



Expression of a defective mouse mammary tumor virus envelope glycoprotein precursor which binds stably to GRP78 within the lumen of the endoplasmic reticulum is associated with decreased glucocorticoid-induced apoptosis in mouse lymphoma cells

Minh Lam¹, Michael Stallcup² and Clark W. Distelhorst^{1,3}

¹ Department of Medicine, Case Western Reserve University, Cleveland, Ohio, USA

² Departments of Pathology and Biochemistry, University of Southern California Health Sciences Center, Los Angeles, California 90033, USA

³ corresponding author: Dr Clark W. Distelhorst, Department of Medicine, School of Medicine (BRB 329), Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4937, USA. tel: 216-368-1175; fax: 216-368-1166; e-mail: cwd@po.cwru.edu

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Abstract

Viral proteins inhibit apoptosis in host cells by a variety of mechanisms. This report proposes an additional mechanism, based on the interaction of a mutant mouse mammary tumor virus (MMTV) envelope glycoprotein precursor, Pr74, with the stress protein GRP78 (BiP) within the lumen of the endoplasmic reticulum (ER) (*J. Biol. Chem.* 268 7482–7488, 1993). We show that WEHI7.2 (W7.2) mouse lymphoma cells, which do not express Pr74, are more sensitive to cell death induction by the glucocorticoid hormone dexamethasone (dex), than W7MG1 cells, which were derived by infecting W7.2 cells with MMTV and therefore express Pr74 under control of the glucocorticoid-inducible MMTV promoter. Moreover, W7-ENV/N cells, derived by stably transfecting W7.2 cells with a constitutively expressed cDNA encoding mutant Pr74, were less sensitive to dex-induced cell death than control transfectant W7-ENV/– cells. Among multiple W7-ENV/N subclones, susceptibility to dex-induced cell death was inversely related to the level of Pr74 synthesis. The interaction of Pr74 with GRP78 induces an increase in GRP78 synthesis. Thus, the repression of cell death associated with Pr74 expression may be secondary to elevated synthesis of GRP78, a stress protein previously implicated in protection against cell death.

Keywords: apoptosis; GRP78; mouse mammary tumor virus; viral envelope glycoprotein precursor

Abbreviations: ICE, interleukin-1 β converting enzyme; EBV, Epstein Barr virus; iap, inhibitor of apoptosis gene; MMTV, mouse mammary tumor virus; GRP78, 78 kDa glucose regulated protein; ER, endoplasmic reticulum; dex, dexamethasone; W7.2, WEHI7.2

Introduction

The decision of a cell to undergo apoptosis, or programmed cell death, can be initiated by a variety of intracellular and extracellular signals. Once initiated, the process of apoptosis is regulated by a genetically conserved array of counterbalancing factors, including proteins such as Bcl-2 that inhibit apoptosis, and proteases, such as homologues of interleukin 1 β -converting enzyme (ICE), that mediate cleavage of specific target proteins (Steller, 1995; White, 1996). Cell death by apoptosis is important for normal development and homeostasis, and serves as a mechanism by which individual cells eliminate themselves for the overall good of the organism.

In lymphocytes and other types of cells apoptosis can be triggered by viruses, including, for example, human immunodeficiency virus-type 1 (Li *et al.*, 1995; Maldarelli *et al.*, 1995; Westendorp *et al.*, 1995), human T lymphocyte virus 1 (Leno *et al.*, 1995), adenovirus (Chiou *et al.*, 1994), and varicella-zoster virus (Sadzot-Delvaux *et al.*, 1995). The host apoptotic response contributes to the cytopathic effect of viruses, but also provides protection against viral infection by limiting viral replication (Clem and Miller, 1993). However, many viruses subvert the host defense mechanism by encoding proteins that inhibit apoptosis, thereby facilitating viral replication, spread, or persistence (Shen and Shenk, 1995). A number of anti-apoptotic viral proteins have been identified that inhibit apoptosis by various mechanisms. For example, Cowpox virus crmA protein and baculovirus p35 protein inhibit ICE and ICE-like proteases, respectively (Tewari and Dixit, 1995; Clem and Miller, 1993; Hershberger *et al.*, 1994). The Epstein-Barr virus (EBV) LMP1 protein upregulates Bcl-2 expression (Okan *et al.*, 1995), whereas the EBV BHRF-1 protein is a member of the Bcl-2 family of proteins and appears to inhibit apoptosis by a mechanism similar to Bcl-2 (Henderson *et al.*, 1993). Other members of the Bcl-2 family include the adenovirus E1B 19K protein and a Bcl-2 homologue encoded by the African Swine Fever virus (Chiou *et al.*, 1994; Neilan *et al.*, 1993). Finally, an antiapoptotic protein encoded by the baculovirus inhibitor of apoptosis (iap) gene contains a zinc finger motif and presumably acts as a transcription factor (Birnbaum *et al.*, 1994).

