Association of Activating Transcription Factor 2 (ATF2) with the Ubiquitin-conjugating Enzyme hUBC9

IMPLICATION OF THE UBIQUITIN/PROTEASOME PATHWAY IN REGULATION OF ATF2 IN T CELLS

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Activating transcription factor 2 (ATF2) is regulated by phosphorylation via the Jun N-terminal kinase, and its binding activity is markedly induced at late stages of T and B lymphocyte activation (Feuerstein, N., Firestein, R., Aiyer, N., Xiao, H., Murasko, D., and Cristofalo, V. (1996) J. Immunol. 156, 4582–4593). To identify proteins that interact specifically with ATF2 in lymphocytes, the yeast two-hybrid interaction system was employed using ATF2 cDNA as a “bait.” In two separate screenings, a clone was identified that revealed a novel sequence with homology to several members of the ubiquitin-conjugating enzyme family. An identical sequence was recently reported as the human homolog of the yeast UBC9, hUBC9. Northern blot analysis revealed a 1.3-kilobase RNA transcript, which showed differential levels of expression in various human tissues and a moderate induction after a 48-h stimulation of peripheral blood T lymphocytes. An antibody that was generated against the bacterially expressed glutathione S-transferase-hUBC9 detected a ~19-kDa protein, which localizes predominantly in the nuclei of T cells. Further quantitative assays using the yeast two-hybrid system confirmed a high and specific level of interaction of hUBC9 with ATF2 and lack of interaction with lamin or control vectors. Two other cyclic AMP-responsive element-binding transcription factors, CREB and ATF1, also showed significant levels of interaction with hUBC9. However, this interaction was severalfold lower as compared with ATF2. Far Western blot analysis confirmed the specific binding of ATF2 and hUBC9 also in vitro. Evidence is presented that indicates a physiological significance for the interaction of hUBC9 with ATF2. (a) We show that ATF2 is ubiquitinated in vivo and in vitro, and (b) ATF2 ubiquitination in vitro is facilitated by addition of purified hUBC9. (c) ATF2 is shown to undergo a proteolytic process, which is rapidly regulated upon T cell activation concomitant with induction of ATF2 phosphorylation. (d) A proteasome inhibitor delays the down-regulation of ATF2 phosphorylation after T cell activation. Taken collectively, these results implicate a role for hUBC9 and the ubiquitin/proteasome pathway in regulation of ATF2 in T cells.

Activating transcription factor 2 (ATF2) is a member of the ATF/CREB family of basic region leucine zipper (bZIP) DNA-binding proteins that regulates transcription by binding to a conserved CRE elements in the promoter of genes (1, 2). We have shown that stimulation of T or B lymphocytes via the antigen receptor is associated with a marked induction of ATF2 binding to consensus CRE elements as well as to CRE elements in the promoter of proliferating cell nuclear antigen at late stages of activation or differentiation (3). In addition, induction of CRE binding activity was observed by us and others in cloned T cells stimulated with IL-2 (4) and in human peripheral blood T cells stimulated by PHA (5). Conversely, inhibition of T cell proliferation by rapamycin, a potent macrolide immunosuppressant, was associated with a marked inhibition of ATF2 binding activity (3, 6). This indicates that transactivation of ATF2 is tightly regulated and may play an important role during lymphocyte activation.

The transcription factor ATF2 has been implicated in the induction of cytokine gene expression after engagement of the antigen receptor in lymphocytes (7, 8). Specifically, ATF2 was implicated in the transcription of tumor necrosis factor γ (7), in tumor growth factor β (8), and in the virally induced interferon β (9). An important role for ATF2 has also been demonstrated in the expression of T cell-specific genes (10–13). ATF2 binds to the conserved CRE decamer motif in the CD8α promoter (10) and in TCR Vβ promoter (11). Isoforms of the CRE-BP1 DNA (ATF2) were found to mediate activity of a T cell-specific enhancer required for expression of the CD3ε gene of the T cell receptor complex (12). Because this motif is also present in the enhancer and promoter of the TCR α and β genes, it has been suggested that ATF2 may mediate expression of other members of the CD3/TCR complex during T cell activation and differentiation (12).

ATF2 was also implicated in the pathogenesis of HTLV-1 (14, 15), the etiologic agent of adult T cell leukemia, an aggressive malignancy of helper T lymphocytes in humans. Specifically, ATF2 was shown to bind and transcriptionally activate the virus-encoded transactivator protein, Tax, leading to induction of HTLV-1 gene expression (9–12). Thus, it appears that pathological processes on oncogenic virus expression in human T lymphocytes utilize cellular processes that up-regulate ATF2.

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transcription factors during T cell activation.

Evidence indicated that full-length ATF2 is inactive due to intramolecular inhibition exerted by its DNA binding domain (16). This led to the hypothesis that ATF2 interaction with other proteins releases this intramolecular inhibition and transactivates the protein (16). Indeed, ATF2 transcriptional activation was shown to involve interaction with several different proteins including: E1A (17–19), the tumor suppressive gene product Rb (20), the high mobility group HMG (21), NF-kB (21), and c-Jun (22). Therefore, the function of ATF2 may be determined by its specific interaction with other inducer proteins. Transcriptional activation of ATF2 was shown to be also induced by phosphorylation mediated by stress-activated protein kinases including Jun N-terminal kinase (JNK), a subgroup of the MAP kinase family (23–26). Furthermore, studies have shown that cell stimulation via pro-inflammatory cytokines or ultraviolet radiation is associated with rapid phosphorylation of ATF2 by JNK, implicating a role for ATF2 in rapid signaling processes via JNK transduction pathways (24). JNK has been implicated in integration of different signaling pathways leading to T cell activation via the TCR/CD3 (27). Taken collectively, this evidence leads to the possibility that transcriptional activation of ATF2 may be tightly regulated by early and late signaling processes during T cell activation.

