed similar maximal increases at 1–2 min in both control (2.0 ± 0.5-fold, mean ± S.E.) and dexamethasone-treated (1.9 ± 0.3-fold) cells. Dexamethasone itself caused no significant increase in Ras binding activity in unstimulated cells (1.4 ± 0.3-fold) although an increase was observed in one of the three experiments. These results suggested that neither the constitutive activity of Ras in unstimulated cells nor the further stimulation of Ras in antigen-stimulated RBL-2H3 cells is impaired by treatment with dexamethasone.

The simplest interpretation of the above results is that Raf-1 is activated largely in the cytosol and then translocated to the membrane in its active form through recruitment by Ras. Current models suggest, however, that Raf-1 is translocated in its inactive form (9) and that both cytosolic and membrane-associated forms of Raf-1 may be activated in a Ras-independent and a Ras-dependent manner, respectively (30). Our data do not distinguish between these two possibilities, but the data suggest that treatment with dexamethasone blocks both activation and association of Raf-1 with Ras, that both processes are dependent on an intact Raf-1-Hsp90 complex, and that dissociation of this complex by treatment with dexamethasone accounts for the suppression of the ERK-2/phospholipase A2 activation pathway and ultimately the generation of arachidonic acid and TNFα by dexamethasone in RBL-2H3 cells (Ref. 2 and this paper).

It is apparent from the present and published studies that glucocorticoids can act via the glucocorticoid receptor to suppress transduction of signals from the plasma membrane to the
nucleus at several steps. Dexamethasone at 0.1 or 1.0 μM concentrations may negatively regulate various cytokine genes either directly, by blocking activity of the transcriptional factors AP-1 and NF-κB (see Ref. 31), or indirectly by inducing production of the inhibitory NF-κB protein, IκB (32, 33). In addition, dexamethasone is a potent suppressant of the ERK-2 (2) and JNK (3) MAP kinase pathways apparently because it impedes the proper assembly of Hsp90 with key kinases such as Raf-1 and the less well studied MEKK-1 (this paper). These pathways have been linked to activation of AP-1 (34), NF-AT (35), and NF-κB (36) in various types of cells, and inhibition of these pathways would likely influence production of a variety of cytokines. Production of TNFα (37) and interleukin-5 (38) in mast cells is reported to be dependent on NF-κB and NF-AT, respectively. In conclusion, the present results reveal an unsuspected action of dexamethasone and point to its possible use in elucidating the role of Hsp90 in Raf-1 and MEKK-1 signaling.

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