IEX-1 (immediate early response gene X-1) is a stress-inducible gene. Its overexpression can suppress or enhance apoptosis depend-ent on the nature of stress, yet the polypeptide does not possess any of the functional domains that are homologous to those present in well characterized effectors or inhibitors of apoptosis. This study using sequence-targeting mutagenesis reveals a transmembrane-like integrated region of the protein to be critical for both pro-apoptotic and anti-apoptotic functions. Substitution of the key hydrophobic residues with hydrophilic ones within this region impairs the capacity IEX-1 to positively and negatively regulate apoptosis. Mutations at N-linked glycosylation and phosphorylation sites or truncation of the C terminus of IEX-1 also abrogated its potential to promote cell survival. However, distinguished from the transmembrane-like domain, these mutants preserved pro-apoptotic activity of IEX-1 fully. On the contrary, mutation of nuclear localization sequence, despite its importance in apoptosis, did not impede IEX-1-mediated cell survival. Strikingly, all the mutants that lose their anti-apoptotic ability are unable to prevent acute increases in pro-duction of intracellular reactive oxygen species (ROS) at the initial onset of apoptosis, whereas those mutants that can sustain anti-death function also control acute ROS production as sufficiently as wild-type IEX-1. These findings suggest a critical role of IEX-1 in regulation of intracellular ROS homeostasis, providing new insight into the mechanism underlying IEX-1-mediated cell survival.

A coordinated balance between cell survival and apoptosis is essential for embryonic development, tissue homeostasis, and cellular responses to various types of stress (1). Defects in this balance may contribute to a variety of diseases, including cancers, autoimmune disorders, and aberrant embryonic development (2). Programmed cell death occurs in mitochondrion-dependent and independent pathways, and the former accounts for most forms of apoptosis in response to cellular stress, loss of survival factors, and developmental cues (3, 4). The mitochondrial pathway triggers cell death as a consequence of alteration in mitochondrial membrane permeability induced by apoptotic effectors like oxidative stress and signaling-mediated translocation of Bax, Bad, Bim, or dATPase, leading to the release of cytochrome c and other proteins contained in the mitochondrial intermembrane space (5, 6). Substantial evidence indicates that prior to an irreparable loss of mitochondrial structural integrity, apoptotic effectors often stimulate an acute increase in the mitochondrial membrane potential $\Delta \Psi_m$ that facilitates the formation of reactive oxygen species (ROS), the amplitude of which determines a cell to die by apoptosis or to adapt (4, 7–11). Conceivably, prevention of ROS production in the initial phase of apoptosis is essential to guard the integrity of mitochondrial membrane, protecting cells from undergoing apoptosis.

IEX-1 (immediate early response gene X-1), also known as IER3, p22/PRG1, Dif-2, or the mouse homologue gly696, is a stress-inducible gene (12–15). It can be rapidly induced in various cells by irradiation, viral infection, inflammatory cytokines, chemical carcinogens, growth factors, and hormones under the control of transcription factors such as NF-kB/reI complexes, p53, Sp1, c-Myc, and Ap-1 (12–19). Like other immediate early response genes, IEX-1 plays a pivotal role in cell survival under conditions of stress (20–22). In vivo targeted expression of IEX-1 to lymphocytes protects activated T cells from apoptosis, giving rise to an extended immune response after antigen stimulation and predisposing to a lupus-like autoimmune disease and T cell lymphoma in mice (23, 24). Gene-targeted deletion of IEX-1 caused hypertension and cardiac hypertrophy in mice, but the underlying mechanisms remain elusive (25).

In the present study, we identify distinct as well as overlapping functional regions for anti- and pro-apoptotic activities of IEX-1. Substituting three key hydrophobic residues with hydrophilic ones in the transmembrane (TM)-like sequence is sufficient to abrogate effects of IEX-1 on inhibition and promotion of apoptosis. Additionally, N-linked glycosylation and phosphorylation as well as the C-terminal sequence of IEX-1, are all crucial for anti-apoptosis activity of IEX-1, but these structures appear to exert no discernible role in pro-apoptosis. On the contrary, mutation of nuclear localization sequence, despite its importance in apoptosis, did not impede IEX-1-mediated cell survival (26). IEX-1-mediated cell survival is found to correlate well with its ability of reducing intracellular ROS formation either at basal levels or immediately after apoptotic stimulation. These findings, in line with IEX-1 localization in mitochondria, suggest a key role for IEX-1 in regulation of ROS homeostasis in cells.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—Sequence-targeting mutagenesis of human IEX-1 was carried out according to a PCR overlap extension technique using primers containing the expected mutations. The primers were forward 5'-ATGGCACAATCTCGAGGCGGCACACC-3' and reverse 5'-TAAAGGCGGCCGCGGTGGTGGTCTC-3' for generating IEX-1 C2AC7A mutant; forward 5'-ACCGAGCACGAGTGCACCGGTTCCTCTCACCC-3' and