In the present report, we propose an additional mechanism by which a naturally occurring mutant viral protein may inhibit apoptosis, involving interaction of the viral protein with GRP78 (BiP), a stress protein implicated in protection against cell death (Little and Lee, 1994). Two previously characterized derivatives of the glucocorticoid-

sensitive WEHI7.2 (W7.2) T cell mouse lymphoma line, W7.MG1 and W7-ENV/N, are employed in this study. W7.MG1 cells were derived by infecting W7.2 cells with mouse mammary tumor virus (MMTV) (Danielsen *et al*, 1983) and express a mutant MMTV envelope glycoprotein precursor Pr74 that is not processed normally, but retained within the ER (Byravan and Stallcup, 1990). W7-ENV/N cells were derived from W7.2 cells by retroviral mediated transfer of a sequence encoding the mutant, nonprocessed form of Pr74 (Bedgood *et al*, 1992; Corey and Stallcup, 1992). In an earlier study, we found that mutant Pr74 expressed in both W7.MG1 and W7-ENV/N cells binds stably to GRP78 within the lumen of the endoplasmic reticulum (ER), thereby inducing an increase in GRP78 synthesis (Ulatowski *et al*, 1993). Because elevated levels of GRP78 have been correlated with resistance to apoptosis (Shen *et al*, 1987; Hughes *et al*, 1989; Sugawara *et al*, 1990; Li and Lee, 1991; Gomer *et al*, 1991; Li *et al*, 1992), the present studies were undertaken to determine if expression of mutant Pr74 in W7.2 mouse lymphoma cells is associated with reduced sensitivity of lymphoma cells to glucocorticoid-induced apoptosis.

Results

The W7.2 and W7.MG1 lines, like all BALB/c mouse derived cell lines, contain MMTV sequences stably integrated within the genome (Horibata and Harris, 1970). Therefore, to determine the expression level of the MMTV glycoprotein precursor, Pr74, dex-treated and untreated cells were labeled with [³⁵S]methionine and Pr74 was immunoprecipitated from cell extracts (Figure 1). These findings indicate that Pr74 expression was not detected in W7.2 cells, whereas both basal and dex-inducible expression of Pr74 were detected in W7MG1 cells. To determine if Pr74 expression affects cell survival, cells were treated with a range of concentrations of dex, followed by assessment of viability (i.e. trypan blue dye exclusion) at periodic time intervals. Comparison of the death response in W7.2 cells versus W7MG1 cells over a broad range of dex concentrations showed W7MG1 cells to be significantly less susceptible to dex-induced cell death (Figure 2). Cell death following dex treatment of W7.2 and W7MG1 cells is associated with nuclear morphological changes typical of apoptosis (Lam *et al*, 1993, 1994). Consistent with the data shown in Figure 2, apoptotic morphological changes induced by dex were much more frequent in W7.2 cells than in

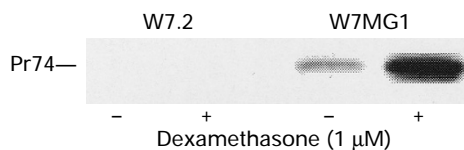


Figure 1 Differential expression of Pr74 in W7.2 and W7MG1 cells. Cells were incubated with or without 1 μM dex for 4 h and then pulse-labeled with [³⁵S]methionine. Pr74 was immunoprecipitated from cell extracts containing equivalent amounts of trichloroacetic acid precipitable radioactivity and was subjected to SDS-gel electrophoresis, followed by autoradiography. Shown is an autoradiogram representative of multiple experiments.

W7MG1 cells (data not shown). Thus, the data in Figure 2 are consistent with the hypothesis that expression of mutant Pr74 inhibits apoptosis induction.

