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Calmodulin Involvement in Stress-Activated Nuclear Localization of Albumin in JB6 Epithelial Cells†

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ABSTRACT: We report that albumin is translocated to the nucleus in response to oxidative stress. Prior measurements have demonstrated that in concert with known transcription factors albumin binds to an antioxidant response element, which controls the expression of glutathione *S*-transferase and other antioxidant enzymes that function to mediate adaptive cellular responses [Holderman, M. T., Miller, K. P., Dangott, L. J., and Ramos, K. S. (2002) *Mol. Pharmacol. 61*, 1174−1183]. To investigate the mechanisms underlying this adaptive cell response, we have identified linkages between calcium signaling and the nuclear translocation of albumin in JB6 epithelial cells. Under resting conditions, albumin and the calcium regulatory protein calmodulin (CaM) co-immunoprecipitate using antibodies against either protein, indicating a tight association. Calcium activation of CaM disrupts the association between CaM and albumin, suggesting that transient increases in cytosolic calcium levels function to mobilize intracellular albumin to facilitate its translocation into the nucleus. Likewise, nuclear translocation of albumin is induced by exposure of cells to hydrogen peroxide or a phorbol ester, indicating a functional linkage between reactive oxygen species, calcium, and PKC-signaling pathways. Inclusion of an antioxidant enzyme (i.e., superoxide dismutase) blocks nuclear translocation, suggesting that the oxidation of sensitive proteins functions to coordinate the adaptive cellular response. These results suggest that elevated calcium transients and associated increases in reactive oxygen species contribute to adaptive cellular responses through the mobilization and nuclear translocation of cellular albumin.

Oxidative stress arises when there is an excess of free radicals and/or a decrease in antioxidant levels within the cell. Cellular responses to oxidative stress occur, in part, through transcriptional regulation via a cis-acting enhancer element known as the antioxidant response element (ARE)[1] (*1*−*3*). Activation of AREs typically involves the nuclear translocation and binding of critical transcription factors, leading to the inducible expression of glutathione *S*-transferase and other genes encoding antioxidative and detoxifying enzymes that function to maintain cellular redox status and guard against oxidative damage (*4*). Recently, albumin, phi AP3, and α-smooth muscle actin have been shown to function as components of a signaling complex involving the known transcription factors Nrf1, Nrf2, and JunD in modulating adaptive cellular responses to oxidative stress through their association with the ARE in vascular smooth muscle (*5*). These results are consistent with earlier measurements that demonstrated albumin to be a specific modulator of cellular glutathione levels (*6*). However, albumin is also known to inhibit apoptosis in macrophages, suggesting a role for albumin in scavenging reactive oxygen species (ROS) that normally are associated with the oxidation of critical proteins that initiate apoptosis (*7*, *8*). Indeed, the ability of albumin to enhance calcium reuptate by calcium pumps, which maintain low calcium levels associated with healthy cells, suggests a functional linkage between albumin, calcium signaling, and oxidative stress (*9*−*14*). Alternatively, albumin may play a signaling role in response to oxidative stress through direct interactions with other proteins to form macromolecular complexes, such as those involving the regulation of the ARE that we have previously identified (*5*).

To understand the mechanisms underlying observed changes in gene transcription and intracellular calcium levels associated with cellular albumin, we have investigated the linkage between cellular calcium levels, oxidative stress, and nuclear

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[1] Abbreviations: ARE, antioxidant response element; CaM, calmodulin; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GST, glutathione *S*-transferase; GSTA1, glutathione *S*-transferase A1; PKC, protein kinase C; ROS, reactive oxygen species; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase; Nrf2, NF-E2-related factor 2; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

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translocation of albumin in JB6 epithelial cells. To identify the possible involvement of calcium-binding proteins, we conducted a proteomic screen for calcium regulatory proteins that associate with albumin using mouse brain lysates. These results identified a structural linkage between albumin and the calcium regulatory protein calmodulin (CaM), which was confirmed following immunoprecipitation and immunoblotting using antibodies against either albumin or CaM. We report that increases in intracellular calcium levels disrupt the cytosolic protein complex containing albumin and calmodulin and promote the transport of albumin into the nucleus under conditions of oxidative stress. Taken together, these results suggest an important regulatory role for albumin in modulating cellular antioxidant defenses.