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*This work was supported by National Institutes of Health Grant AI050822, Research Scholar Grant RSG-01-178-01-MG0 from the American Cancer Society, and a Moran foundation award (Grant PRU 00–114) from the Baylor College of Medicine (to M. X. W). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: ROS, reactive oxygen species; TM, transmembrane; WT, wild type; GFP, green fluorescent protein; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; Ab, antibody; DCF, 2',7'-dichlorofluorescein; CM-H2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase.
reverse 5'-GGTAGAGAACGCGTCACATGCTGTCGGTTG-3' for K60AR63A mutant; and forward 5'-CTAACACTACAGGCTTCTCTCTGTCAATACCATACGGCC'-3' and reverse 5'-GCCATGTTGATTTGACAGAGCGCTGTTAGTGTAG-3' for transmembrane segment mutation (TM-mutant). To substitute N-glycosylated asparagine at position 133, the following primers were employed to insert an Nhel restriction enzyme site: forward 5'-GGTAGAGAACGCGTGCACTGCGTGCTCGGTG-3' and reverse 5'-CGATGCGACGCAGCTGGCGTTG-3' for amplification of cDNA sequence 1–414; and forward 5'-TGTACGAGCTTCCGACCTCAGGTCTGC-3' and reverse 5'-CGATGCGACGCAGCTGGCGTTG-3' for sequence 402–471. The two PCR products were then fused in pBluescript using an Nhel restriction enzyme site. Truncation of 18 residues at the C terminus was obtained by amplification of IEX-1 cDNA sequence ranging from 1 to 414 by PCR. All the IEX-1 mutants were verified by DNA sequencing, T18A mutant was a kind gift from Dr. F. Porée (Universite Rene Descartes, Paris, France) (21). Wild-type (WT) IEX-1 and its variants were cloned into mammalian cell-expression plasmid pcDNA3 (Invitrogen). For localization studies, WT IEX-1 and its TM-mutant were cloned in-frame into pEGFP-N3 (Clontech) to generate GFP fusion proteins.

Transfection and Apoptosis Assays—Chinese Hamster Ovary (CHO) and p65KO3T3 cells were seeded into gelatinized 12-well plates and cultured overnight in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO2. p65KO3T3 is a mouse embryonic fibroblast cell line derived from NF-κB p65-knock out mice (27). The cells were transfected in duplicate with various constructs, along with a LacZ-expressing reporter plasmid at a 4:1 ratio of IEX-1 construct to reporter plasmid, using Polyfect transfection reagent (Qiagen). After 36 h, the cells were treated with apoptosis inducers for indicated times, followed by fixation of the cells with 2% formaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline for 5 min at room temperature, and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) overnight at 37 °C. Percentages of apoptosis were blindly evaluated by counting a minimum of 200 lacZ-positive cells in three randomly selected areas of each well in duplicate samples using an inverted microscope. Apoptotic cells were determined by characteristic morphology of apoptosis (28). Alternatively, relative caspase-3 activities, indicative of apoptosis, were measured in cell lysates using a caspase-3 fluorometric substrate. Briefly, the cells were transfected with WT IEX-1 or its variant constructs and stimulated as above, followed by lysis of the cells on ice for 30 min in a cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with 1% protease inhibitor mixture (Sigma). After centrifugation at 13,000 × g for 5 min at 4 °C, the supernatant (50 μl) was incubated in triplicate for 10 min at room temperature with a caspase-3 fluorometric substrate (Upstate, Charlottesville, VA) at a final concentration of 50 μM in the lysis buffer. The caspase-3 fluorometric substrate, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin was hydrolyzed by active caspase-3 specifically, releasing fluorescent 7-amido-4-methylcoumarin that was measured at 0 and 10 min, with the fluoroMax-3 spectrophotometer using 380/460 nm as excitation/emission wavelength. A blank was measured in parallel without substrate and subtracted from all the measurements.