To elucidate the mechanisms that regulate ATF2 in T cell activation, we searched for proteins that interact with ATF2 in vivo. The genetic approach of the yeast two-hybrid was employed using the full-length human ATF2 cDNA used as a “bait.” The search was performed using a cDNA library of PHA-activated human peripheral blood leukocytes. We report here the isolation and characterization of a cDNA clone that encodes a new member of the ubiquitin-conjugating enzyme family, which specifically interacts with ATF2 in the yeast two-hybrid system and in vitro in Far Western analysis. An identical sequence was recently reported as the human homolog of the yeast ubiquitin-conjugating enzyme 9, hUBC9 (28–30). We demonstrate that hUBC9 protein localizes predominately in the nuclei of control and stimulated T cells. hUBC9 belong to a family of ubiquitin-conjugating enzymes (E2) that participate in the linking of C-terminal glycine residues of ubiquitin to specific lysine residues of target proteins (for review, see Refs. 31–33). This ubiquitination leads to target protein degradation by the 26 S proteasome. We demonstrate that ATF2 is ubiquitinated in vivo and in vitro and provide evidence that hUBC9 facilitates the ubiquitination of ATF2 in vitro. We further demonstrate that changes in abundance of degradation products of ATF2 occur rapidly upon T cell activation concomitant with induction phosphorylation of ATF2. These results taken collectively implicate the proteasome pathway and specifically hUBC9 in the regulation of ATF2 during T cell activation.

MATERIALS AND METHODS

Reagents—Affinity-purified antibody specific for conjugated ubiquitin was a kind gift of Dr. Arthur L. Haas (Medical College of Wisconsin, Milwaukee, WI) and Dr. C. Pickart (Johns Hopkins University, Baltimore, MD). Anti-ATF2 polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and a gift from Dr. Michael Green, University of Baltimore, MD). Anti-ATF2 polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY, and a gift from Dr. Michael Green, University of Baltimore, MD). Anti-ATF2 polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY, and a gift from Dr. Michael Green, University of Baltimore, MD). Anti-ATF2 polyclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY, and a gift from Dr. Michael Green, University of Baltimore, MD).

Generation of a Polyclonal Antibody to hUBC9—The hUBC9 cDNA was subcloned into the pGEX2T vector in frame with the glutathione S-transferase (GST) moiety. The pGEX2T-hUBC9 construct was transformed into BL21 pLysS Escherichia coli and induced to express a GST-hUBC9 fusion protein by addition of isopropyl-1-thio-β-D-galactopyranoside. The fusion protein was isolated and purified by affinity chromatography using glutathione-agarose beads. The fusion protein was then eluted from the beads and was sent for production of a monoclonal antibody to hUBC9 (the GST moiety was cleaved using thrombin cleavage assay), demonstrated a titer of ≥1:204,800.

Cloning of Full-length hUBC9 Gene Transcripts from xgt10 Human Peripheral Blood Leukocyte cDNA Library—A 670-bp probe was prepared from the coding region of the hUBC9 cDNA by EcoRI/SpeI digestion. This 32P-labeled probe was used in Southern blot, Northern blot, and screening of xgt10 human peripheral blood leukocyte cDNA library. cDNA inserts were excised from purified phage DNA by EcoRI digestion. From 1 × 106 plaque-forming units that were screened with the 670-bp probe, two phages were obtained that were carried through primary, secondary, and tertiary screenings. These phages were grown in liquid culture, and a DNA extraction was performed. The cDNA inserts were released by EcoRI digestion of purified phage DNA and subjected to Southern blot analysis utilizing the 670-bp coding region probe. Both phages carried an identical insert of 1.3 kb that hybridized strongly to the probe. The inserts were sequenced using automated sequencing analysis (ABI sequencer, Kimmel Cancer Institute, Jefferson University).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using the guanidinium isothiocyanate method as described previously (6). 10 µg of total RNA was analyzed on glyoxal-agarose gel and transformed human multiple tissue Northern blots (CLONTECH) contained 2 µg of poly(A)+ RNA per lane of different human tissues run on a denaturing formaldehyde 1.2% agarose gel, transferred to a charged modified nylon membrane, and fixed by UV irradiation. A 670-bp probe was prepared from the coding region of the hUBC9 cDNA by EcoRI/SpeI digestion and labeled by random priming using [32P]dCTP. Hybridization was performed as described previously (6).

Southern Blot Analysis—Southern blot (Zoo Blot, CLONTECH) contained 4 µg of genomic DNA per lane from eukaryotic different species. DNA was digested with EcoRI, run on a 7% agarose gel, transferred to a charged modified nylon membrane, and fixed by UV irradiation. A 670-bp probe was prepared from the coding region of the hUBC9 cDNA by EcoRI/SpeI digestion and labeled by random priming using [32P]dCTP.
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.blit analysis detected specifically an ~18–20-kDa protein.

Far Western Overlay Binding Assay—This assay was performed using the protocol described previously (34). pGEXXX–ATF2 construct was transformed into BL21 E. coli and induced to express GST–ATF2. ATF2 was cleaved from the GST moiety by incubation with factor Xa (ICN) at 0.4 mg/ml for 3 h at 4 °C in buffer containing 1% Nonidet P-40, 50 mM Tris, pH 8.0, 1 mM CaCl2. The GST moiety was pelleted using glutathione-agarose beads, and the supernatant was collected as purified ATF2. Purified ATF2 and purified actin (Sigma) were analyzed on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated in blocking solution (5% bovine serum albumin in phosphate-buffered saline) and incubated with human tumor tissues. The nitrocellulose membranes were stripped by incubation in medium containing 2% SDS, 0.6% β-mercaptoethanol, 62.5 mM Tris, pH 6.7, at 50 °C for 30 min with shaking. After extensive washing, the membrane was rebotted with anti-ATF2.