EXPERIMENTAL PROCEDURES

Materials. Mouse brains were purchased from Pel Freez Biologicals (Rogers, AR). Rabbit polyclonal anti-calmodulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against albumin family members were generated against the conserved peptide (i.e., E^{25}AHKSEIAHRFKDLGEQ^{41}) (*15*). UltraLink Immobilized Protein G was obtained from Pierce (Rockford, IL). CaM−Sepharose 4B was purchased from Amersham Bioscience (Piscataway, NJ). Complete EDTA-free protease inhibitor cocktail tablets (catalog no. 1 836 170) were obtained from Roche (Indianapolis, IN). Sepharose 4B, Triton X-100, buffers, and other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO). Sequencing grade modified trypsin was obtained from Promega (Madison, WI). Recombinant CaM was expressed and purified essentially as previously described (*16*).

Cell Culture. JB6 P+ cells (clone 41-5a) were maintained in minimum essential medium (MEM; JRH Biosciences, Lenexa, KS) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B in 5% CO_2:95% air at 37 °C. Cells were subcultured by trypsinization, and all experiments were conducted with cells at passage levels 99−113.

Lysis and Preparation of Cell Homogenates. Frozen mouse brains were thawed on ice and placed into a precooled tissue grinder. Samples and buffers were kept on ice throughout the preparation procedure. Tissue was homogenized following addition of lysis buffer [3 mL/brain of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, EDTA-free Roche complete protease inhibitor cocktail, and 1 mM $CaCl_2$ or 1 mM EGTA]. Alternatively, JB6 cells were grown to 80% confluence, washed three times with phosphate-buffered saline (PBS), scraped in lysis buffer, and then homogenized using a Dounce homogenizer. The homogenate was transferred into Eppendorf tubes and centrifuged at 20800*g* for 30 min in a tabletop centrifuge at 4 °C to remove cytoskeletal elements. Supernatants were carefully removed without disturbing the pellet and the top lipid layer and were transferred to clean tubes.

Immunoprecipitation. Prior to the isolation of protein complexes, samples were precleared with protein G-Sepharose beads for 30 min at 4 °C with rotation. Protein G beads were pelletted and discarded prior to the addition of primary antibodies. Primary antibodies against either CaM or albumin were then added, following by incubation for 1 h at 4 °C with gentle rotation. Protein G−Sepharose beads were added to the samples to capture primary antibodies and incubated overnight at 4 °C. The antibody−bead complex was sedimented by centrifugation and washed three times with lysis buffer, and protein associations were analyzed by Western blot analysis.

Affinity Isolation of CaM-Binding Proteins and MS Analysis. Per manufacturer's recommendations, brain lysates were incubated for 2 h at 4 °C with Sepharose beads with and without (control) bound CaM with gentle rotation in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. Beads were washed 5 times with lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100] containing 1 mM $CaCl_2$ or 1 mM EGTA, and bound proteins were resolved by SDS−PAGE. To elute samples, 50−100 μL of elution buffer (lysis buffer plus 4.0 M urea, 10 mM EGTA, and 5 mM DTT) was added to beads, and the samples were incubated at 90 °C for 5 min with occasional vortexing. The eluted material was separated from the beads by centrifugation at 20800*g* for 1 min and either resolved by SDS−PAGE or prepared for analysis by mass spectrometry. Prior to MS analysis, eluted proteins were exhaustively digested with trypsin (Promega, Madison, WI), and the resulting peptides were analyzed by tandem mass spectrometry and evaluated by SEQUEST (ThermoFinnigan) using the annotated mouse database, essentially as previously described (*17*−*19*). Briefly, microcapillary reversed-phase HPLC was performed using an Agilent 1100 series capillary LC system (Agilent Technologies, Inc., Palo Alto, CA) coupled to a LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) using an in-house manufactured ESI interface. The reversed-phase separation was performed using a 360 μm o.d. × 150 μm i.d. × 60 cm length capillary column (Polymicro Technologies Inc., Phoenix, AZ) packed with 5 Tm Jupiter C_{18} stationary phase (Phenomenex, Torrence, CA). SEQUEST results were filtered with criteria developed by Yates and co-workers (*20*).