Isolation of Mitochondria—293T cells stably transfected with either WT IEX-1 or TM-mutant were suspended on ice for 10 min in homogenizing buffer (H buffer; 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM HEPES, and 1:100 protein inhibitor mixture, pH 7.4) and Dounce-homogenized. The resultant homogenate was centrifuged at 600 × g for 10 min at 4 °C to remove nuclei and unbroken cells. The remaining supernatant was spun at 7,000 × g for 15 min at 4 °C to obtain a crude mitochondrial pellet and an ER-enriched supernatant as described (28). The mitochondridrion-enriched pellet was washed twice by centrifugation at 7,000 × g for 15 min at 4 °C, re-suspended in H buffer, and loaded onto a continuous sucrose gradient from 0.8 M to 1.5 M in H buffer, followed by centrifugation at 35,000 rpm for 2 h at 4 °C using an SW41-Ti rotor (L-90K Ultracentrifuge, Beckman Coulter). The visible mitochondrial band in the sucrose gradient was carefully collected and subjected to two more rounds of sucrose gradient fractionation as above. After a final centrifugation, samples were collected consecutively from the bottom of the centrifuge tube at 0.5 ml per fraction. Each fraction was diluted with 1 ml of H buffer, pelleted by centrifugation as above, and analyzed by Western blotting using a polyclonal antibody (Ab) specific for IEX-1.

Detection of Intracellular ROS—Intracellular ROS were assayed by measuring intracellular oxidation of 2',7'-dichlorofluorescein (DCF) as described (29). The substrate is 5-(and-6)-chloromethyl-2',7'-dichlorofluorescin diacetate, acetyl ester (CM-H2DCFDA, Invitrogen). It is nonfluorescent until removal of the acetate groups by intracellular oxidation and thus serves as a cell-permeate indicator for ROS. To measure intracellular ROS, the substrate was added directly to cell culture at a final concentration of 5 μM and incubated for 15 min at 37 °C followed by two washes with phosphate-buffered saline. DCF fluorescence intensity was measured randomly at six different fields per well with a Spectra MAX GEMINI EM microplate fluorometer using 488/525 nm as excitation/emission wavelength, and the average fluorescence intensity was given by the instrument. Background controls were cells transfected with the same construct without substrate loading, and two wells for each construct were measured in parallel.

Western Blot Analysis—Cells transfected with indicated constructs were lysed in cell-lysis buffer and centrifuged to remove nuclei and cell debris. Approximately 50 μg of proteins of whole cell lysate were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with a polyclonal Ab specifically recognizing IEX-1, which was raised in rabbits against IEX-1 peptide sequence 51–75 (30). IEX-1 and its mutants were detected by incubation of the membrane with horseradish peroxidase-linked goat anti-rabbit Ab. The x-ray films were developed using a SuperSignal West Pico Kit (Pierce). The membrane was stripped and re-probed by anti-glyceraldehyde-3-phosphate dehydrogenase Ab or anti-cytokine C Ab (BD Sciences) for protein loading controls.

Confocal Laser Scanning Microscopy—To determine intracellular localization of IEX-1, MCF-7 and CHO cells on gelatinized coverslips were transfected with GFP vector control, IEX-1-GFP, or TM-mu-GFP construct for 36 h and examined directly by confocal laser-scanning microscopy. To determine localization of IEX-1 in mitochondria, cells expressing GFP, IEX-1-GFP, or TM-mu-GFP were counterstained for 30 min at 37 °C in complete culture medium with MitoTracker red580 (Molecular Probes Inc.), a red fluorescence specific for mitochondria, at a concentration of 50 nM, followed by five washes with phosphate-buffered saline. The stained samples were mounted and analyzed on a Leica TCS4D confocal laser scanning microscope equipped with Leica Confocal Software Version 2.5.

Statistical Analysis—Two-tailed Student’s t tests were used to analyze the statistical significance of experimental samples compared with relevant controls.

