Identification and Characterization of a Novel Cytoplasm Protein ICF45 That Is Involved in Cell Cycle Regulation*

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A novel cytoplasm protein, interphase cytoplasm foci protein 45 kDa (ICF45), was identified by screening the cDNA expression library for HeLa cells with serum from an autoimmune patient. The complete cDNA sequence of ICF45 was determined to be 1.32 kb and to encode 298 amino acids with an apparent molecular mass of 45 kDa. The ICF45 transcripts were detected in different tissues and were relatively rich in human liver and lung tissues but scarce in brain tissue. Immunofluorescence with anti-ICF45-specific antibodies demonstrated that ICF45 is strongly expressed in interphase and cannot be seen in mitosis. The subcellular localization of ICF45 and fusion proteins GFP-ICF45, ICF45-GFP, and HA-ICF45 showed ICF45 centralized into 1–2 dots in the cytoplasm and always near the nuclear membrane. The staining foci of ICF45 appeared to be slightly larger than centrosomes and in some cases were found to colocalize with centrosomes. After effectively silencing the ICF45 by RNAi, the growth and proliferation of the cells were significantly inhibited, and p53 was detected to be up-regulated. The silencing of ICF45 also resulted in an appearance of polycentrosome and multinuclear cells, which finally went to apoptosis. Our results suggest that ICF45 is a highly conserved novel protein, which is expressed in a cell cycle-dependent manner and seemed to be involved in cell cycle progression and cell proliferation.

In the past a few decades, the autoantisera from patients with certain autoimmune diseases, such as systemic lupus erythematosus and scleroderma polymyositis, have been a valuable and important resource for finding new proteins, including some well known proteins (1–4). Centromere-associated proteins (5–8), RNA polymerase I (9), DNA topoisomerase I (10), and 70-kDa autoantigen of U1-small nuclear ribonucleoprotein (11) are only a few examples. The development of some newer techniques, such as RACE,1 GFP, and RNAi, has been dramatically speeding up the process of identifying new proteins and their function. Moreover, it is to be expected that some nontarget proteins might be caught during screening cDNA library with autoimmune patient sera, and some of these proteins could also be completely new and important.

It was during our screening of HeLa cells from the cDNA expression library λEXlox with the autoantisera EJ, which recognizes mammalian centromeres (8, 12), when 11 positive clones were selected in the first round, and one of them was recognized as a centromere nonrelated protein with an apparent molecular mass of 45 kDa. What interested us the most about the protein is that the presence of this novel protein was highly cell cycle-dependent. It exists specifically in interphase cells and cannot be seen in mitosis. More interestingly, this protein was found to centralize into 1 or 2 sharp dots near the nuclear membrane at the cytoplasmic site. It was thus named ICF45 (Interphase Cytoplasmic Foci protein 45 kDa). In this study, we investigated the cellular dynamic distribution of ICF45 by using GFP fusion protein, and we probed the possible role of ICF45 with RNAi. The results suggested that this novel protein ICF45 is an essential factor in cell proliferation and cell cycle progression.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum and were maintained at 37 °C in 5% CO2. The reagents used were Lipofectamine™ 2000 (Invitrogen), G418 (Sigma), FITC-conjugated goat anti-rabbit and goat anti-mouse IgG, and rhodamine-conjugated goat anti-rabbit and goat anti-mouse IgG (Jackson ImmunoResearch). Oligonucleotides were synthesized from Takara. Autoantisera EJ used for the cDNA library screening was from a patient with gastric antral vascular ectasia or watermelon stomach disease (12).

Cloning of ICF45 cDNA—The positive cDNA clones were obtained by screening HeLa cells from the cDNA expression library λEXlox (NovaGen) with EJ serum. The cDNA fragment was constructed into pEXlox plasmid by automatic subclone according to the manufacturer’s instructions and was sequenced by Takara. The cDNA clone contained 130–1320 bp of full-length cDNA.