Preparation of Nuclear Extracts and Detection of Albumin. Nuclear extracts were prepared essentially as previously described (*21*). Nuclear extract was subjected to Western blot analaysis for albumin following separation of 40 μg of lysate by SDS−PAGE, as previously described (*22*). Anti-albumin antibody was diluted 1:5000 (1 h at room temperature), and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) was diluted 1:10 000 (30 min at room temperature). Detection was by enhanced chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL), and images were captured using a Roche LumiImager.

RESULTS

Complex Formation between Cellular Albumin and CaM. Oxidative stress is associated with calcium dysregulation, suggesting a possible linkage between activation of transcriptional regulation through ARE and the functional activation of calcium signaling proteins. In this respect, CaM functions to coordinately regulate energy metabolism in all eukaryotic cells and has been implicated as a primary target of oxidative stress (*13*, *23*). Further, increases in CaM

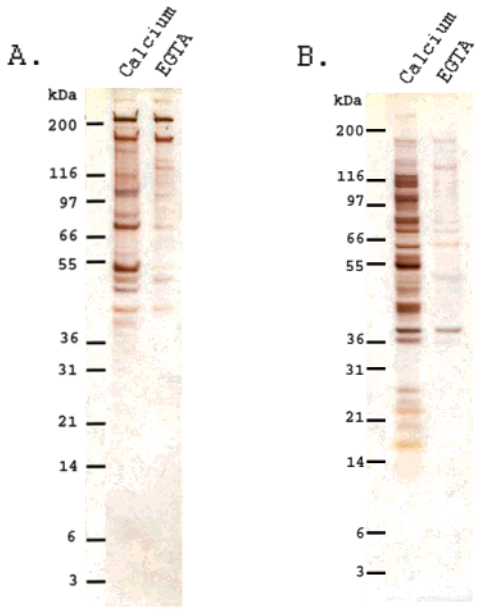

FIGURE 1: Isolation of calmodulin binding proteins. CaM−Sepharose beads were incubated with cell lysate prepared from JB6 cells (A) or mouse brains (B) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. After extensive washing, bound proteins were eluted, resolved by SDS−PAGE, and visualized using silver stain. There was negligible binding to Sepharose beads alone (control) whether in the presence of $CaCl_2$ or EGTA (data not shown). Protein standards are depicted at the left of each panel. Among the known Ca^{2+}−CaM binders that were identified following online reversed-phase microcapillary liquid chromatography coupled with ion trap mass spectrometry were calcium/calmodulin-dependent protein kinases II & IV, calcineurin, calpain, glyceraldehyde-3-phosphate dehydrogenase, inositol 1,4,5-trisphosphate receptor type 1, myelin basic protein, plasma membrane calcium-ATPase, α-spectrin, and β-spectrin. In addition, albumin was also identified to bind to CaM.

expression levels have been correlated with cell transformation, suggesting that CaM itself is associated with alterations in transcriptional regulation (*24*). We have, therefore, investigated the possible association between CaM and proteins known to participate in the formation of transcriptional regulatory complexes that recognize AREs involved in the regulation of cellular antioxidant levels (e.g., glutathione levels) (*4*).

To accomplish this, we isolated CaM-binding proteins from cellular lysates from both JB6 cells and whole mouse brain using Sepharose-4B beads with covalently bound CaM. JB6 cells represent a standard model system used to investigate linkages between oxidative stress and cell transformation, while whole mouse brain was chosen as an abundant source of tissue to identify CaM-binding proteins using mass spectrometry. Following affinity purification, selected binding proteins are observed that respectively associate with apo-CaM (in the presence of EGTA) or calcium-activated CaM (in the presence of saturating amounts of calcium) for both JB6 cells and brain homogenates (Figure 1). No protein is visualized on SDS−PAGE gels from control experiments involving only the Sepharose beads (data not shown). Irrespective of the tissue source, significantly more proteins associate with CaM in the presence of calcium, consistent with the known ability of CaM to selectively bind to more than 30 different target proteins following calcium activation (*25*−*27*). A similar distribution of isolated proteins are observed on SDS−PAGE gels following isolation of