RESULTS

Target Mutagenesis of IEX-1—IEX-1 does not share any of the functional domains significantly homologous with those presented in well characterized pro- or anti-apoptotic effectors, despite its pivotal role in...
regulation of apoptosis. To delineate functional domains of IEX-1, a series of mutants were made on the basis of residue charge, hydrophobicity, conservation, and demonstrated functional significance in other systems, as depicted in Fig. 1A. A C2A/C7A mutant has an alanine substitution in the place of two cysteines at positions 2 and 7, on the assumption that they may be involved in formation of an intra- or inter-peptide disulfide bond and are required for IEX-1-mediated regulation of apoptosis. IEX-1 contains a polybasic sequence HKR5RR, spanning amino residues from 58 to 64, respectively. The sequence matches a canonical nuclear localization sequence motif, K(K/R)X(K/R) (13, 26, 31). Targeting Lys and Arg at positions 60 and 63 for Ala replacement generated a K60A/R63A mutant. Like Bcl-2 and Bcl-xL, IEX-1 has a TM-like integrated region at positions 86–101 that can potentially target the protein to the ER, Golgi, and the mitochondria as predicted by the program PSORT. In an attempt of disrupting intracellular localization of IEX-1, three hydrophobic residues at positions 90, 93, and 99 were converted to hydrophilic Thr or Ser, respectively, within the TM-like domain. Four mutants were generated: A K60AR63A, TM-mutant. B, expression of IEX-1 and its derivatives. Equal amounts of cell lysates prepared from CHO cells transfected with indicated constructs were analyzed by immunoblotting using anti-IEX-1 Ab. Protein loading control is shown in the lower panel using anti-glyceraldehyde-3-phosphate dehydrogenase Ab (GAPDH), and molecular mass markers (MW) are indicated in kDa on the right. One representative result of three experiments performed is shown.

**FIGURE 1.** A schematic diagram of IEX-1 and its variants (A) and their protein expression (B). A, unfilled rectangles and hatched areas represent a nuclear localization sequence and a TM-like domain (TM), respectively. The numbers indicate relative sequence positions in IEX-1 polypeptide. C, cysteine; T, threonine; K, lysine; R, arginine; L, leucine; N, asparagine; I, isoleucine; A, alanine; S, serine; D, deletion; and TM-mu, TM-mutant. B, expression of IEX-1 and its derivatives. Equal amounts of cell lysates prepared from CHO cells transfected with indicated constructs were analyzed by immunoblotting using anti-IEX-1 Ab. Protein loading control is shown in the lower panel using anti-glyceraldehyde-3-phosphate dehydrogenase Ab (GAPDH), and molecular mass markers (MW) are indicated in kDa on the right. One representative result of three experiments performed is shown.

The TM-like Domain Is Required for Both Anti- and Pro-apoptosis—To determine effects of IEX-1 mutations on cell survival, WT IEX-1 and its variant constructs were transfected, along with or without a β-galactosidase-expressing reporter plasmid, into CHO cells, followed by stimulation of the transfectants for 6 h with staurosporin. Apoptosis was evaluated by counting apoptotic cells among β-galactosidase-positive cells (Fig. 2) or measurement of caspase-3 activity (Fig. 3). Staurosporin induced ~50% of cell death in vector-transfected cells over background controls (Fig. 2A). The staurosporin-triggered apoptosis was diminished by half in cells expressing WT IEX-1, an observation similar to the early investigation (21). Expression of C2A/C7A and K60A/R63A mutants of IEX-1 also prevented cells from staurosporin-induced cell death to a degree comparable to WT IEX-1 (Fig. 2A). The result suggests that the two cysteines at positions 2 and 7 as well as the nuclear localization signal are not required for anti-apoptotic activity of IEX-1. In contrast, CHO cells expressing the TM-mutant underwent apoptosis indistinguishable from the cells transfected with a control plasmid (Fig. 2A). Deletion of the entire TM segment, ranging from 86 to 101 residues, yielded a similar result (data not shown). These data suggest a critical role for the TM-like region in IEX-1-regulated cell survival. As a positive mutation control, we included IEX-1 T18A mutant in the assay, in which a threonine at position 18, a potential ERK phosphorylation site, was replaced by alanine (21). As reported previously, expression of T18A mutant was also ineffective in preventing cells from apoptosis triggered by staurosporin. Similar results were obtained when these transfectants were treated with varying concentrations of H2O2 (Fig. 2B) or when p65KO3T3 cells were transfected with these IEX-1 variants and stimulated by TNF-α (Fig. 2C).