The 5′ end of the transcript was derived from 5′-RACE by using the SMART RACE cDNA amplification kit (Clontech) according to the manufacturer’s instructions. The cDNAs for 5′-RACE were synthesized by RT-PCR from HeLa total RNA. The primers used for PCR were GP51, 5′-CACCACGACTCTTCCGTCAAAGCCTG-3′ and GP3, 5′-TCGGAGAAGCAGCGTGTACATTGGGT-3′ for first PCR amplification, and NOS51, 5′-CTCAGAGAGGAGGACTTGTGGAC-3′ for nest PCR. The PCR products were inserted into TA cloning vector (Takara) and sequenced. The 5′-RACE product contains 88–371 bp of full-length cDNA. The DNA sequence was compared with NCBI GenBankTM nonredundant, expressed sequence tag (EST) and the genome data base by BLASTN. The protein sequence of ICF45 was used to search homologous proteins of identical and differential species in the NCBI/EMBL protein data base by BLASTP.

Polyclonal Antibody Production—The entire encoding sequence of ICF45 was amplified by PCR using pEXlox-ICF45 recombination plasmid as a template and inserted into the BamHI site of pGEX-2Z (constructed by pGEX-2T), and the primers were 5′-CAGGATCCCTGACCTGACTCTTCCGTCAAAGCCTG-3′ and 3′-CTCAGAGAGGAGGACTTGTGGAC-5′.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY463216.

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‡ The abbreviations used are: RACE, rapid amplification of cDNA ends; PI, propidium iodide; GFP, green fluorescent protein; HA, hemagglutinin; RNAi, RNA interference; FITC, fluorescein isothiocyanate; RT, reverse transcriptase; nt, nucleotide; siRNA, small interfering RNA; PBS, phosphate-buffered saline; EST, expressed sequence tag.

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The complete nucleotide and amino acid sequences of human ICF45. The two TGA and TAG stop codons before the ATG start codon and the polyadenylation signal are shown in boldface. The N-linked glycosylation sites (GATMAK and GTLAAD) are boxed. The N-linked glycosylation sites (GDSR and NQTL) are underlined. Two fragments (224–242 and 498–516 bp) chosen as hairpin siRNA are highlighted.

GGGCGCCTGTAAA-3' and 5'-CGCGGATCCCGCAAAAGGTCGAG-5'. The cDNA fragment was then cleaved with NdeI/XhoI enzymes from pGEX-2Z-ICF45 and ligated into the same sites of pAVU6 (Clontech). The primers used were 5'-CATCAGTACAAGGGAGATTACA-3' and 5'-CGCGGATCCCGCAAAAGGTCGAG-3', corresponding to 725–752 and 1020–1033 bp of ICF45 cDNA. The PCR was carried out at 94 °C for 20 s, 68 °C for 20 s, and 72 °C for 40 s using 0.4 μM primer per reaction for 30 cycles. The expression of glyceraldehyde-3-phosphate dehydrogenase was used as a control.

Construction and Transient Transfection of Fusion Protein Expression Plasmids—GFP-ICF45 and HA-ICF45 fusion protein mammalian expression vectors were constructed by cleaving cDNA fragment ICF45 from pGEX-2Z-ICF45 with BamHI and ligating into the same sites of pEGFP-C1 and pCMV5-HA vectors (gifts from Dr. Yeguang Chen) separately and were confirmed by sequencing. The ICF45-GFP expression vector was constructed by PCR amplification of the entire encoding region Plasmids—Semiquantitative reverse transcription-PCR (RT-PCR) of various human tissues was conducted by using Human Multiple Tissue cDNA Panels (Clontech). The primers used were 5'-CAGCCAGTACACGGGAGATTACA-3' and 5'-CGCGGATCCCGCAAAAGGTCGAG-3', corresponding to 725–752 and 1020–1033 bp of ICF45 cDNA. The PCR was carried out at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min using 0.4 μM primer per reaction for 30 cycles.

Flow Cytometry—Stable RNAi cells and control cells were trypanosized and washed three times with cold PBS and were then fixed with 70% ethanol at 4 °C overnight. After RNase digestion for 30 min at 37 °C, PI was added in a final concentration of 65 μg/ml and a flow cytometric analysis was performed on a FACS Calibur (Coulter EPICS XL).