RESULTS

Cloning and Isolation of an ATF2-binding Protein in the Yeast Two-hybrid System Identifies a Novel Human Member of the Ubiquitin-conjugating Enzyme Family—The two-hybrid interaction system in yeast was employed in an effort to isolate proteins that interact with the transcription factor ATF2. To this end, full-length ATF2 cDNA was subcloned in frame with the GAL4 DNA binding domain in the yeast expression vector pGBT9 at the BamHI site. The ATF2-pGBT9 plasmid was transformed into the HF7C strain of yeast containing stable constructs of the reporter genes LacZ and HIS3. The presence of ATF2-pGBT9 in the yeast was stably maintained by selection for the pGBT9 selection marker TRP1, which allows yeast growth in the absence of tryptophan. Expression of ATF2-pGBT9 only in the yeast cells transfected with ATF2-pGBT9 was confirmed by Western blot analysis using specific antibody to ATF2. HF7C yeast cells transformed with pGBT9–ATF2 were then tested for false activation of the yeast two-hybrid reporter genes (HIS3 and LacZ). pGBT9–ATF2 alone or co-transformed with the activation domain (pGAD10) alone did not activate either the HIS3 or LacZ reporter genes. This indicated that the ATF2 bait was not falsely activating at the GAL4 binding sites of these reporter genes. Thus, further experiments were performed to screen a human cDNA expression library of PHA-activated lymphocytes for proteins that interact with ATF2.

Using a standard LiCl transformation procedure, the yeast cells were transformed with a PHA-stimulated human peripheral leukocyte Matchmaker™ cDNA. Transformants were grown in the absence of tryptophan, leucine, and histidine (selective minimal media) at 30 °C for 8 days. The library was screened twice by this method with a total of 3 × 10⁶ clones analyzed. Eight His® colonies that grew on the selective media were analyzed for β-galactosidase activity by the filter assay method. Two of the eight His® colonies screened positive for β-galactosidase activity. DNA inserts from these positive clones were extracted and transferred into bacteria. Transformants were then grown in liquid culture, and plasmid DNA was extracted. Inserts were released from the pGAD10 vector by EcoRI restriction enzyme digestion and analyzed on a 1% agarose gel. The two positives encoded a 600-bp cDNA and a 2.1-kb cDNA.

Purification of Nuclei and Western Blot Analysis of hUBC9—Subcellular fractionation of nuclei and cytosolic fraction were performed as described previously (6, 35, 56). Jurkat T cells were suspended in RPMI 1640 containing 10% fetal bovine serum and incubated with the p53–mutated concanavalin A (5 µg/ml) and PMA (10 ng/ml). At the end of the incubation period, the cells were washed with phosphate-buffered saline and resuspended in 100 µl/× 10⁶ cells of RSB buffer (0.01 M Tris-HCl, 0.01 mM NaCl, 1.5 mM MgCl2, pH 7.2) supplemented with 0.1% Nonidet P-40 and protease inhibitors: 1 µg phenylmethylsulfonyl fluoride, leupeptin, 0.25 mM lactacystin, 0.25 mM leupeptin, 0.6 units/ml inorganic phosphate from yeast (Sigma), 5 mM creatine phosphokinase from rabbit muscle (Sigma), 0.1 mM dithiothreitol, leupeptin, phenylmethylsulfonyl fluoride, 0.25 mM TPCK, 0.25 mM TLCK, 5 mM N-ethylmaleimide, 50 mM N-acetyl-Leu-Leu-norleucine, 0.1% Triton X-100, and 0.5% sodium deoxycholate supplemented with 5 mM EDTA, 1% dithiothreitol, leupeptin, phenylmethylsulfonyl fluoride, 0.25 mM TPCK, 0.25 mM TLCK, 5 mM N-ethylmaleimide, and 0.5% sodium deoxycholate. The cells were incubated at 4 °C. After 7 min of incubation at 4 °C, the beads were pelleted by centrifugation and washed three times with RIPA buffer, 10 µl of protein G-agarose (Sigma) for 1 h. The agarose beads were pelleted by centrifugation and washed three times with RIPA. The beads were suspended in 2× SDS sample buffer, boiled, and analyzed on 10–12% acrylamide gel. Proteins were transferred onto nitrocellulose membrane and blotted with anti-ATF2 as described previously. In brief, after blocking with 5% nonfat dry milk, the membrane was incubated with anti-ATF2 (1:1000 dilution) and the antibody was detected using ECL detection system as described previously (4). In certain experiments, as indicated, the Western blot membrane was probed by incubation in medium containing 2% SDS, 0.6% β-mercaptoethanol, 62.5 mM Tris, pH 6.7, at 50 °C for 30 min with shaking. After extensive washing, the membrane was rebotted with anti-ATF2.

Preparation of Cell Extract for in Vitro Ubiquitination—Jurkat T cells at logarithmic growth were washed with phosphate-buffered saline and lysed in buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, and 1% Triton X-100, and 1 mM dithiothreitol, leupeptin, 0.25 mM lactacystin, 0.25 mM leupeptin, 0.6 units/ml inorganic phosphate from yeast (Sigma), 5 mM creatine phosphate, 10–50 µg of Jurkat T cell protein extract (as indicated in the specific experiments), purified hUBC9 (~150 ng), and 1 µg lactacycin (purchased from Dr. E. Corey, Harvard University, Cambridge MA). The reaction mixture was incubated at 37 °C for various periods of time as indicated and stopped by adding 20 µl of glutathione-agarose beads and 2 µl of proteinase K. After 7 min of incubation at 4 °C, the beads were pelleted by centrifugation at 4 °C, and washed twice more with Tris-buffered saline. The pelleted beads were dissolved in 30 µl of 2× SDS sample buffer, boiled, and analyzed on 7.5% acrylamide gels. The proteins were electrophoresed onto nitrocellulose membrane, and the ubiquitin-conjugated ATF2 forms were detected by Western blot analysis with anti-ATF2 antibody or with affinity-purified antibody, which detect specifically ubiquitin conjugate. Western blot analysis of anti-UB was done at 1:4000 dilution, and detection was performed with ECL.