The described percentages of cell death were consistent with increased caspase-3 activity that detects apoptotic cell death in a more objective manner as compared with counting apoptotic cells in the basis of their morphology. As shown in Fig. 3A, staurosporin treatment increased caspase-3 activity in CHO cells by ~60% in vector-transfected cells as compared with dimethyl sulfoxide solvent-containing background controls. The increment was, however, reduced to 20% or <20% over background after expressing WT IEX-1, C2A/C7A, or K60A/R63A mutants (Fig. 3A). Mutation of the TM-like domain in IEX-1 again severely impaired its ability to prevent caspase-3 activation induced by staurosporin. This also was true for H2O2-induced caspase-3 activation in CHO cells (Fig. 3B) as well as TNF-α-stimulated caspase-3 activation in p65KO3T3 cells (data not shown). These results indicate that the TM-like domain is indispensable for IEX-1-mediated protection against apoptosis induced by both intrinsic and extrinsic stimuli.
Apart from anti-death effects, IEX-1 has also been shown to facilitate apoptosis under conditions of serum deprivation (32). To see whether these mutations had any effect on pro-apoptotic activity of IEX-1, CHO cells were harvested, lysed, and reacted with a caspase-3-specific fluorometric substrate. Fluorescence intensity was measured immediately (0 min) or 10 min after addition of the substrate by FluoroMax-3 spectrometer using 380/460 nm as excitation/emission wavelength. A blank was measured in parallel without substrate and subtracted from all the measurement. Relative caspase-3 activity is expressed as percentage increases (means ± S.D.) in fluorescence intensity at 10 min relative to 0 time point in the cells transfected with the same construct. **, statistical significance (p < 0.01) in the presence versus absence of overexpressed IEX-1 or its variants after apoptotic stimulation. One representative result of three independent experiments performed is shown.

**FIGURE 3.** Effects of IEX-1 and its variants on caspase-3 activation. CHO cells were transfected with indicated constructs and stimulated in triplicate for 6 h with or without 400 nM staurosporin (STS) (A) or 1 mM H2O2 (B). At the end of stimulation, cells were harvested, lysed, and reacted with a caspase-3-specific fluorometric substrate. Fluorescence intensity was measured immediately (0 min) or 10 min after addition of the substrate by FluoroMax-3 spectrometer using 380/460 nm as excitation/emission wavelength. A blank was measured in parallel without substrate and subtracted from all the measurement. Relative caspase-3 activity is expressed as percentage increases (means ± S.D.) in fluorescence intensity at 10 min relative to 0 time point in the cells transfected with the same construct. **, statistical significance (p < 0.01) in the presence versus absence of overexpressed IEX-1 or its variants after apoptotic stimulation. One representative result of three independent experiments performed is shown. The finding stresses an indispensable role for the TM-like domain in regulation of both anti- and pro-apoptotic activities of IEX-1.

Although important in anti-apoptotic activity, T18A mutant, similar to its WT counterpart, significantly enhanced apoptosis upon serum withdrawal, suggesting that ERK-induced phosphorylation at position 18 is not essential for the observed pro-apoptotic activity of IEX-1. Perhaps the ERK signaling pathway does not work appropriately under this condition. Conversely, a K60A/R63A mutant with an altered nuclear localization sequence showed no apoptosis-enhancing effects over background (Fig. 4A), in contrast to its insignificance in cell survival, confirming early investigation (26). Thus, our results delineate distinct regions of IEX-1 for its anti- and pro-apoptotic activities and as a corollary, discrete nonoverlapping mechanisms are likely to mediate these two opposing activities conferred by IEX-1.

**N-Glycosylation and the C Terminus Are Crucial for Anti-apoptosis but Not for Pro-apoptosis**—We next tested a role for the lumen tail portion of the protein in anti- and pro-apoptotic activities of IEX-1. Sequence