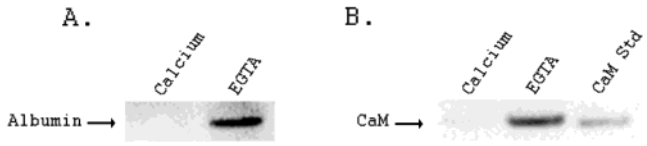

FIGURE 2: Co-immunoprecipitation of a protein complex containing CaM and albumin. (A) CaM−Sepharose beads were incubated with JB6 cell lysate in the presence of 1 mM $CaCl_2$ or 1 mM EGTA, and CaM binders were eluted, resolved by SDS−PAGE, transferred onto nitrocellulose paper, and probed for albumin with a polyclonal anti-albumin peptide antibody. (B) Mouse brain lysates were immunoprecipitated with polyclonal anti-albumin peptide antibody in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. After being washed, immunoprecipitated proteins were eluted, and the presence of co-immunoprecipitated CaM was analyzed by Western blotting. Purified recombinant CaM (0.25 μg) was used as a standard.

binding partners using an antibody against CaM covalently bound to an affinity matrix (data not shown), indicating that irrespective of the method of isolation that similar CaM-binding proteins are observed.

Using mass spectrometry to screen the CaM binders in lysates obtained from whole brain, which provided sufficient material for MS analysis, we identified a number of known CaM-binding proteins (see legend to Figure 1). Thus, our affinity purification methodology has selectively isolated CaM-binding proteins. In addition, albumin was also identified as one of the CaM binders and was present following affinity purification of proteins that bind to the apo- and calcium-bound forms of CaM. While an estimate of the relative stoichiometries of bound albumin is not possible from the peptide identification using mass spectrometry, these results suggest the presence of a protein complex involving albumin and CaM. Furthermore, since albumin has previously been implicated to be part of a protein complex associated with ARE regulation (*5*), these results suggest a possible linkage between calcium signaling and the modulation of adaptive cellular responses through transcriptional activation of antioxidant genes (e.g., glutathionine) under the control of the ARE.

Confirmation of the association between albumin and CaM was assessed in JB6 cells using an immobilized CaM Sepharose-4B affinity matrix to isolate CaM-binding proteins (Figure 2). Following incubation with JB6 cellular lysate, CaM-binding partners were eluted, and the presence of albumin was assessed by a Western immunoblot using primary antibodies against albumin. One observes that albumin selectively associates with apo-CaM (Figure 2A). No association between albumin and CaM is observed if calcium is added to the cellular homogenate prior to complex isolation. Thus, the association between apo-CaM and albumin is diminished following calcium activation of CaM. Likewise, immunoprecipitation involving incubation of primary antibodies against albumin in whole brain cell lysates, followed by the capture of the antibody protein complex using protein-G−Sepharose beads and a Western blot analysis probing for the presence of CaM, indicates a selective association between albumin and apo-CaM that is also diminished following calcium activation of CaM (Figure 2B).

Taken together, these results demonstrate that albumin and apo-CaM selectively associate in a protein complex in both whole brain and JB6 cell lysates. In both cases, calcium activation of CaM disrupts the protein complex, suggesting

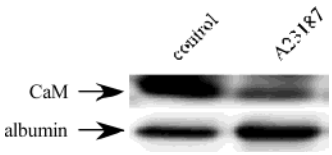

FIGURE 3: Calcium-dependent reduction in association between albumin and CaM. Western immunoblot against CaM bound to albumin following immunoprecipitation using a polyclonal antibody against albumin using total cellular lysates (40 μg) obtained from serum-starved JB6 cells (see Experimental Procedures) treated with DMSO alone (control) or calcium ionophore (1 μM A23187) for 30 min (top). Blots were stripped and reprobed for albumin (bottom). Similar results were observed in two separate experiments.

specificity of the formed complex and that calcium signaling modulates the association between CaM and albumin. Since calcium activation of CaM exposes hydrophobic binding surfaces within each binding domain (*28*, *29*), it is apparent that the protein complex between apo-CaM and albumin is specific and does not involve hydrophobic binding surfaces that become exposed following calcium activation of CaM. Thus, disruption of the protein complex involving apo-CaM and albumin is the result of calcium activation, which has the potential to influence the formation of a protein complex involving albumin and other proteins previously identified to be involved in adaptive cellular responses to oxidative stress through association with ARE (*5*).