Immuno precipitation and Western Blot Analysis of ATF2—Immuno precipitation and Western blot were done as described previously (6, 55). Jurkat T cells were suspended at 1 × 10⁶ cells/ml and stimulated under different conditions as indicated. At the end of the incubation, the cells were washed with phosphate-buffered saline and lysed with RIPA buffer (0.15 M NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 5 mM EDTA, 1% dithiothreitol, leupeptin, phenylmethylsulfonyl fluoride, 0.25 mM TPCK, 0.25 mM TLCK, 5 mM N-ethylmaleimide, 50 mM N-acetyl-Leu-Leu-norleucine, 0.1% Triton X-100, and 0.5% sodium deoxycholate. The cells were incubated at 4 °C. After 7 min of incubation at 4 °C, the beads were pelleted by centrifugation and washed three times with RIPA. The beads were suspended in 2× SDS sample buffer, boiled, and analyzed on 10–12% acrylamide gel. Proteins were transferred onto nitrocellulose membrane and blotted with anti-ATF2 as described previously. In brief, after blocking with 5% nonfat dry milk, the membrane was incubated with anti-ATF2 (1:1000 dilution) and the antibody was detected using ECL detection system as described previously (4). In certain experiments, as indicated, the Western blot membrane was probed by incubation in medium containing 2% SDS, 0.6% β-mercaptoethanol, 62.5 mM Tris, pH 6.7, at 50 °C for 30 min with shaking. After extensive washing, the membrane was rebotted with anti-ATF2.
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Sequence analysis of the two clones identified above revealed that the 600-bp clone was 100% homologous to the 5’ end of the 2.1-kb cDNA. Thus, it was concluded that the two cDNAs are derived from the same gene that encodes an ATF2-binding protein. Further experiments were carried to clone the full-length cDNA of this gene from a human peripheral blood leukocyte cDNA library. Two inserts of 1.3 kb were obtained that hybridized strongly to the probe. Sequencing of these cDNAs showed 100% identity to the cDNA of the clone originally found in the yeast two-hybrid system.

The predicted amino acid sequence of the cDNA (158 amino acids) was compared with protein sequences deposited in GenBank™. Homology was found with several ubiquitin-conjugating enzymes as indicated in Fig. 1. A conserved sequence motif (amino acids 82–97), which contains the active cysteine residue that is required for ubiquitin-thioester formation, was found in the predicted protein. This identified a novel human homolog of the yeast ubiquitin-conjugating enzyme as an ATF2-binding protein in the yeast two-hybrid system (Fig. 1). Several months after we had completed the sequence of this protein, an identical sequence was published by other groups and was identified as the human homolog of the yeast ubiquitin-conjugating enzyme 9 (UBC9) (28–30). We have determined that hUBC9 is a conserved gene in various species (Fig. 3). The presence of multiple DNA bands in certain species after 48 h of stimulation with PHA (Fig. 2A) further demonstrate a significantly higher affinity of hUBC9 and pGBT9-ATF2 for protein interaction. This assay was previously demonstrated significant interaction with CREB and ATF1 in both filter assay and the liquid assay, this interaction was significantly weaker as compared with its interaction with ATF2 (10-fold lower). These results indicate that hUBC9 may also target the transcription factors CREB and ATF1 in vivo and further demonstrate a significantly higher affinity of hUBC9 interaction with ATF2.

Demonstration of hUBC9 Binding to ATF2 in the Filter Overlay Binding Assay—The filter overlay binding assay is an assay for protein-protein interaction. This assay was previously shown to correlate with protein-protein interactions detected in the two-hybrid system (34). Thus, we have utilized this assay to examine the interaction of hUBC9 with ATF2. To this end, hUBC9 cDNA was subcloned into the pGEX2T vector in frame with the GST moiety. The pGEX2T-hUBC9 construct was expressed in E. coli strain SFY526 containing the Lacz reporter gene were cotransformed with the hUBC9-pGAD10 plasmid and pGBT9-CREB, pGBT9-ATF1, pGBT9-ATF2, or pGBT9-TAF2 as well as control plasmids. Transformants were grown on -Trp/-Leu yeast dropout plates at 30 °C for 4 days. Individual colonies were taken from each plate and grown overnight in liquid dropout medium at 30 °C. Equal amounts of cells were collected, and the activation of the reporter gene was measured by the quantitative liquid β-galactosidase assay (Fig. 4A) as well as by β-galactosidase filter assay (Fig. 4B).

Fig. 4 shows that only yeast cells co-transformed with pGAD-hUBC9 and pGBT9-ATF2, and not cells cotransformed with the control null vectors, demonstrated a marked β-galactosidase activity indicating the specificity of the interaction of hUBC9 with ATF2. Interestingly, hUBC9 also demonstrated a low but significant interaction with two other transcription factors, CREB and ATF1, although it did not bind at all to the nuclear protein lamin. Notably, although hUBC9 demonstrated significant interaction with CREB and ATF1 in both filter assay and the liquid assay, this interaction was significantly weaker as compared with its interaction with ATF2 (~10-fold lower). These results indicate that hUBC9 may also target the transcription factors CREB and ATF1 in vivo and further demonstrate a significantly higher affinity of hUBC9 interaction with ATF2.