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Functional Domains of IEX-1

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**FIGURE 2.** Effects of IEX-1 and its variants on apoptosis induced by various stimuli. A, staurosporin-induced apoptosis. CHO cells were transfected with indicated constructs, along with a β-galactosidase-expressing reporter for 36 h, and then either left untreated or treated with dimethyl sulfoxide (DMSO) alone or together with 400 nM staurosporin (STS) for 6 h. Percentages (means ± S.D.) of apoptotic cells were obtained by averaging the results from randomly selected three areas of each well of 12-well plates with each construct in duplicate (n = 6). **, statistical significance (p < 0.01) in the presence versus absence of overexpressed IEX-1 or its variants after apoptotic stimulation. One representative result of three independent experiments performed is shown. B, H2O2-induced apoptosis. CHO cells were transfected as above and then either left untreated (data not shown) or treated in duplicate with 15 μM H2O2 for 7 h. Apoptosis and statistical significance were determined as described in A. One representative result of three independent experiments performed is shown. C, TNF-α-induced apoptosis. p53KO3T3 cells were transfected with indicated constructs, along with a β-galactosidase-expressing reporter for 36 h, and then either left untreated (data not shown) or treated in duplicate with 15 μg/ml cycloheximide (CHX) alone or along with 100 units/ml TNF-α for 8 h. Apoptosis and statistical significance were determined as described in A. One representative result of two independent experiments performed is shown.
Functional Domains of IEX-1

Analysis using the PSORT program predicts IEX-1 to be a type II TM protein with the N terminus exposed to the cytoplasm while the C terminus is in the lumen of the endomembrane compartment. Although WT IEX-1 protected >50% apoptosis induced either by staurosporin or by H$_2$O$_2$ (data not shown) in CHO cells or by TNF-α in p65KO3T3 cells (Fig. 5, A and B), a mutation at the putative N-linked glycosylation site or truncation of the C terminus both abrogated the protection against apoptosis induced by these stimuli. In accordance to this, the two mutants displayed little influence on caspase-3 activation stimulated by staurosporin or H$_2$O$_2$ (Fig. 3, A and B) or by TNF-α (data not shown). These observations demonstrate the necessity of these structures in IEX-1-induced cell survival, in contrast to their little effect on pro-apoptotic activity. Cells expressing these two mutants underwent apoptosis as efficiently as the cells harboring IEX-1 under serum deprivation (Fig. 4B).

The TM-like Domain Is Not Required for Transporting IEX-1 to Mitochondria—The importance of the TM-like domain in both pro- and anti-apoptosis regulated by IEX-1 prompted us to further address whether the hydrophobic segment played a determinant role in localizing IEX-1 in mitochondria. To this end, WT IEX-1 and TM-mutant were fused in-frame with GFP at the N terminus of GFP and introduced into MCF-7 cells (Fig. 6A) or CHO cells (data not shown). Cells expressing GFP alone had a diffuse green fluorescent pattern throughout a cell, whereas cells transfected with IEX-1-GFP expressed a punctuate fluorescence pattern in the cytosol and a few fluorescent scatters in the nucleus, clearly suggesting IEX-1 to be an intracellular membrane-associated protein or to be associated with other proteins in subnuclear compartment of the nucleus (Fig. 6A) (26). Counterstaining of cells expressing IEX-1-GFP with MitoTracker, a red fluorescence marker specific for the mitochondria, revealed that IEX-1 expression was partially co-localized with the mitochondria, probably due to the presence of mitochondrial heterogeneity (Fig. 6A) (33).

To our surprise, a similar co-localization with the mitochondria was also seen in cells expressing TM-mu-GFP, albeit with a slightly lesser sufficiency, suggesting that the TM-like region is not essential for entrance of IEX-1 to mitochondrial compartment (Fig. 6A). To verify this, mitochondria were purified from IEX-1-transfected and TM-mutant transfected 293T cells as detailed under "Experimental Procedures." All IEX-1 isoforms were recovered in mitochondria as IEX-1, in agreement with the fluorescence and data not shown). Identical distribution of IEX-1 and cytochrome c in a continuous sucrose gradient indicates an association of IEX-1 with mitochondria biochemically. Through a similar mitochondrial purification procedure, we also detected TM-mutant in purified mitochondria as IEX-1, in agreement with the fluorescence pattern of cells transfected with a TM-mu-GFP construct (Fig. 6C). We did not see N133A/L134S and the C-terminally truncated IEX-1 in purified mitochondria, although the proteins were in association with cytosolic membrane fractions (data not shown).
IEX-1-regulated Cell Survival through Control of Intracellular ROS Production—Our data showed that IEX-1 protected cells from apoptosis induced by both intrinsic and extrinsic stimuli. In agreement with this, IEX-1 was found in mitochondria, the central machinery for regulation of apoptosis. To address the mechanism whereby IEX-1 protected cells from apoptosis, we evaluated intracellular ROS production in cells harboring WT IEX-1 or its variants. In the absence of any stimulation, overexpression of IEX-1 reduced intracellular ROS production by >60% (Fig. 7A). Similar reductions in ROS production were also observed in CHO cells transfected with a C2A/C7A mutant (~50%) or a K60A/R63A mutant (~30%). In sharp contrast, cells carrying mutants that were unable to protect cells from apoptosis, including TM-mutant and the N133A/L134S and A139–156 mutants, produced intracellular ROS at levels either higher than or similar to that of vector-transfected cells (Fig. 7A). Moreover, after stimulation with staurosporin, ROS was acutely increased in 15 min, an event occurring prior to alteration in mitochondrial membrane permeability, followed by a rapid decline in vector-transfected cells as well as cells harboring a TM-mutant or a N133AL134S mutant. However, ROS levels in cells transfected with WT IEX-1 or C2A/C7A or K60A/R63A mutant remained relatively constant under similar conditions (Fig. 7B). The correlation of the ability of IEX-1 to suppress apoptosis with ROS modulation underscores a role of IEX-1 in regulation of ROS homeostasis in cells.