Western Blot Analysis—Total protein extracts were loaded on 12% SDS-PAGE. After the proteins were transferred to a nitrocellulose membrane, the proteins were probed with anti-ICF45-specific antibodies (1:100). The membrane was developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega).
and mouse anti-p53 monoclonal antibody (1:100, Santa Cruz Biotechnology). The cells were washed three times for 10 min each in PBS followed by a 1-h incubation at 37 °C with the second antibody. The secondary antibodies used were FITC-conjugated goat anti-rabbit IgG or rhodamine-conjugated goat anti-rabbit (1:100 dilution) and rhodamine-conjugated goat anti-mouse IgG (1:100) or FITC-conjugated goat anti-mouse IgG (1:100) (all the four antibodies were from Jackson Immunoresearch). The cells were then washed three times with PBS and/or followed by counterstaining with propidium iodide (PI) (Molecular Probes) and mounted antifade solution. Samples were observed with Olympus laser-scanning confocal microscopy (Olympus Fluoview FV300).

RESULTS

Identification and Characterization of ICF45 Gene—We obtained 11 positive clones by screening HeLa cells from the cDNA expression library AEX/ox with EJ serum from an auto-immune patient, and the cDNA fragments were constructed into pEX/ox vector and sequenced. Then the 11 sequences were searched against the EST and GenBank™ nonredundant data bases using BLASTN, and one of the 11 clones was identified as a novel gene. We later named it the ICF45 gene. The cDNA of the ICF45 contains a polyadenylation signal consensus sequence (ATAAA) (14). For determining the complete cDNA and the translation initiation site of the ICF45 transcript, we used the 5′-RACE method, and we obtained a 283-bp fragment. The RACE product was further used to search the human EST data base using BLASTN, and we obtained an EST clone (GenBank™ accession number N72931). We aligned these sequences of the original cDNA fragment 130–1320 bp from the cDNA library, the 5′-RACE product 88–371 bp, the new EST clone 1–289 bp, and the human genome data base with Vector NTI 6.0 software, and we finally obtained the complete ICF45 cDNA sequence.

The complete ICF45 cDNA has 1320 bp and encodes a 298-amino acid polypeptide (Fig. 1). Its theoretical pl is 8.12, and the predicted molecular mass is 34.8 kDa (predicted by PeptideMass) (15). Analysis of the amino acid sequence of ICF45 by Simple Modular Architecture Research Tool (smart.embl-heidelberg.de) (16, 17) revealed an unknown conserved function domain DUF549 (Pfam accession number PF04446). In addition, two potential N-glycosylation sites (underlined in Fig. 1), a tyrosine sulfation site, three cAMP- and cGMP-dependent protein kinase phosphorylation sites, three protein kinase C phosphorylation sites, four casein kinase II phosphorylation sites, two N-myristoylation sites (boxed in Fig. 1), and an amidation site were suggested by searching the PROSITE data base (18).

High Conservation of ICF45 in Eukaryotes—To investigate the homologous proteins of ICF45 in other species, a BLAST search of the NCBI/EMBL protein data base nonredundant with the ICF45 protein sequence was performed and revealed the existence of a series of ICF45 homologs from yeast to human. However, these homologs were all hypothetical proteins predicted (with no experimental evidence) from corresponding cDNA sequences. These homologous proteins were mouse AK010876 with identities of 89% (GenBank™ accession number BAB27240); Drosophila melanogaster BG:DS00929 with identities of 55% (GenBank™ accession number AAF44908); A. gambiae Pst st. PEST agCP9855 with identities of 52% (GenBank™ accession number EA000037); Arabidopsis thaliana AC005700 with identities of 52% (GenBank™ accession number EAA000700); Saccharomyces cerevisiae RGR024C with identities of 52% (GenBank™ accession number RGR024C); Schizosaccharomyces pombe with identities of 49% (GenBank™ accession number CAB40011); Plasmodium yoelii yoelii with identities of 48% (GenBank™ accession number EAA21470); and Plasmodium falciparum 3D7 with identities of 47% (GenBank™ accession number EAA00037). These hypothetical proteins were highly homologous with human ICF45. In S. cerevisiae, homolog of ICF45, the hypothetical protein RGR024C was essential for cell viability, as deleting the gene from the genome rendered the yeast unable (19). This result suggests that ICF45 might play an important role in the growth and proliferation in most eukaryotic cells.

Tissue-specific Distribution of ICF45 mRNA—To determine the transcriptional levels of the human ICF45 gene in various tissues, a human multiple tissue cDNA panel (Clontech) was used for the quantification (Fig. 2A). The primers used were designed to amplify the 309-bp fragment (725–1033 bp). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was examined as a control. Lane M shows the DNA marker. B, ICF45 expressions in E. coli and HeLa cells were probed with affinity-purified anti-ICF45 antibodies.