Cytosolic Calcium Disrupts the Protein Complex between CaM and Albumin. CaM is known to regulate the nuclear trafficking of redox responsive transcription factors, including NFAT and members of the NF−κB family (*30*−*32*). Thus, the calcium-dependent interaction between apo-CaM and albumin, in conjunction with the suggested involvement of albumin in the transcriptional regulation of AREs, raises the possibility that CaM participates in transcriptional regulation of antioxidant genes by sequestering albumin in the cytosol prior to calcium activation. To test this hypothesis, we asked whether increasing levels of intracellular free calcium, through the treatment of live cells with a calcium ionophore, affected the formation of a protein complex between albumin and CaM.

Co-immunoprecipitation was performed to investigate the association between CaM and albumin under resting conditions. We find that CaM is associated with albumin following immunoprecipitation of albumin (Figure 3), suggesting the formation of a cellular protein complex involving CaM and albumin. The mobilization of calcium stores through the addition of the calcium ionophore A23187 functions to diminish the association between CaM and albumin. As a control the immunoblot was stripped and reprobed for albumin, whose abundance was not diminished following calcium mobilization. Likewise, under these conditions, total expression levels of cellular albumin and CaM are not significantly changed (data not shown), suggesting that the diminished CaM detected in association with albumin was not secondary to the activation of proteases and protein degradation. These observations demonstrate that CaM and albumin exist as part of a specific *in vivo* protein complex.

Oxidative Stress Induces Nuclear Translocation of Albumin. Albumin has previously been identified in the modulation of the adaptive cellular response through a direct assessment of its binding to the ARE involved in the regulation of antioxidant genes (*5*). To clarify whether this

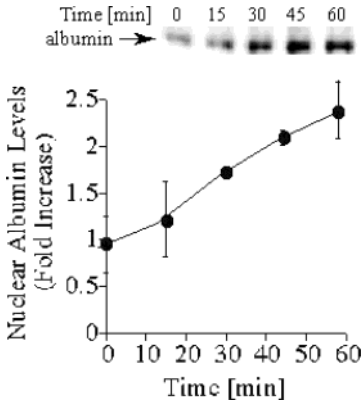

FIGURE 4: Oxidative stress induces nuclear translocation of albumin. Nuclear concentrations of albumin were determined following exposure of serum-starved JB6 cells to 35 μM H_2O_2 for the indicated times. Nuclear extracts were prepared, and 40 μg of total protein was subjected to a Western immunoblot using primary antibodies against albumin (top). Time-dependent increases in nuclear albumin levels were assessed by a densitometric analysis of the blots (bottom).

interaction might be modulated by oxidative stress, we investigated the translocation of albumin into the nucleus under conditions of oxidative stress through an assessment of the abundance of albumin in nuclear extracts (Figure 4). Under resting conditions, there is minimal albumin detected in the nuclear extract of JB6 cells. Following exposure of cells to hydrogen peroxide for varying periods of time, there is a progressive increase in the amount of albumin in the nuclear extract. The nuclear translocation of albumin following exposure to hydrogen peroxide is consistent with its ability to interact with proteins that bind to the ARE. The time course of albumin translocation, furthermore, occurs on the same time scale associated with the induction of other cellular antioxidant responses (*33*, *34*). Thus, our results suggest a possible involvement of the nuclear translocation of albumin in modulating cellular antioxidant responses.

Cellular Activation by Phorbol Ester Induces Nuclear Localization of Albumin. Other essential ARE-binding components (e.g., Nrf2) are mobilized and undergo nuclear translocation on a time scale similar to that of albumin and appear to be under the control of protein kinase C (PKC), since they are activated by the tumor promoting phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (*33*). To assess possible linkages between albumin and other members of the complex that participate in the transcriptional regulation of ARE, we assessed the possible involvement of PKC. In this respect, PKC isoforms (including α, β, γ, ϵ, δ, and ξ) are phosphorylated and catalytically activated upon treatment of cells with H_2O_2 (*35*), suggesting that PKC activation may play a central role in the nuclear translocation of albumin in response to both H_2O_2 and TPA. Furthermore, PKC activation is associated with increases in intracellular calcium levels—suggesting possible cross-talk mechanisms that may involve CaM.