DISCUSSION

Our current studies, in line with early investigations, suggest that pro- and anti-apoptotic activities of IEX-1 are conducted by distinct functional domains (21, 26, 34, 35). As a corollary, the underlying mechanisms whereby IEX-1 promotes or blocks apoptosis must differ. T18 phosphorylation, N-glycosylation, and the C terminus of IEX-1 are critical for anti-death function of IEX-1, but these structures display no significant effects on pro-apoptotic activity. On the contrary, nuclear localization sequence mutation abrogates IEX-1-facilitated apoptosis and disrupts its nuclear localization (26). Yet, the mutant can protect cells from apoptosis as sufficiently as WT IEX-1. These observations suggest that although pro-apoptosis is associated with localization of IEX-1 in subnuclear structure of the nucleus (26), its anti-apoptosis activity is apparently related to its localization in other cell organelles such as mitochondria than the nucleus.

IEX-1 is expressed as multiple protein bands as have been observed previously in cells overexpressing IEX-1 and in endogenous IEX-1 expression (Fig. 1B) (15, 24, 30). Because IEX-1 contains more than ten O-glycosylation sites as predicted by the NetOGlyc 3.1 Server or the
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YinOYang 1.2 program, in addition to one N-linked glycosylation, these multiple isoforms may result from glycosylation at varying degrees or other post-translational modifications. As shown in Fig. 1B, the two major isoforms of IEX-1 are usually observed around 23–25 and 18–20 kDa in IEX-1-transfected cells. Removal of the N-linked glycosylation site gave rise to two protein bands of ~29 and 31 kDa, migrating slower than the two WT isoforms (Fig. 1B). Mutation of the TM-like region also gave rise to one isoform that is larger than the two WT isoforms. The increases, rather than decreases, in the molecular weight of IEX-1 protein after mutation of the N-glycosylation site or the TM-like segment probably resulted from altered glycosylation, because the mutations introduce three Ser/Thr residues into the TM-like segment or one Ser into the N-glycosylation site that may serve as potential sites for O-glycosylation (Fig. 1A). The glycosylation may play a role in the stability of the mutants (Fig. 1A). In accordance with this, when transfected cells were cultured in the presence of O-glycosylation inhibitor, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-α-GalNAc) for 48 h (36), the TM-mutant and N133AL134S mutant were barely or no longer detected by Western blot analysis (data not shown). Notably, the C-terminally truncated form of IEX-1 also yielded one isoform. Perhaps failure of forming the two major isoforms of IEX-1 may account for the functional loss of the TM-, N133AL134S, and Δ139–156 mutants.