FIG. 2. Comparison of ICF45 mRNA transcription in various tissues and protein expressed in prokaryotic and eukaryotic cells. A, human multiple tissue cDNA panels were used to quantify the levels of ICF45 transcripts. The primers used were designed to amplify the 309-bp fragment (725–1033 bp). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was examined as a control. Lane M shows the DNA marker. B, ICF45 expressions in E. coli and HeLa cells were probed with affinity-purified anti-ICF45 antibodies.
30a expression vectors and induced to express in *Escherichia coli*. The prokaryote expression protein all came into the inclusion body. The inclusion bodies were then further purified and used to immunize rabbits to produce anti-ICF45-specific antibodies.

To characterize this novel protein in eukaryotic cells, the total protein extracts of HeLa cells and prokaryote expressed protein were analyzed by Western blotting with anti-ICF45-specific antibodies and control of preimmune rabbit serum. The results revealed that the ICF45 protein in eukaryote form and prokaryote form migrates on SDS-PAGE differently and yields the apparent molecular masses of about 45 and 34 kDa, respectively (Fig. 2B). The full-length cDNA of ICF45 encoded 298 Aa with a predicted molecular mass of 34.7 kDa. The ~10-kDa difference between the observed and predicted molecular mass was most probably because of post-translation modification, such as multiphosphorylation, N-glycosylation, or N-myristoylation. Such modifications have been predicted by protein sequence analysis and speculated to slow down the mobility of ICF45 and increase its apparent molecular mass on SDS-polyacrylamide gels.

Subcellular Localization of ICF45—To determine the subcellular location of ICF45, an indirect immunofluorescence assay was performed with the affinity-purified anti-ICF45 antibodies in HeLa cells. The results showed that the novel protein ICF45 centralized into a 1–2 sharp dot pattern in cytoplasm (Fig. 3A), and these cells were all in interphase. The number of cells containing one dot protein was 58.6 ± 3.4% and with two dots was 40.6 ± 3.3%. For the latter, the two dots in many cells were inter-connected and seemed to be dividing from one dot into two separate dots. This observation was further confirmed with the GFP-ICF45 and ICF45-GFP assay that allowed us to get more dynamic information on its subcellular distribution (Fig. 3B). A very small number of cells contained no dots or more than two dots. It was clearly shown that ICF45 presented a cell cycle-dependent expression fashion. It was specifically expressed in interphase, located close to the nuclear envelope, and was followed by its disappearance when cells reached mitosis (Fig. 3A). The cell cycle expression of ICF45 implied that ICF45 might play an active role in cell cycle progression.

To determine further the subcellular localization and dynamic distribution of ICF45 in living cells, GFP-ICF45 and ICF45-GFP were transfected and expressed separately in HeLa cells. After transient transfection for 24–48 h, GFP fusion proteins had been richly expressed in cells and showed green fluorescent dots and centralized into bright 1–2 dots in the cytoplasm (Fig. 3B). The two GFP fusion proteins displayed a clear dynamic process of the “division” of ICF45-rich dot structure. At first, a single dot emerged in the most transfected cells,
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interphase cells, the localization of ICF45 was close to the centrosomes (Fig. 4A), and the proportion of cells displaying co-localization of ICF45 and centrosome was 4.8 ± 0.4% of total cells, and this result was further testified by tracing the dynamic distribution of GFP-ICF45 combined with anti-γ-tubulin immunostaining at chosen time points (Fig. 4B). In the cells that contain a single centrosome (a sign of G1 or early S phase), GFP-ICF45 mostly appeared as one dot or a pair of closely connected dots and adjacent to the centrosomes. When centrosomes were divided (showing the cells are in late S or G2 phase), GFP-ICF45 also revealed two separate dots in most cases. When centrosomes moved to the two poles of the cells, which marked when the cells came into mitosis, the green fluorescent dot of GFP-ICF45 began to disappear. The results suggest that GFP-ICF45 presented typical cell cycle-dependent dynamics and showed a coordinate relation with the centrosome, which is far from random or by accident.

HA-tagged ICF45 fusion protein was used in a similar experiment to localize ICF45 in HeLa cells since HA-ICF45 seems more close to the native protein in size compared with the GFP fusion protein. The results were as expected, except for some cells, where HA-ICF45 was overexpressed, and it displayed a larger speckle near the nuclear membrane (Fig. 3C). In many cells, the two dots of HA-ICF45 were found very close and inter-connective to each other, suggesting the HA fusion protein is capable of carrying out the “normal” functions in division and redistribution.