Quantitative Analysis of hUBC9 Binding to ATF2 in the Yeast Two-hybrid Assay: Comparison with CREB and ATF1—Further studies in the yeast two-hybrid system were pursued to quantitatively compare the level of interaction of hUBC9 with ATF2 to its interaction with other proteins (lamin, CREB, and ATF1) and to null vectors expressing the GAL4 DNA binding domain or the activation domain alone. CREB and ATF1 are members of the ATF/CREB family of transcription factors, which bind to CRE sequences in the promoters of genes but, in contrast to ATF2, may be regulated by the cAMP-dependent pathway (38). CREB and ATF1 full-length cDNA (kindly provided by Dr. C.-Z. Giam, Case Western Reserve University, Cleveland, OH) were cloned into the yeast expression vector pGBT9 within the open reading frame of the GAL4 amino acids 1–147) DNA binding domain. Yeast cells of strain SFY526 containing the LacZ reporter gene were cotransformed with the hUBC9-pGAD10 plasmid and pGBT9-CREB, pGBT9-ATF1, or pGBT9-ATF2 as well as control plasmids. Transformants were grown on -Trp/-Leu yeast dropout plates at 30 °C for 4 days. Individual colonies were taken from each plate and grown overnight in liquid dropout medium at 30 °C. Equal amounts of cells were collected, and the activation of the reporter gene was measured by the quantitative liquid β-galactosidase assay (Fig. 4A) as well as by β-galactosidase filter assay (Fig. 4B).
transformed into BL21 pLysS E. coli and induced to express a GST-hUBC9 fusion protein. Purified GST-hUBC9 fusion protein was radiolabeled with \(^{32}\text{P}\)ATP in the presence of heart muscle kinase (Fig. 5A). The GST moiety was cleaved from the hUBC9 protein by utilizing a thrombin cleavage site engineered between GST and hUBC9 (Fig. 5B). Thus, in this way, we have determined that hUBC9 has an apparent molecular mass of \(\sim 19\) kDa as estimated by SDS-PAGE, which is consistent with the predicted amino acid sequence (Fig. 5B).

Purified ATF2 and purified actin were analyzed on SDS-PAGE and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was probed with the purified \(^{32}\text{P}\)hUBC9 in Far Western analysis as described under “Materials and Methods.” After extensive washing, the membrane was dried and autoradiographed. Fig. 5C showed that hUBC9 binds specifically to purified ATF2 but does not bind at all to actin, confirming the specific binding of hUBC9 to ATF2 also in a biochemical assay in vitro.

### Nuclear Localization of hUBC9

We generated an antibody to GST-hUBC9 that detected purified hUBC9 by enzyme-linked immunosorbent assay at a titer of $1:205,000$. This antibody detected a \(\sim 19\)-kDa protein in Western blot assays of protein extracts derived from murine lymph node, spleen, and thymus tissues and from Balb C 3T3 fibroblast cells (data not shown). To determine whether hUBC9 is found in the nuclei or translocated to the nuclei upon T cell activation, we purified nuclei from Jurkat T cells that were stimulated for 30 min with ConA + PMA. Equal amounts of the nuclear proteins and soluble proteins were analyzed on SDS-PAGE, transferred onto nitrocellulose, and blotted with anti-hUBC9 antibody. Fig. 6A demonstrates that hUBC9 has a molecular mass of \(\sim 19\) kDa and is more abundant in the nuclei as compared with the soluble fraction in both control and stimulated T cells. Stimulation of Jurkat T cells with ConA + PMA for 30 min was not associated with any change in the subcellular localization of hUBC9. A further subcellular fractionation experiment was performed to determine the possibility of cross-contamination of the nuclear and soluble fractions. Fig. 6B demonstrates equal protein staining of the nuclear and soluble fractions of Jurkat T cells. Using Western blot analysis, we have demonstrated that actin is significantly more abundant in the soluble fraction. Conversely, numatrin (also called B23 and nucleo-
phosphmin), which we have shown previously to be exclusively localized in the nuclear fraction (35), was completely absent from the soluble fraction (even in higher exposure numatrin could not be detected in the soluble fraction (data not shown). Reprobing the blot with anti-hUBC9 demonstrates the presence of this protein in both fractions (whereas numatrin could only be detected in the nuclei fraction). Densitometry analysis showed that the amount of hUBC9 in the nuclei is 3.9-fold higher as compared with the amount of hUBC9 in the soluble fraction. This verifies that hUBC9 is predominantly a nuclear protein. The evidence of the presence of hUBC9 in the nuclei is important because it corroborates with a potential physiological significance to the interaction of hUBC9 with ATF2 or nuclear proteins.

Evidence That ATF2 Is Ubiquitinated in Vivo and in Vitro—Further experiments were undertaken to examine whether ATF2 is ubiquitinated in vivo. To this end, Jurkat T cells were incubated for 17 h with various doses of a compound frequently used as an inhibitor of the proteasome LLnL (39–41). ATF2 was then immunoprecipitated from protein extracts, analyzed by SDS-PAGE, and blotted with affinity-purified antibody specific for conjugated ubiquitin. Fig. 7A demonstrates a dose-dependent increase in ubiquitination of ATF2 in Jurkat T cells treated with LLnL. To enable detection of the nonubiquitinated

FIG. 4. Comparison of hUBC9 binding to ATF2, CREB, ATF1 and lamin. Yeast cells SFY526 containing the LacZ reporter gene were cotransformed with the hUBC9-pGAD10 plasmid and pGBT9-CREB, pGBT9-ATF1, pGBT9-lamin, or pGBT9-ATF2, as well as null plasmids. Transformants were grown on −Trp−Leu yeast dropout plates at 30 °C for 4 days and then assayed for β-galactosidase activity in filter assay (B). Individual colonies were taken from each plate and grown overnight in liquid dropout medium at 30 °C, equal amounts of cells were collected and further assayed for β-galactosidase activity in liquid assay (A). Results represent the mean of (OD_{420}/OD_{600}) × 1000 of quadruplicate samples ± standard deviation. The experiment was performed two times with similar results.
ATF2 the membrane was stripped and rebotted with anti-ATF2. This analysis showed that the increase in the amounts of ubiquitinated forms of ATF2 was associated with a parallel dose-dependent decrease in the amounts of the major nonubiquitinated form of ATF2 (indicated as a in Fig. 7A), and a concomitant dose-dependent accumulation of degradation products of ATF2 (indicated as b and c in Fig. 7A). None of the various ATF2 forms were detected when protein extracts were precipitated with nonimmune IgG (see in Fig. 8C). The evidence of LLnL-induced ubiquitination of ATF2 in vivo and the concomitant accumulation of intermediate degradation products indicates that ATF2 may be a target of proteolytic processes involving the proteasome pathway.