One of the earliest detectable events in cells destined to die in response to glucocorticoid treatment is a generalized repression of protein synthesis (Borthwick, 1978). To determine the effect of Pr74 expression on the repression of protein synthesis by dex, W7.2 and W7.MG1 cells were pretreated with dex for 4 h prior to measuring the incorporation of [³⁵S]methionine into newly synthesized protein (Table 1). A significant decrease in [³⁵S]methionine

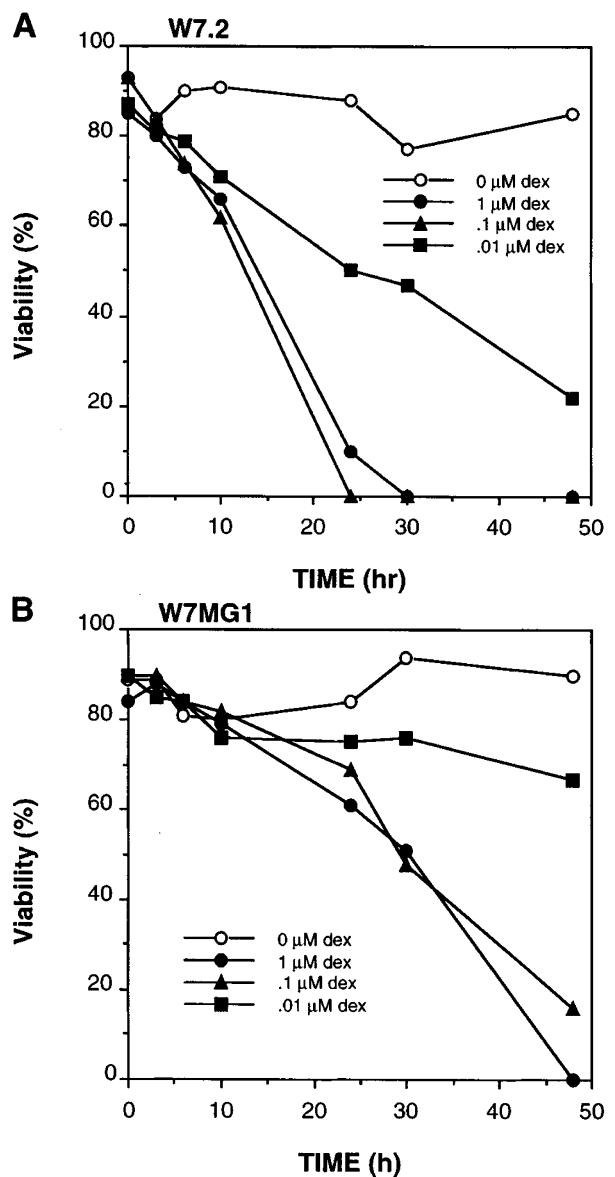


Figure 2 Effect of dex on viability of W7.2 and W7MG1 cells. Cells were cultured with or without 0.01–1 μM dex and, at various time points, cell viability was measured by trypan blue dye exclusion. Symbols are the mean of duplicate determinations for a single experiment that is representative of five experiments.

incorporation was detected in W7.2 cells but not in W7MG1 cells following dex treatment. These data confirm that dex has a greater catabolic effect in W7.2 cells than in W7MG1 cells. One factor known to regulate the sensitivity of lymphoid cells to glucocorticoids is glucocorticoid receptor content (Vanderbilt *et al*, 1987). W7.2 and W7MG1 cells have similar glucocorticoid receptor levels (Table 2).

Table 1 Effect of dexamethasone treatment on protein synthesis

dex	W7.2	W7MG1	W7ENV/-	W7-ENV/N
	[³⁵ S]methionine incorporation (cpm/million cells)			
-	149 800	167 600	491 300	509 300
	± 8500	± 12 500	± 16 400	± 200
+	99 800	159 500	379 000	512 800
	± 18 000	± 21 500	± 6500	± 12 200
± × 100	66%	95%	77%	100%

Cells were incubated with or without 1 μM dexamethasone (dex) for 4 h prior to pulse-labeling with [³⁵S]methionine. The incorporation of [³⁵S]methionine into protein was quantified by measuring trichloroacetic acid precipitable radioactivity. Results represent the mean ± S.E. for four separate experiments.

Table 2 Glucocorticoid receptor levels

Cell line	Specific binding
	dpm/40 million cells (mean ± S.E.)
W7.2	22 090 ± 1898
W7MG1	16 129 ± 4153
W7-ENV/-	15 534 ± 1118
W7-ENV/N	17 884 ± 413

The specific binding of [³H]triamcinolone was measured by a standard whole cell competitive binding assay in which cells were incubated with a concentration of [³H]triamcinolone sufficient to saturate receptor sites, in the presence or absence of 100-fold excess unlabeled hormone.

Therefore, the differential sensitivity to glucocorticoids does not lie at the glucocorticoid receptor level.