Following treatment of JB6 cells with TPA, albumin is translocated to the nucleus, with kinetics similar to those resulting from hydrogen peroxide exposure (Figure 5). These results suggest coordination in the nuclear translocation of albumin and other components of the protein complex associated with ARE transcriptional regulation. Furthermore, since there is currently a limited understanding of the

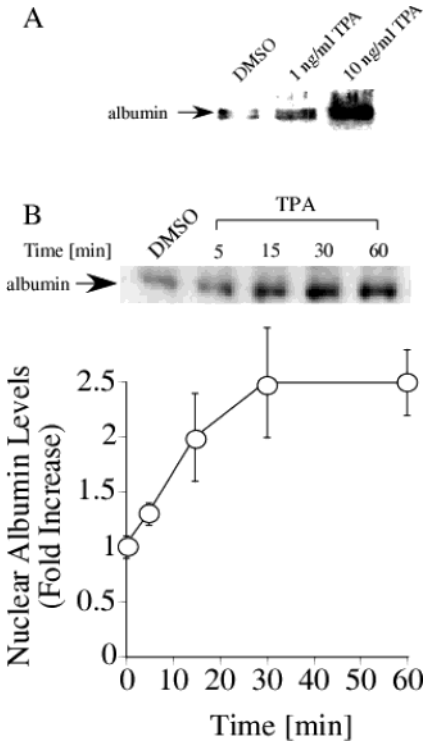

FIGURE 5: TPA-dependent nuclear translocation of albumin. (A) Concentration dependence of albumin nuclear localization following incubation for 1 h with vehicle alone (DMSO) or using the indicated concentrations of TPA. (B) Time dependence of albumin nuclear localization following treatment with 10 ng/mL TPA and quantification by a densitometric analysis of the blots (bottom). In all cases, nuclear extracts were prepared from serum-starved JB6 cells, and a equal amount of protein (40 μg) was subjected to a Western immunoblot analysis for albumin.

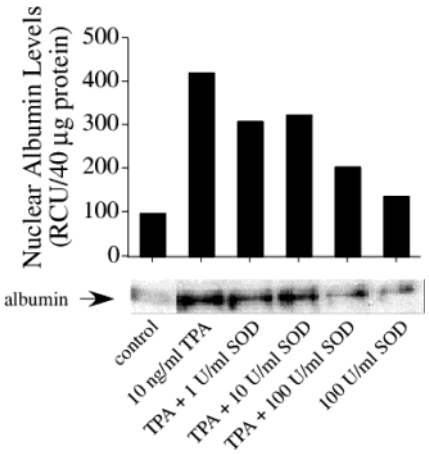

FIGURE 6: SOD inhibition of TPA-induced nuclear translocation of albumin. Western immunoblots against albumin in nuclear extracts (40 μg) obtained from serum-starved JB6 cells treated for 1 h with 10 ng/mL TPA (when indicated) in the absence and presence of variable amounts of SOD, and associated densitometric determination of relative amounts of albumin in the nucleus. RCU, relative chemiluminescent units.

relationship between ROS generation and the activation of specific signaling pathways associated with antioxidant responses (*36*), these results suggest a possible coordination between ROS generation, calcium signaling, and the activation of PKC.

Antioxidant Enzymes Block Nuclear Translocation of Albumin. Under normal cellular conditions, it is generally thought that 1−2% of cellular oxygen is reduced to form superoxide through cellular respiration (*37*). Increases in cytosolic free calcium or activation of protein kinases results in the up-regulation of cellular metabolism (and the generation of superoxide) through respiratory control mechanisms, suggesting the possible cross-talk between multiple signaling mechanisms in the integration of the cellular response to conditions of oxidative stress (*13*). Therefore, to differentiate between direct effects involving changes in calcium levels or PKC activation from cellular responses resulting from generation of superoxide associated with alterations in cellular metabolism, we have assessed the effect of superoxide dismutase (SOD) on the TPA-dependent nuclear translocation of albumin. In this respect, prior results have demonstrated that cells rapidly take-up extracellular SOD under conditions of oxidative stress (*38*). We report that the TPA-dependent nuclear translocation of albumin is blocked following incubation with SOD (Figure 6), demonstrating that the modulation of albumin translocation into the nucleus involves the redox modulation of key regulatory proteins.