To examine whether ATF2 can be ubiquitinated in vitro, purified GST-ATF2 fusion protein was exposed to standard ubiquitin conjugation assay using Jurkat T cell protein extracts and purified hUBC9 as a source of enzymes. After incubation at 37 °C for 40 min, the GST-ATF2 was purified using glutathione beads, analyzed on SDS-PAGE, and blotted with anti-ubiquitin antibody. Fig. 7B shows that ATF2 was extensively ubiquitinated in vitro (lanes 2 and 4), and that this process was significantly inhibited in the presence of EDTA as reported previously (39). No ubiquitination of ATF2 was detected in the absence of ubiquitin and source of enzymes (Fig. 7B, lane 1). The membrane was then further blotted with anti-ATF2 without stripping the anti-Ub antibody. This analysis showed that lanes 1 and 3, which lacked any reactivity with anti-Ub antibody, contained the nonubiquitinated forms ATF2, thus, confirming that ATF2 can be ubiquitinated in vitro. Notably, a condition that includes Jurkat cell extract without GST-ATF2 was not included in this experiment or in Fig. 7C because it was found in other experiments performed...
Evidence That Degradation and Phosphorylation of ATF2 Are Co-regulated in T Cells—The finding that ATF2 interacts with a ubiquitin-conjugating enzyme and is ubiquitinated in vivo implicates the ubiquitin-proteasome pathway in regulation of ATF2. To explore this possibility, we investigated whether ATF2 is exposed to proteolytic processes during T cell activation. In Fig. 8 (A–C), cells were stimulated for various periods of time with ConA + PMA and the proteins were immunoprecipitated with anti-ATF2 or with nonimmune IgG. The immunoprecipitates were analyzed on gels and further blotted with anti-ATF2 antibody. It was seen that there are no detectable changes in the abundance of the major form of ATF2 (indicated as a in Fig. 8A) in Jurkat T cells stimulated with ConA + PMA for various periods of time. However, minor lower molecular weight forms of ATF2 were detected specifically by ATF2 antibody in ATF2-immunoprecipitates and not in nonimmune IgG immunoprecipitates (Fig. 8A). These bands were found previously to be accumulated by LLnL (see Fig. 8A) and, thus, represent intermediate degradation products of hUBC9 in ubiquitination of ATF2.

FIG. 7. Ubiquitination of ATF2 in vivo and in vitro. A, ubiquitination of ATF2 in vivo. Jurkat T cells were incubated for 17 h with various amounts of LLnL: lane 1, 0; lane 2, 25 μM; lane 3, 50 μM; lane 4, 100 μM. Cells were lysed in RIPA supplemented with 5 mM EDTA and 50 μM LLnL as described under “Materials and Methods.” After incubation at 37 °C for 40 min, the GST-ATF2 was pelleted using glutathione beads, analyzed on SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with antibody to ubiquitin conjugates. To determine the presence and position of nonubiquitinated ATF2, the nitrocellulose membrane was further stripped from the anti-Ub antibody as described under “Materials and Methods” and then reblotted with anti-ATF2. Arrows indicate the positions of the nonubiquitinated forms of ATF2: the major form (a) and the degradation forms (b and c). WB, Western blot. B, ubiquitination of ATF2 in vitro. Purified GST-ATF2 fusion protein (~15 μg) was exposed to standard ubiquitin conjugation assay using Jurkat T cell protein extracts (25 μg) and purified hUBC9 (150 ng) as a source of enzymes as described under “Materials and Methods.” After incubation at 37 °C for 40 min, the GST-ATF2 was pelleted using glutathione beads, analyzed on SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-Ub antibody (left panel). To determine the presence and position of the nonubiquitinated ATF2, the nitrocellulose membrane was further reblotted with anti-ATF2 without stripping the Ub antibody (right panel). Arrows indicate the position of the nonubiquitinated forms of ATF2. C, ubiquitination of ATF2 in vitro is facilitated by hUBC9. Ubiquitin conjugation assay was performed as described under “Materials and Methods” using 5 μg of purified GST-ATF2 in the presence or absence of purified hUBC9 (150 ng) supplemented with 10 μg of Jurkat T cell extracts as a source of enzymes. The reaction was carried on for various periods of time from 0 to 180 min. GST-ATF2 was pelleted with the glutathione beads, analyzed on SDS-PAGE, and blotted with anti-ATF2 antibody. z indicates a protein that reacted nonspecifically in GST-ATF2 preparation with anti-ATF2 antibody.

by us that such conditions result in a completely empty lane with no reactivity at all with anti-ATF2 (data not shown).