The preceding findings suggest that Pr74 expression may protect cells against the catabolic action of glucocorticoids. To test this hypothesis, we compared the dex sensitivity of two additional W7.2 derivatives, W7-ENV/N and W7-ENV/-, prepared by stable transfection of W7.2 cells with a retroviral vector encoding Pr74 or an empty retroviral vector respectively (Bedgood *et al*, 1992). We showed previously that W7-ENV/N cells constitutively express defective Pr74 and that nascent Pr74 accumulates within the ER in stable association with GRP78 (Ulatowski *et al*, 1993). Here we show that W7-ENV/N cells were less sensitive than W7-ENV/- cells to dex-induced repression of protein synthesis (Table 1) and cell death (Figure 3). The difference in dex sensitivity was not regulated at the level of the glucocorticoid receptor, as W7-ENV/N and W7-ENV/- cells have the same intracellular concentration of glucocorticoid receptors (Table 2). Analysis of multiple W7-ENV/N subclones reveals an inverse relationship between the level of Pr74 synthesis and sensitivity to dex-induced cell death (Figure 4). These data confirm that increased expression of mutant Pr74 is associated with decreased sensitivity to dex-induced cell death.

Discussion

Viruses subvert the apoptotic program of mammalian cells by a variety of mechanisms (Shen and Shenk, 1995). The observations reported here suggest an additional mechanism, involving synthesis of a naturally occurring, defective viral protein that accumulates within the ER in stable association with GRP78. The evidence is based on an inverse correlation between the level of expression of a defective MMTV

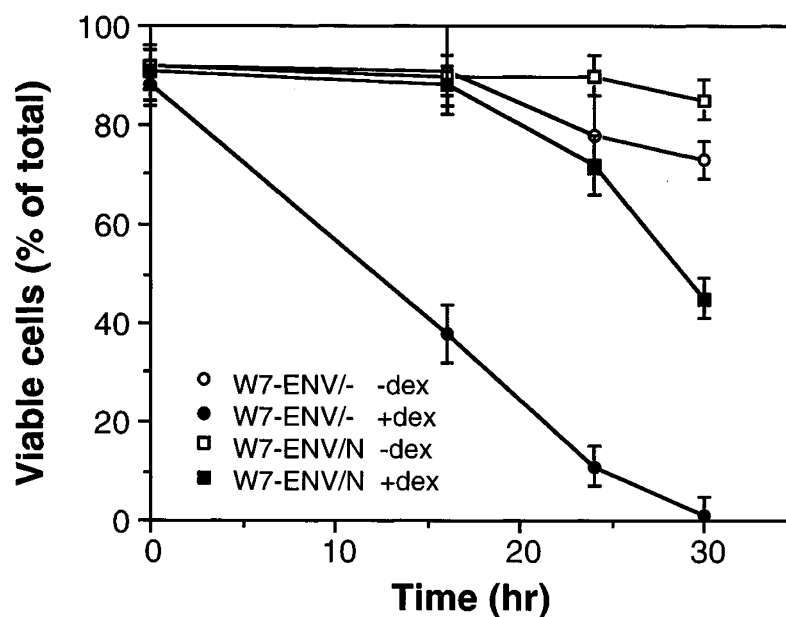


Figure 3 Effect of dex on viability of W7-ENV/- and W7-ENV/N cells. Cells were cultured with or without 1 μM dex and cell viability was measured at various times by Trypan blue dye exclusion. Symbols are the mean ± S.E. of three experiments.