It is noteworthy that activation of PKC through the addition of TPA is thought to induce the selective generation of superoxide through the assembly and activation of NADPH oxidases at specific membrane locations (*39*). This physiological induction of superoxide is blocked by SOD (Figure 6), consistent with the ability of SOD inhibitors to block TPA-induced oxidative stress and inflammatory responses in epithelial model systems (*47*). In contrast, addition of a bolus of hydrogen peroxide induces a nonspecific oxidative stress that overwhelms antioxidant defenses, which leads to observable translocation of albumin (Figure 4). Thus, spatial constraints on the generation and activity of ROS as well as the ability of the cell to effectively detoxify the physiological generation of ROS appear to be important in modulating the albumin:CaM complex.

DISCUSSION

Summary of Results. We have demonstrated that albumin is selectively translocated to the nucleus under conditions of oxidative stress (Figure 4), consistent with the proposed role of albumin in modulating cellular antioxidant levels in response to oxidative stress through the formation of a protein complex associated with AREs. Furthermore, like Nrf2, albumin is translocated to the nucleus in response to PKC activation following cellular exposure to phorbol esters (*33*, *34*) (Figure 5), suggesting coordination in the recruitment of albumin with other components of the protein complex involved in the transcriptional regulation of ARE. These results are consistent with earlier suggestions that albumin is a prosurvival factor whose action involves the modulation of NF−κB activation and up-regulation of cellular glutathione levels through the transcriptional regulation of ARE responsive elements (6, *8*, *32*, *40*−*42*).

The coordinate regulation associated with calcium activation of CaM and the mobilization and associated nuclear translocation of albumin is, furthermore, consistent with other evidence, suggesting that CaM directly associates with members of the NF−κB family (i.e., c-Rel and RelA) to modulate their nuclear translocation (*32*). Specifically, CaM binding differentially affects nuclear translocation within the NF−κB family since CaM-binding facilitates the nuclear translocation of RelA, while CaM association with c-Rel

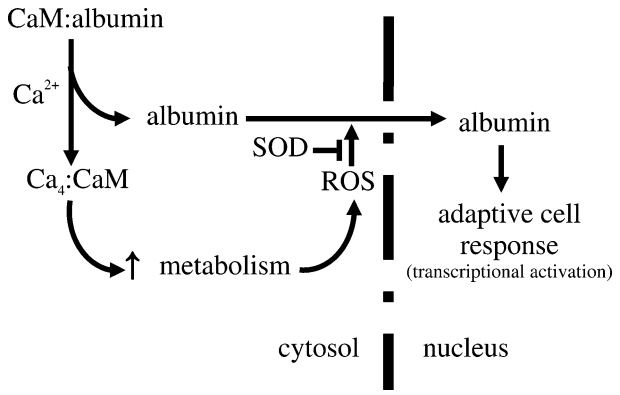

FIGURE 7: Model depicting relationships between calcium signaling, oxidative stress, and the nuclear translocation of albumin. Albumin exists as part of a protein complex with apo-CaM in the cytosol. Following calcium activation, the CaM:albumin protein complex dissociates, resulting in the coordinate up-regulation of metabolism by Ca_4:CaM and the associated generation of reactive oxygen species (ROS) promotes the nuclear localization of albumin. Once in the nucleus, albumin has been shown to have the potential to form a complex with other proteins (e.g., Nrf2) associated with the activation of the ARE element and associated transcriptional activation of antioxidant genes (e.g., glutathione) underlying adaptive cellular responses to oxidative stress (*5*).

blocks its nuclear translocation. The current results suggest a role for CaM in modulating the nuclear translocation of albumin, which has previously been implicated in the modulation of ARE gene expression (Figure 7). These findings are consistent with earlier data suggesting a central role of CaM in modulating cellular antioxidant responses through the control of other gene regulatory elements (*43*, *44*). Given the central role of albumin in protecting the vasculature against oxidative damage and the rapid uptake of serum albumin into cells with a half-time of approximately 10 min (*45*, *46*), these results suggest that cellular albumin may have a critical role in sensing the extracellular environment and facilitating adaptive cellular responses.

ROS Generation and Cellular Antioxidant Responses. Phorbol esters, via activation of PKC, are specifically associated with the phosphorylation of target proteins and function to up-regulate cellular metabolism and the associated generation of ROS (*47*). Thus, the ability of antioxidant enzymes (i.e., SOD) to block the TPA-induced nuclear localization of albumin suggests that the generation of ROS rather than direct phosphorylation underlies the targeting of albumin to the nucleus (Figure 6). In this latter respect, phorbol esters and the associated generation of ROS also function to up-regulate cytosolic calcium levels, in part because of the sensitivity of critical calcium regulatory proteins (i.e., pumps and channels) to oxidative and nitrative modifications (*12*, *23*, *48*−*50*). Taken together, these results suggest coordination between calcium signaling, PKC activation, generation of ROS, and adaptive cellular responses associated with activation of ARE responsive elements.