To examine whether hUBC9 can potentiate the ubiquitination of ATF2 in vitro, the ubiquitin conjugation assay was performed in the presence or absence of purified hUBC9 supplemented with suboptimal amounts of Jurkat T cell extracts (10 μg) as a source of enzymes. GST-ATF2 was pelleted with the glutathione beads, analyzed on SDS-PAGE, and blotted with anti-ATF2 antibody. Fig. 7C demonstrates a time-dependent induction in ubiquitination of ATF2, as seen by the appearance of higher molecular weight forms of ATF2. It was demonstrated previously that hUBC9 facilitated the appearance of the ubiquitinated-ATF2 forms as compared with control samples, which were incubated with Jurkat T cell extract alone at 20 and 40 min. Notably, after incubation for 180 min in the presence of hUBC9 and Jurkat cell extract, it was seen that the majority of ATF2 was in the ubiquitinated form whereas most of the nonubiquitinated ATF2 was not present. This evidence, taken collectively with evidence of ATF2 interaction with hUBC9 in yeast two-hybrid, supports a functional role for hUBC9 in ubiquitination of ATF2.
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ATF2. We now report that ATF2 degradation products are constitutively present in unstimulated T cells and reproducibly demonstrated a rapid decrease in abundance 30 min after T cell activation (Fig. 8A).

In further experiments, the kinetics of the changes in ATF2 products was examined in the presence of LLnL (25 μM) and the proteins were analyzed on lower percentage acrylamide gel to enable better resolution of the various ATF2 forms (Fig. 8B). This experiment demonstrated rapid disappearance of the ATF2 degradation products (indicated as b and c in Fig. 7B) after 20 min of stimulation and further showed that this event was very transient. Specifically, it is seen that the degradation products started to accumulate again after 60 min and their abundance returned to control level after 5 h. The accumulation of an additional degradation product after 12 h of incubation with LLnL (but not in the absence of LLnL, Fig. 8A) corroborate with the previous observation in Fig. 8A. The LLnL-induced degradation product as well as the other ATF2 forms were not detected in immunoprecipitates of nonimmune IgG, confirming that these are ATF2-specific forms (Fig. 8C).

Intriguingly, the rapid disappearance of the ATF2 degradation products (b and c) after T cell activation was concomitant with appearance of slow mobility shift in the major ATF2 form, which is indicative of ATF2 phosphorylation (see low exposure in Fig. 8B). Indeed phosphorylation of ATF2 was shown to be detected with the appearance of slow mobility forms of ATF2 (24) and to occur in response to extracellular signals (23, 25, 26), including antigen stimulation of splenic B lymphocytes (59). Taken collectively, these results indicate that ATF2 is constitutively degraded in unstimulated Jurkat T cells, and that this degradation process is rapidly and transiently regulated (induced or reduced) upon T cell activation concomitantly with induction of phosphorylation of ATF2.

Previous studies have shown that inhibition of the proteasome pathway up-regulated the amounts of phosphorylated STAT transcription factor, implicating the ubiquitin proteasome pathway in negative regulation of phosphorylated STAT (42). Thus, in further experiments, we investigated the kinetics of the phosphorylation of the major ATF2 in the presence or absence of LLnL. Cells were preincubated for 1 h with LLnL (100 μM) and then stimulated with conA + PMA for 15 min, 45 min, or 4 h. Proteins were analyzed on SDS-PAGE without prior immunoprecipitation and blotted with anti-ATF2 antibody. Fig. 9 shows rapid induction of phosphorylation of the major ATF2 form (which is resolved into three distinct forms). This phosphorylation was apparent in the presence as well as in the absence of LLnL. After 4 h of stimulation with ConA + PMA in the absence of LLnL, the phosphorylated forms of ATF2 were not present, indicating negative regulation of the phosphorylated forms to control levels. However, in cells stimulated in the presence of LLnL the two higher phosphorylated forms of ATF2 were still detected after 4 h (see arrows on right side in Fig. 9), indicating that LLnL interfered in the down-regulation of ATF2 phosphorylation. This implicates a role for degradation processes in the negative regulation of the phosphorylation of ATF2 in activated T cells. Although further studies will be required to unravel the precise nature and cross-talk of these processes, the data presented may implicate degradation processes in regulation of signaling of ATF2 in T cells.

**Discussion**

The yeast two-hybrid interaction system was employed to elucidate the mechanisms that regulate ATF2 during lymphocyte activation. We report a novel ATF2-interacting protein that is a new member of the ubiquitin-conjugating enzyme family, hUBC9. We demonstrate nuclear localization of hUBC9 and its interaction with two other CRE-binding transcription factors: CREB and ATF1. Evidence is presented to demonstrate that ATF2 is exposed to degradation processes in T cell and that it is ubiquitinated in vivo and in vitro. Taken collectively, this evidence implicates a role for hUBC9 and the proteasome...
pathway in regulation of ATF2 in T cells.

The ubiquitin-dependent degradation system is a major pathway in the selective degradation of proteins in eukaryotes. Evidence is accumulating to show that ubiquitination is involved in regulation of gene expression, DNA repair, cellular stress response, cell cycle progression, signal transduction, and programmed cell death (for review, see Refs. 31–33 and 43). In the ubiquitination reaction, ubiquitin, a highly conserved protein of 76 amino acids, is first activated by an activation enzyme, E1. It is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2 class). The E2 enzyme, either alone or together with a ubiquitin-protein ligase, E3, catalyzes transfer of ubiquitin to a lysine residue of a target protein. The ubiquitinated protein is recognized and degraded by a multisubunit 26 S proteasome complex. Ubiquitination of some proteins, such as calmodulin, histones, and certain membrane receptors, may serve a regulatory function without targeting them to cytosolic degradation (44).

The ubiquitin-conjugating enzymes (E2) are a family of proteins characterized by a highly conserved catalytic site containing an invariant active cysteine site (31–33). Previous studies in yeast Saccharomyces cerevisiae have identified at least 10 different UBCs (32) that are involved in various cellular processes such as DNA repair, cell cycle progression and in heat shock resistance, suggesting that E2 enzymes may be key players in establishing the diversity and the specificity of the ubiquitin-proteolytic system. Yeast UBC9 protein and its homolog, Husc5, have been shown to be essential for cell viability (32) and for normal mitosis (45). Repression of UBC9 synthesis in S. cerevisiae results in cell cycle arrest in G2/M phase (46). In this case, UBC9 was implicated in the degradation of mitotic B-type cyclins, Cdc5 and Cdc2 (43). Although the sequence of the human homolog of UBC9 has been reported recently (28–30), the function of hUBC9 is not yet known.