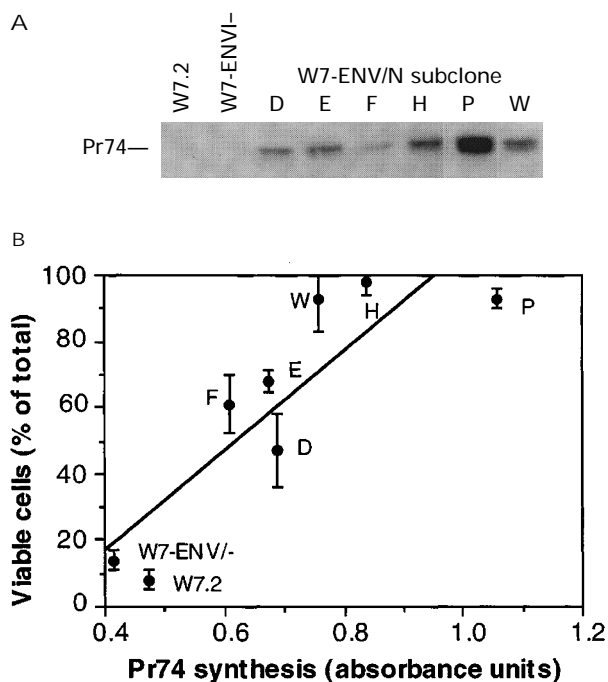


Figure 4 Relationship between dex-induced cell death and levels of nascent Pr74. (A) W7.2 cells, W7-ENV/- cells and W7-ENV/N subclones were pulse labeled with [35 S]methionine and then Pr74 was immunoprecipitated from cell extracts containing equivalent amounts of trichloroacetic acid precipitable radioactivity. Immunoprecipitated proteins were subjected to SDS-PAGE, followed by autoradiography. (B) Cells were incubated for 24 h in the presence or absence of 1 μ M dex prior to measuring cell viability by Trypan blue dye exclusion. Relative levels of nascent Pr74 were determined by scanning densitometry of the autoradiogram shown in (A) and plotted versus cell viability. Symbols represent mean \pm S.E. cell viability in three experiments.

envelope glycoprotein precursor, Pr74, and the susceptibility of lymphoid cells to glucocorticoid-induced apoptosis. How the stable interaction between Pr74 and GRP78 reduces the susceptibility of mouse lymphoma cells to glucocorticoid-induced apoptosis is currently unknown. One possibility is that protection against apoptosis is mediated through elevated synthesis of GRP78, which occurs secondary to the stable interaction between Pr74 and GRP78 within the ER lumen. Indeed, we reported previously a direct relationship between levels of defective Pr74 synthesis and GRP78 synthesis (Ulatowski *et al*, 1993).

GRP78 (BiP) is a highly conserved 78 kDa protein that resides within the ER lumen (Munro and Pelham, 1986; Hendershot *et al*, 1988). GRP78 has a high degree of amino acid sequence homology to the 70 kDa heat shock protein (HSP70) and is, therefore, considered a member of the HSP70 family of stress proteins (Hunt and Morimoto, 1985). GRP78 functions as a molecular chaperone, associating transiently with nascent proteins, facilitating their translocation into the ER (Sanders *et al*, 1992) and aiding in their folding and assembly into oligomeric structures (Bole *et al*, 1986; Dorner *et al*, 1987). For example, in B lymphocytes, nascent immunoglobulin heavy chains and light chains associate with GRP78 until they assemble to form complete immunoglobulin molecules (Haas and Wabl, 1983). Thus, like other members of the HSP70 family GRP78 is thought to function as a catalyst of

protein assembly, binding and hydrolyzing ATP as it associates and dissociates from peptides and proteins (Flynn *et al*, 1989; Gething and Sambrook, 1992). Normal polypeptides bind only transiently to GRP78 (Olden *et al*, 1979; Welch *et al*, 1983). However, abnormal proteins, such as Pr74 in the present study, bind irreversibly to GRP78, causing their retention within the ER and inducing an elevation in the level of GRP78 synthesis (Bole *et al*, 1986; Dorner *et al*, 1987; Kassenbrock *et al*, 1988; Hendershot *et al*, 1988; Kozutsumi *et al*, 1988).

Cells that express high levels of GRP78 resist the repression of protein synthesis that results from depletion of ER-associated calcium stores by calcium ionophore treatment (Brostrom *et al*, 1990; Brostrom and Brostrom, 1990). Moreover, induction of GRP78 synthesis by anoxia, calcium mobilization by the calcium ionophore A23187, or inhibition of protein glycosylation by 2-deoxyglucose or tunicamycin impedes the cell death response to a variety of apoptosis inducers, including calcium mobilizing agents (Li and Lee, 1991; Li, 1992), oxidative stress (Gomer, 1991), cytotoxic T cells (Sugawara *et al*, 1990), tumor necrosis factor (Sugawara *et al*, 1990), and a number of cytotoxic agents (Shen and Shenk, 1987; Hughes *et al*, 1989). Indeed, abrogation of the GRP78 stress response increases the cytotoxic response to calcium mobilizing agents (Li and Lee, 1991; Li *et al*, 1992; Little and Lee, 1995) and cell mediated cytotoxicity (Sugawara *et al*, 1993).