Albumin and Cellular Signaling. In this paper, we demonstrate for the first time that albumin undergoes nuclear translocation under conditions associated with the nuclear recruitment of transcription factors (i.e., Nrf2) known to modulate ARE transcriptional regulation through the formation of an activated protein complex (*34*). These results support earlier suggestions that albumin functions as part of a protein complex that modulates ARE signaling (*5*). Furthermore, these results are consistent with recent evidence that albumin functions as a signaling molecule in modulating cell function (*51*). Indeed, specific albumin binding proteins (such as gp60) mediate the endocytosis of albumin into caveolae and are coupled to G-protein activation in a process that is under tight hormonal and enzymatic regulation (*45*, *52*−*54*). In addition, cellular antioxidant and inflammatory responses have been linked to post-translational oxidative modifications to albumin (*45*, *55*). While specific mechanisms have not previously been elucidated, this latter association may be related to prior measurements that demonstrate cellular albumin to enhance ROS generation and to modulate cytosolic calcium levels and functions as a prosurvival factor (*10*, *11*, *40*, *55*, *56*).

Prior work has identified the transcription factor Nrf2 to play a central role in the chemoprotective response through the transcriptional up-regulation of 16 known antioxidant and phase II detoxification enzymes, which are under the control of an ARE consensus sequence in their promoter (*4*, *57*, *58*). These enzymes, which include GST, NAD(P)H:quinine oxidoreductase (NQ01), and heme oxygenase-1 (HO-1), all play an important role in maintaining celluar redox status and protecting cells from oxidative damage. Furthermore, while the TPA-response element (TRE) is mediated by different signaling pathways, it is sometimes embedded within the ARE promoter sequence (*59*−*61*), suggesting a possible linkage between the integration of divergent signaling pathways through the transcriptional regulation of these gene elements and the induction of cellular transformation. Indeed, overexpression of transcription factors (i.e., c-Jun and c-Fos), which are associated with the activation of TPA response elements (TRE), have been suggested to diminish activation of ARE elements (*4*).

Physiological Relevance. Cell transformation in response to environmental and physiological agents, including phorbol esters and the reactive oxygen species superoxide, have been linked to alterations in the responsiveness of ARE and TPA-transcriptional elements (*4*, *47*, *62*, *63*). The integrated responsiveness of these signaling pathways has been suggested to contribute to cell transformation (*4*). In this respect, JB6 cells represent an important model system that has been extensively used to define underlying mechanisms associated with the cell transformation response (*64*−*67*). ROS have been implicated in this process, since addition of CuZnSOD during the first 2 h of tumor promoter treatment inhibits the cell transformation response (*47*). Further evidence for the requirement of superoxide in transformation comes from studies using JB6 cell lines stably overexpressing MnSOD, which fail to undergo transformation when treated with TPA (*67*). Likewise, albumin translocation to the nucleus is inhibited by SOD, and translocation is detected during the first 2 h of TPA treatment (Figure 6). Thus, our results suggest that the nuclear translocation of albumin in response to increases in cellular calcium levels and generation of ROS has the potential to modulate the ARE responsive element through its ability to associate with known transcriptional factors (such as Nrf2), affecting the nuclear recruitment of critical transcriptional complexes that are known to associate with albumin and to be responsive to tumor promoters in JB6 cells.

Conclusions and Future Directions. We have identified a structural linkage between calcium signaling, oxidative stress,

and formation of a protein complex between albumin and apo-CaM. Under conditions of oxidative stress, cellular calcium and ROS levels increase, promoting the dissociation of this protein complex and the nuclear localization of albumin. Future measurements should seek to identify other members of the protein complexes involving albumin in both the cytosol and the nucleus and the coordinate regulation of the 16 different known enzymes under control by ARE relative to alternate signaling pathways (e.g., TRE elements) that integrate environmental signals to determine cell fate. An understanding of the signaling pathways associated with adaptive cellular responses to environmental stressors will facilitate the construction of predictive models for therapeutic interventions.

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