Cell Growth and Proliferation Was Inhibited after Silencing of ICF45—By transfecting the pAVU6 + 27-ICF45 hairpin siRNA expression vector into HeLa cells, we obtained a clonal cell line in which ICF45 was stably silenced by G418 selection and was identified with anti-ICF45-specific antibodies; shown in Fig. 5, B and D, the hairpin fragment was 224–242 bp of ICF45, but a control vector clonal cell line and the second hairpin fragment of 498–516 bp had no specific silencing response.

In the cell line of stable silencing of ICF45, the cell morphology had dramatic changes and formed large numbers of aberrant cells. The cells became much larger and contained multiple nuclei (Fig. 5C). The cell number was 15.1 ± 1.9% of total cells, and the control cells were 0.8 ± 0.3%. Cell growth and proliferation of the stable ICF45 knock down cells were greatly inhibited by the cell growth curve (Fig. 5E), and by the 5th day, the growth speed of the control cell line was 10 times higher than the RNAi cell line, and many cells were found to have stopped growing and became quiescent.

Polycentrosomal and Multinuclear Cells Formed after Silencing of ICF45—To find out whether centrosomes also had changes in the stable silencing of the ICF45 cell line, immunostaining analysis was performed with anti-γ-tubulin and anticentrin-specific antibodies. Many cells displayed an overduplication of centrosomes, and this resulted in polycentrosomal morphology (Fig. 6, A and B). The proportion of multcentrosome cells was about the same as that of multinuclear cells, i.e. 15.7 ± 1.6%, and this proportion in the control cells was 0.8 ± 0.3%. Further immunostaining assay with anti-α-tubulin monoclonal antibodies also showed a dramatic change in spindle structure and organization (Fig. 6B), and for the resulting multipole mitotic spindles (Fig. 6C), the proportion of abnormal spindle cells was 19.2 ± 2.6% in total mitosis cells and 1.0 ± 0.3% in control cells. The multipole spindles and the failure after cytokinesis seemed to be the reason for the formation of many coenocytes. Furthermore, these large multinuclear cells were found to stop growing; their DNA began to condense and fragmentize, and finally they went to apoptosis (Fig. 6A). In these apoptotic multinuclear cells, centrosomes became

![Figure 4. Subcellular distribution of ICF45 and centrosome.](image)

A, cells in interphase (lanes 1 and 2) and metaphase (lane 3) were stained with purified anti-ICF45-specific antibodies (green) and anti-γ-tubulin monoclonal antibodies (red). As shown in the merge images, ICF45 was close to the centrosomes and only expressed in interphase cells (lane 1); in some cells, ICF45 co-localized with centrosomes (lane 2). B, GFP-ICF45 expressed in HeLa cells and stained with anti-γ-tubulin monoclonal antibodies. As shown in the merged images, GFP-ICF45 (green) and centrosomes (red) localized separately (lane 1) and co-localized in some cells (lane 2). Scale bars correspond to 10 μm.

and as the division began, the resulting two dots demonstrated a connection to each other (Fig. 3B), and finally became two well separated or widely separated dots, whereas their position remained close to the nuclear membrane. After the cells came into mitosis, the green fluorescent dots of GFP-ICF45 and ICF45-GFP began to fade out quickly. The dynamic distribution patterns of ICF45 ligated to C-terminal and N-terminal ends of GFP in HeLa cells were basically the same.

The only known cellular structure that displays a more or less similar structure is the centrosomes, which show 1–2 dot patterns in the cytoplasm (Fig. 3 and Fig. 4). To analyze the relation of ICF45 and centrosome, double immunostaining was performed with anti-γ-tubulin and anti-ICF45 antibodies. In interphase cells, the localization of ICF45 was close to the centrosomes, but was usually significantly larger than the latter (Fig. 4A). In some cells, ICF45 was found co-localized with centrosomes (Fig. 4A), and the proportion of cells displaying co-localization of ICF45 and centrosome was 4.8 ± 0.4% of total cells, and this result was further testified by tracing the dynamic distribution of GFP-ICF45 combined with anti-γ-tubulin immunostaining at chosen time points (Fig. 4B). In the cells that contain a single centrosome (a sign of G1 or early S phase), GFP-ICF45 mostly appeared as one dot or a pair of closely connected dots and adjacent to the centrosomes. When centrosomes were divided (showing the cells are in late S or G2 phase), GFP-ICF45 also revealed two separate dots in most cases. When centrosomes moved to the two poles of the cells, which marked when the cells came into mitosis, the green fluorescent dot of GFP-ICF45 began to disappear. The results suggest that GFP-ICF45 presented typical cell cycle-dependent dynamics and showed a coordinate relation with the centrosome, which is far from random or by accident.