The mechanism by which elevated GRP78 synthesis maintains cell survival is unknown. A major function of the ER is to sequester and release calcium for use in intracellular signaling (Carafoli, 1987). Moreover, the ER calcium pool is essential for a number of vital cellular functions which include protein processing (Lodish, 1992; Gething, 1992), maintaining high translation rates of newly transcribed messages (Brostrom *et al*, 1990; Brostrom and Brostrom, 1990), preserving the structural integrity of the ER (Koch *et al*, 1988; Booth and Koch, 1989), and regulating cell proliferation and cell cycle progression (Short *et al*, 1993). In a previous report, we showed that viral protein-mediated induction of GRP78 synthesis is accompanied by decreased calcium efflux out of the ER lumen following inhibition of ER-associated calcium-ATPase activity by thapsigargin (Lam *et al*, 1993). Thus, it is possible that elevated levels of GRP78 may maintain cell viability by interfering with intracellular calcium signals involved in mediating apoptosis or by maintaining ER functions necessary for cell survival.

The defective MMTV envelope glycoprotein Pr74 that binds stably to GRP78 is only one of several viral proteins reported to bind stably to GRP78 within the ER lumen. For example, a misfolded influenza virus hemagglutinin (Hurtley *et al*, 1989) and a vesicular stomatitis virus G protein (Machamer *et al*, 1990) bind stably to GRP78. Moreover, infection of cells with paramyxoviruses stimulates synthesis of GRP78 (Peluso *et al*, 1978). Expression of these viral proteins is not associated with cell death, but effects on cell survival following apoptotic signals have not been investigated. Thus, the possibility exists that a range of viruses may regulate cell death by expressing proteins that interact with GRP78 and thereby induced GRP78 synthesis.

Materials and Methods

Materials

Acrylamide and other chemicals for gel electrophoresis were from Bio-Rad. Molecular weight standards were from Pharmacia LKB Biotechnology. Dexamethasone (dex) and other chemicals were from Sigma. [³⁵S]methionine (1100 Ci/mmol) was from ICN.

Cell lines and cell culture

W7.2 is a T cell line derived from a BALB/c mouse lymphoma (Horibata and Harris, 1970). W7MG1 cells were derived by infecting W7.2 cells with MMTV (Danielsen *et al*, 1983). W7-ENV/N cells were derived from W7.2 cells by retroviral mediated transfer of a sequence encoding a defective form of Pr74 that is not processed normally, but retained within the ER (Bedgood *et al*, 1992; Corey and Stallcup, 1992). W7-ENV/- cells were prepared by infecting W7.2 cells with the empty retroviral vector. Multiple subclones of the W7-ENV/N line were derived by a limiting dilution technique described previously (Reid, 1979). Cells were maintained in complete medium (Dulbecco's Modified Eagle's Medium supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 0.4 mM non-essential amino acids, and 10% (v/v) heat inactivated horse serum) at 37°C in a 7% CO₂ atmosphere and diluted into fresh medium three times per week to maintain a maximum cell density below 1.5 million cells/ml.

Cell viability

Cell viability was assessed by light microscopy after suspension in trypan blue dye. Cells that excluded trypan blue dye were considered viable.

Cell treatment and labeling

Dex, dissolved in ethanol and diluted into complete medium, was added to cells to give final concentrations ranging from 0.01 µM to 1 µM; an equivalent amount of ethanol in complete medium was added to untreated cells. Cells were then incubated at 37°C prior to labeling with [³⁵S]methionine. For labeling, cells were gently pelleted and resuspended in complete medium lacking methionine; [³⁵S]methionine was then added to give a final concentration of 50 µCi/ml and the cells were incubated for an additional 30 min at 37°C.

Immune precipitation

Methods of cytosol preparation, and immune precipitation were previously described in detail (Ulatowski *et al*, 1993). After treatment with dex and labeling with [³⁵S]methionine as described above, cells were lysed by resuspension and incubation for 15 min at 4°C in the following buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM iodoacetamide, 1% Triton X-100. Cell extracts were centrifuged at 14 000 × g for 20 min at 4°C and the concentration of radiolabeled protein in the clear supernatant cytosol was measured by trichloroacetic acid precipitation. Cytosol samples containing equivalent amounts of radiolabeled protein were incubated for 60 min at 4°C with a rabbit polyclonal antibody to Pr74 (Corey, 1992), or an equivalent concentration of a control IgG (Sigma). Immunoreactive proteins were adsorbed to goat anti-mouse IgG-agarose, analyzed by SDS-polyacrylamide gel electrophoresis and exposed to Kodak XAR-5 film at -80°C.

Receptor assay

The number of glucocorticoid receptors per cell was measured by a previously described whole cell competitive binding assay (Distelhorst *et al*, 1984).

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