In this work, the specificity and affinity of hUBC9 interaction with ATF2 in vitro was markedly demonstrated in a quantitative two-hybrid assay showing a high affinity of hUBC9 to ATF2 and lack of any interaction with lamin or with the GAL4 DNA binding domain. Intriguingly, hUBC9 demonstrated lower but significant interaction with CREB and ATF1, indicating the potential involvement of hUBC9 also in regulation of these transcription factors. In this respect, it is noteworthy that hUBC9 is demonstrated to localize primarily in the nucleus. Indeed, hUBC9 was originally identified via its interaction in yeast two-hybrid with other nuclear proteins: W1 transcriptional repressor (29), Rad51 (28), and the human papillomavirus type 16 E1 (30). Although the nature and the function of this interaction remains to be investigated, it is possible that hUBC9 is an important ubiquitin-conjugating enzyme in the nucleus that plays a crucial role in regulation of nuclear proteins and particularly transcription factors.

Notably, the interaction of hUBC9 with ATF2 was ~10-fold higher as compared with CREB and ATF1, indicating that hUBC9 may have a more significant role in interaction with ATF2. The marked specificity of hUBC9 binding to ATF2 was further confirmed in vitro in the Far Western blot, showing that purified [32P]hUBC9 binds to ATF2 protein, whereas it does not bind at all to actin.

Several lines of evidence indicate that the interaction of hUBC9 and ATF2 may have an important physiological significance. Most significant, by using an inhibitor of the proteasome, LLnL, we demonstrate that ATF2 is ubiquitinated in vivo. LLnL also induced a marked dose-dependent accumulation of intermediate degradation products of ATF2. We further demonstrate that the abundance of the ATF2 degradation products is tightly regulated during T cell activation. Specifically, we show that the ATF2 degradation forms are expressed constitutively in nonstimulated Jurkat T cells and that T cell activation is associated with a rapid and transient disappearance of these ATF2 degradation products. Because the presence of these degradation products represents a steady state equilibrium between the rate of their formation and the rate of their final processing, the evidence of their disappearance indicates that, upon T cell activation, there is an interference in this steady state equilibrium. Thus, upon T cell activation, there is either a marked increase in the final processing of these degradation forms or a marked decrease in the formation of these degradation products via degradation of the major ATF2 forms.

Intriguingly, the change in the abundance of the ATF2 proteolytic products was concomitant with the induction of phosphorylation of the major ATF2 form. Thus, it is tempting to speculate that induction of phosphorylation of ATF2 may have an effect on its rate of degradation. ATF2 was shown to be rapidly phosphorylated in response to several external stimuli such as growth factors and UV irradiation (23–26). This phosphorylation is mediated by JNK, a member of the MAP kinase family, in a process that was shown to involve physical binding of JNK to ATF2. Thus, it is possible that binding of ATF2 to hUBC9 may affect its ability to bind to JNK and vice versa. Indeed, a recent study demonstrated that phosphorylation of c-Jun by JNK or MAP kinase is accompanied by a reduction in c-Jun ubiquitination and consequently degradation (47). Thus, phosphorylation of c-Jun stabilizes the protein by inhibiting its ubiquitination (47). This evidence may be particularly pertinent in view of several lines of similarity between c-Jun and ATF2. Jun and ATF2 interact via heterodimer complexes in binding to either CRE or TPA-responsive element consensus sequences in the promoter of genes (see Refs. 38 and 48 and references therein). The activation of both c-Jun (Ref. 49; see also Ref. 26 and references therein) and ATF2 (23–26, 50) is mediated via phosphorylation, which occurs after activation by growth factors and UV radiation and is catalyzed by the MAP kinase family pathways. In addition, extensive evidence indicates that c-Jun is regulated by ubiquitination (47, 53, 54). Most recently, it has been reported that hUBC9 interacts specifically with the C-terminal half of c-Jun but not with c-Fos in yeast two-hybrid (57), indicating that hUBC9 may be the enzyme that regulates the ubiquitination of c-Jun. Thus, our studies indicate another line of similarity between ATF2 and c-Jun; both may be regulated by ubiquitination, which may involve hUBC9. The evidence that c-Jun phosphorylation stabilizes the protein by inhibiting its ubiquitination may corroborate with the observations of this work and suggests that a cross-talk between phosphorylation and degradation processes may be another similar characteristic of both c-Jun and ATF2. This possibility should be investigated in future studies.

Because there is no change in the abundance of the major ATF2 form during T cell activation, a question of importance is: what is the physiological function of the apparent proteolytic process of ATF2? Recent studies have brought to attention the crucial role of the ubiquitin-dependent degradation in regulation of signal transduction of discrete transcription factors (for review, see Ref. 51). Specifically, studies demonstrated the role of ubiquitination in regulation of the transcription inhibitor IκB (Ref. 52; see Ref. 43, and references therein), the transcription factors p53 (Ref. 51, and references therein), STAT (42), and c-Jun (47, 53, 54). A special cross-talk between ubiquitination and phosphorylation has been demonstrated in previous studies related to IκB, Jun, STAT, and TCR (42, 47, 52, 58). Ligand-dependent ubiquitination of T cell antigen receptor was shown to be dependent on its tyrosine phosphorylation by p56 lck (58). Furthermore, the tyrosine phosphatase CD45 is
also necessary for antigen occupancy-stimulated TCR ubiquitination (58). Ubiquitination of the transcription factor STAT has been suggested to be responsible for negative regulation of the phosphorylation signal (42). In this respect, it is interesting that LLnL was found to inhibit the down-regulation of the phosphorylation signal (42). In this respect, it is interesting

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