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smaller and distinctly diminished (Fig. 6A), which indicated that cell apoptosis resulted in the cessation of centrosome duplication and the diminishment of the centrosomes themselves.

Silencing of ICF45 Causes p53 Up-regulation—The expression of p53 as one of the major candidate factors that may influence the apoptosis susceptibility was observed in the stably transfected cell line by Western blot and anti-p53 immunostaining. The Western blot showed that p53 was prominently up-regulated (Fig. 5G), and this up-regulation was extremely obvious in the abnormal multinuclear cells as shown by immunostaining (data not shown). Flow cytometry also showed the apoptotic rate of ICF45 stable silenced cells increased to 5.2 ± 0.2% from 0.7 ± 0.2% of the control cells (Fig. 5F). In time, an increasing number of the multinuclear cells showed classic signs of apoptosis such as shrinking cytoplasm, membrane blebbing (data not shown), and chromatin condensation (Fig. 6A), and finally cells detached from the culture substrate.

DISCUSSION

In the present study we report the identification of a novel cytoplasm protein by screening the cDNA expression library for HeLa cells with EJ serum from an autoimmune patient with the rare disease gastric antral vascular ectasia (12). Our original goal was to clone the gene of a constitutive centromere protein CENP-G, which was recognized by this serum (8). During the early rounds of this screening, we harvested many positive clones including some nontarget novel genes. As we checked these new genes, one of them attracted our attention because of its unique distribution and its specific cell cycle-dependent expression. We later named it ICF45.

This new gene showed no significant similarities to any known gene in the BLAST NCBI/EMBL data base. It has been found that several unknown homologous mRNA sequences are presented in the EST data base. As all these sequences were incomplete and had no certain translation initiation sites, it...
Using the protein sequence of ICF45 to search the NCBI/EMBL protein data base revealed that its homologous proteins exist in many eukaryote species from yeast to human. Although these were hypothetical proteins (with no experimental evidence), this high evolutionary conservation implies that ICF45 may have an important function. Indeed, the experimental deletion of the homologous gene from yeast revealed it serves as an essential factor for yeast proliferation and viability (19). This was also confirmed in our present observation that human cell growth and proliferation were strongly inhibited after silencing of ICF45 (Fig. 5E).

The protein sequence analysis of ICF45 predicts that it has a molecular mass of 34.7 kDa, which is quite different from the apparent molecular mass of 45 kDa on SDS-PAGE. ICF45 was also predicted to have two N-glycosylation sites and some other modification sites (Fig. 1). These possible post-translation modifications might explain in part the difference between the predicted and apparent molecular mass, and the predicted multiphosphorylation sites most likely could be involved in the cell cycle regulation functions such as described here.

The unique localization of ICF45 is one of the most exciting aspects that require further study. The predicted subcellular localization of ICF45 by TargetP version 1.0 (www.cbs.dtu.dk/services/TargetP) (20) was to mitochondria (probability 68.2%), the secretory pathway (7.1%), and others (25.5%). However, our immunostaining analysis with anti-ICF45-specific antibodies showed 1–2 sharp and round dots near the nuclear membrane in the cytoplasm, which only presents in interphase and is lacking in mitosis (Fig. 3A). The dynamic distribution visualized using both GFP C-terminal and N-terminal ICF45 fusion proteins obtained the same result (Fig. 3B). These results suggested that neither the N nor the C terminus of ICF45 had any effect on the distribution of ICF45. The key domain affecting the subcellular localization should be in the middle of the ICF45 protein sequence.