Although it is clear that IEX-1 is an intracellular membrane-associated protein and it is localized at nuclear membrane, Golgi, and ER, its localization in mitochondria has never been clearly shown. Considering its importance in regulation of apoptosis stimulated by both intrinsic and external apoptosis effectors, demonstration of its mitochondrial localization would help us to understand the mechanism underlying IEX-1-induced cell survival. Our study with confocal laser scanning microscopy shows a partial co-localization of IEX-1 with mitochondria in cells expressing IEX-1-GFP fusion protein. Furthermore, we are able to detect all IEX-1 isoforms in purified mitochondria, concluding its mitochondrial association, which is consistent with regulation of intracellular ROS homeostasis by IEX-1, because mitochondria are the major source of intracellular ROS production. The finding adds IEX-1 to the ever-growing list of apoptotic regulatory molecules that localize to the mitochondria and explains the broad protection offered by IEX-1 against apoptosis induced by various stimuli. Yet, how IEX-1 is transported into mitochondria is not known at present. The N terminus of IEX-1 harbors a positively charged and highly conserved polypeptide, and it is predicted by the PSORT program to be a potential mitochondrial localization signal. To test this, the N-terminal sequence, ranging from residues 1 to 30, was fused in-frame with GFP at the C terminus of the peptide and expressed in MCF-7 cells. The resulting fusion protein gave rise to a pattern of fluorescence indistinguishable from that obtained when GFP protein alone was expressed (data not shown). Moreover, substitution of three positively charged residues, histidines and arginine at positions 3, 5, and 8, into Ala, or truncation of either 12 or 25 residues at its N terminus also failed to disturb the mitochondrial distribution of IEX-1 (data not shown). Thus, the postulated mitochondrial localization signal at its N terminus appears to have little role in directing IEX-1 into mitochondria.

It is known that some mitochondrial membrane-associated proteins lack a putative mitochondrial targeting signaling peptide at their N termini but possess TM-like segments that interact with the mitochondrial inner membrane (37, 38). IEX-1 also contains a TM-like region, mutation of which abrogates both pro- and anti-apoptosis activities of IEX-1, emphasizing its importance in regulation of apoptosis. However, mutation of the TM-like domain affects only slightly, if any, mitochondrial localization of IEX-1. The finding that TM-mutant can still enter mitochondria raises the intriguing possibility that the TM-like domain is not required for transportation of IEX-1 to mitochondria but may be essential for interaction of IEX-1 with the mitochondrial inner membrane where IEX-1 can control ROS production in cells through a yet unknown mechanism. Presumably, TM-mutant is unable to interact with the mitochondrial inner membrane and thus loses its ability to protect cells from apoptosis, a possibility that is under current investigation.

Our studies show that IEX-1 overexpression significantly reduces basal levels of intracellular ROS production as well as at the initial phase of apoptosis, suggesting a role of IEX-1 in regulation of intracellular ROS homeostasis. In accordance with this, IEX-1-mediated reduction of intracellular ROS production is correlated to its ability to suppress cell death. Mutants like C2A/C7A and K60A/R63A can modulate ROS production in the same way as WT IEX-1, and their ability to protect cells from apoptosis is also similar. Conversely, mutants, including TM-mutant, N-linked glycosylation mutant, and the C-terminally truncated mutant, that all fail to control intracellular ROS production are unable to prevent cells from proceeding to apoptosis. A body of evidence indicates that ROS homeostasis is critical in maintaining mitochondrial membrane integrity (4, 39, 40). An acute increase in ROS production often causes irreversible damage of mitochondrial membrane integrity, leading to the release of cytochrome c and other proteins inside mitochondria (4, 7–11). Modulation of intracellular ROS production by IEX-1, in line with its association with mitochondria, explains multiple effects of IEX-1 on cellular responses to stress, because ROS can act as intracellular signaling molecules in a stress response in a variety of cell types, regulating apoptosis, proliferation, and transformation, besides a direct damage of mitochondrial membrane integrity. Deregluation of intracellular ROS homeostasis may also account for hypertension and cardiac hypertrophy development in mice with gene-targeted deletion of IEX-1, because cardiac vasculature is a rich source of mitochondrial-derived ROS. Lack of IEX-1 may cause chronic oxidative stress, which reduces endothelial nitric oxide bioavailability by direct inactivation of endothelium-derived NO, or by oxidation of tetrahydrobiopterin leading to endothelial NO synthase uncoupling (41). Consistent with this is the increased level of mRNA for NO synthase in the mice that may be necessary to compensate for a decreased level of NO production in the absence of IEX-1 (25). Further investigation of how IEX-1 reduces intracellular ROS production is likely to shed new light on the mechanism for ROS homeostasis maintenance and for apoptosis onset in mitochondria.

Acknowledgments—We thank members in Dr. Wu’s group for stimulating discussion, Dr. F. Förteu for IEX-1 T18A mutant construct, Dr. Prasad Kanteti for critical reading of the manuscript, and Lu Zhang for technical assistance.

REFERENCES