Such a distinct and highly centralized distribution of ICF45 strongly suggests some kind of corresponding subcellular structure or some solid and huge molecular complexes. It has been widely believed that because the electron microscope was intensively used, every cellular organelle had been discovered. Indeed, during last few decades, there were few new structures added to the list. One of them is the coiled body, which was also first detected by auto-antiserum, and most interestingly, it was also recognized as a few (~1–6 dots) round dots, which appeared in interphase cells and disappeared in mitosis as ICF45, but only inside nucleus (21). It is particularly exciting and challenging that to our knowledge there is no organelle that could correspond to the ICF45 distribution pattern. We did some gold immunoelectron-microscopic experiments, and we failed to resolve any detectable ultrastructure behind the gold labeling (data not shown). The extensive analysis of the interacting partners of ICF45 is still ongoing in our laboratory in order to determine its possible structure or molecular complex.

Another obvious characteristic of ICF45 is the cell cycle-dependent manner of its expression. Here we provide some observations showing that ICF45 could also play an active role in cell cycle regulation. After stable silencing of ICF45 by RNAi, cell growth and proliferation were strongly inhibited (Fig. 5E); the inter-dependence of growth in the cells was greatly increased, and cells became unable to grow in soft agar. Furthermore, silencing of ICF45 induced wide morphological changes, including an increase in the cell volume and the emergence of multinuclear cells (about 15.1 ± 1.9% of total cells) that contained 2–10 nuclei in one cell (Fig. 5C and Fig. 6A). This shows that the cell cycle progression was not only inhibited but also seriously disrupted in more than one aspect.

**Fig. 6.** Polycentrosomal cells and multipole spindles in stable RNAi against ICF45 cells. A, centrosomes were shown with anti-γ-tubulin monoclonal antibodies (green) and counterstaining with PI (red). Overduplication and cytokinesis failure of the centrosomes of some ICF45-HP1 siRNA cells led to coenocytes (middle panels). The bottom panels showed apoptosis of an RNAi cell (multinuclear, chromatin condensing, and fragmentizing), in which the centrosomes were prominently diminished. The upper panels showed the normal control cells. B, centrosomes were shown with anti-centrin polyclonal antibodies (red), and co-immunostaining was performed with anti-α-tubulin monoclonal antibodies (green). As shown in the merge images, ICF45-HP1 siRNA cells emerged from many large and abnormal cells and formed polycentrosomes (bottom panels), compared with the normal cells (upper panels). All the scale bars correspond to 10 μm. C, cells were stained with anti-α-tubulin monoclonal antibodies (green) and PI (red). As shown in the merge images, ICF45-HP1 siRNA cells formed some multipole spindle (bottom panels), and the upper panels showed normal control. Scale bars correspond to 10 μm.

was necessary to use the 5′-RACE and bioinformation methods to determinate the 5′ end of ICF45 transcription and to obtain the full-length cDNA of ICF45.
Most interestingly, ICF45 showed a close but unclear relationship with the centrosome. First, the subcellular distribution pattern of ICF45 was very similar to that of the centrosome (Fig. 4). The location of ICF45 was often found adjacent to the centrosomes, and in some cases, they were co-localized, the proportion was 4.8 ± 0.4% in total cells (Fig. 4).

Recently, research has suggested that the centrosome plays active roles not only in microtubule nucleation but also in the cell cycle regulation (22). Centrosome duplication must occur in coordination with other cell cycle events, mainly DNA synthesis (23). Abrogation of the regulation that coordinates centrosome duplication and DNA duplication will likely increase the frequency of centrosome hyperamplification and lead to cytokinesis failure (24). In our experiments, the silencing of ICF45 causes polycentrosome formation, followed by multiple spindles (Fig. 6C), and finally results in the formation of coenocytes (Fig. 5C and Fig. 6A) which is probably due to cytokinesis failure.

There are two possible ways by which ICF45 could affect the cell cycle and centrosomes. ICF45 could regulate cell cycle progression, which in turn affects centrosome function, and vice versa. Because we have detected a strong up-regulation of p53, and cell growth and proliferation were effectively inhibited in all cells, whereas the polycentrosomes accumulate in a part of the cell population, we think ICF45 may first influence the cell cycle regulation and further affect the centrosome cycle. At the same time, p53 elevation could also be responsible for the increase of apoptotic rate seen in the RNAi cells as suggested by many earlier reports (25).

In summary, our research has identified a novel cytoplasmic protein, ICF45, a novel and conservative protein with unusual subcellular localization and cell cycle dynamics. It may represent a new group of proteins, and its investigation could probably provide a new connection among centrosome, nuclear dividing, and cytokinesis.

REFERENCES
1. Pollard, K. M., Reimer, G., and Tan, E. M. (1989) Clin. Exp. Rheumatol. 7, 57–62
2. Tan, E. M. (1989) Adv. Immunol. 44, 93–151
3. Chan, E. K. L., and Andrade, L. E. C. (1992) Rheum. Dis. Clin. N. Am. 18, 535–570
4. Ditzel, H. J. (2000) Immunol. Rev. 215, 185–193
5. Morii, Y., Peebles, C., Fritzier, M. J., Steigerwald, J., and Tan, E. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1627–1631
6. Earnshaw, W. C., and Rothfield, N. (1985) Chromosoma (Berl.) 91, 313–321
7. Craig, J. M., Earnshaw, W. C., and Vagnarelli, P. (1999) Exp. Cell Res. 246, 249–262
8. He, D., Zeng, C., Woods, K., Zhong, L., Turner, D., Busch, R. K., Brinkley, B. R., and Busch, H. (1998) Chromosoma (Berl.) 107, 189–197
9. Kpnis, R. J., Craft, J., and Hardin, J. A. (1990) Arthritis Rheum. 33, 1431–1437
10. D’Arpa, P., Machin, P. S., Ratrie, H., III, Rothfield, N. F., Cleveland, D. W., Earnshaw, W. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2543–2547
11. Spritz, R. A., Strunk, K., Surowy, C. S., Hoch, S. O., Barton, D. E., and Franke, U. (1987) Nucleic Acids Res. 15, 10373–10391
12. Watson, M., Hall, R. J., Mceue, P. A., Varga, J., and Jimenez, S. A. (1996) Arthritis Rheum. 39, 341–346
13. Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002) Nat. Biotechnol. 20, 505–508
14. Sheets, M., Ogg, S., and Wickens, M. (1992) Nucleic Acids Res. 18, 5799–5805
15. Wilkins, M. R., Lindskog, J., Gasteiger, E., Bairoch, A., Sanchez, J. C., Hochstrasser, D. F., and Appel, R. D. (1997) Electrophoresis 18, 403–408
16. Schulz, J., Milpete, F., Bork, P., and Ponting, C. (1998) Proc Natl Acad Sci U S A 95, 5857–5864
17. Letunic, I., Goodstadt, L., Dickens, N. J., Doerk, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R. R., Ponting, C. P., and Bork, P. (2002) Nucleic Acids Res. 30, 242–244
18. Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C. J., Hofmann, K., and Bairoch, A. (2002) Nucleic Acids Res. 30, 235–238
19. Gaevner, G., Gnevev, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucena-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astra-moff, A., El-Bakkoury, M., Bangham, R., Benito, R., Broatch, S., Campanaro, S., Curtiss, M., Davis, K., Deischbaumer, A., Entian, K. D., Flaherty, P., Fourny, F., Garlink, D. J., Gerstein, M., Gotte, D., Goldberg, U., Hege-mann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kotter, P., LaRonde, D., Lamb, D. C., Lang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Oei, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shaffer, B., Shoemaker, D. D., Stokk-shai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Wolkeart, G., Wang, C. Y., Ward, T. R., Wilhelm, J., Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philipp, P., Davis, R. W., and Johnston, M. (2002) Nature 418, 387–391
20. Emanuelsson, O., Nielsen, H., Brunak, S., and Heijne, G. V. (2000) J. Mol. Biol. 300, 1005–1016
21. Andrade, L. E. C., Chan, E. K. L., Raska, I., Peebles, C. L., Roos, G., and Tan, E. M. (1991) J. Exp. Med. 173, 1407–1419
22. Lange, B. M. (2002) Curr. Opin. Cell Biol. 14, 35–43
23. Okuda, M., Horo, H. F., Tazakure, P., Tokuyama, Y., Smulian, A. G., Chan, P. K., Knudsen, E. S., Hofmann, I. A., Snyder, J. D., Beve, K. E., and Fukasawa, K. (2000) Cell 103, 127–140
24. Brinkley, B. R., and Geopfert, T. M. (1998) Cell Motil. Cytoskeleton 41, 281–288
25. Vousden, K. H. (2000) Cell 103, 691–694