The Human Protein HSPC021 Interacts with Int-6 and Is Associated with Eukaryotic Translation Initiation Factor 3*

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The Int-6 protein has been shown to be a subunit of eukaryotic translation initiation factor 3 (eIF3) and to play a role in the control of cell growth. By immunoprecipitation experiments and mass spectrometry analyses, we identified a human protein previously known as HSPC021 that is associated with Int-6. Exposure of Jurkat cells to the phosphatase inhibitor H2O2 triggers a marked phosphorylation on tyrosine of HSPC021. Several experiments were performed to evaluate whether this protein is associated with eIF3. It was observed that HSPC021 coelutes with Int-6 and eIF3 in gel filtration, coimmunoprecipitates with eIF3, and is incorporated into eIF3 both in rabbit reticulocyte lysates and in COS7 cells. A direct protein-protein interaction occurs between HSPC021 and Int-6, but the analysis of different mutants of HSPC021 indicated that a larger region of the protein is necessary for incorporation into eIF3 as compared with binding to Int-6. Taken together, our results establish that HSPC021 is tightly associated with the mammalian translation initiation factor eIF3. Analysis of the primary sequence of HSPC021 from different species revealed the presence of a tetratricopeptide repeat, a proteasome-COP9 (constitutive photomorphogenesis 9) signalosome-initiation factor 3 domain along with a Pumilio FBF repeat. These protein motifs are also present in subunits of eIF3, of the lid of the 26 S proteasome, and of the COP9 signalosome.

The Int-6 protein has been identified by three independent approaches. Studies in mice infected by the mouse mammary tumor virus have shown that the int-6 gene is a common site of integration of the provirus in preneoplastic and neoplastic mammary lesions (1). This integration within the gene causes production of truncated transcripts. Hence, either loss of expression of the modified allele or production of shortened forms of the protein alters control of cell growth. The human cDNA coding for Int-6 was isolated in a two-hybrid screen using the Tax transforming protein of human T-cell leukemia virus type 1 as bait (2). Finally, the Int-6 protein was characterized as a subunit of the translation initiation factor eIF3 (3). This factor represents a complex assembly of various subunits of which 10 have already been cloned for the mammalian factor: p170, p116, p110, p66, Int-6/p48, p47, p44 (also known as p42), p40, p36, and p35 (3–9). The eIF3 factor plays a central role in translation initiation by maintaining the 40 S ribosomal subunit dissociated from the 60 S and by promoting the association of the former with mRNA and initiator Met-tRNA (10). This complex establishes multiple protein-protein interactions with other translation initiation factors. For example, it has been shown that eIF3 contacts eIF5 and eIF1 (8, 11, 12) and that it binds cooperatively with eIF4A to the central domain of eIF4G1 (13). Recently, Asano et al. (14) have shown the existence of a complex including the yeast initiation factors eIF1, eIF2, eIF3, eIF5 and initiator Met-tRNA. The presence of Int-6 in eIF3 purified from various organisms is now well established (3, 15–17), but several observations show that Int-6 is also present in other cellular protein complexes. Int-6 includes both a nuclear export signal at its N terminus and a nuclear localization signal, suggesting that the protein shuttles between nucleus and cytoplasm (18). In agreement with this notion, endogenous Int-6 of primary lymphocytes has been observed in the nuclear compartment, partly associated with the nuclear bodies containing the promyelocytic leukemia protein (19). This localization is related to the ability of Int-6 to interact with the Ret finger protein (19) that binds to promyelocytic leukemia protein (20). A nuclear localization of Int-6 has also been observed in the plant Arabidopsis thaliana. In this organism, it has been shown that Int-6 together with eIF3-p110 interacts with the COP9 (constitutive photomorphogenesis 9) signalosome, which plays an important role in the control of photomorphogenesis (16). In cells of the root, Int-6 is seen predominantly within the nucleus. In mammalian cells also, the localization of Int-6 might differ from one cell type to another. Recently, data on the disruption of the gene encoding the Int-6 ortholog in Schizosaccharomyces pombe have been reported by several groups (17, 21, 22). Yeast strains lacking Int-6 were found to grow slowly in minimal medium and to exhibit an increased sensitivity to various drugs including caffeine, actinomycin D, and cycloheximide (21, 22). Conversely, the overexpression of Int-6 in this organism leads to resistance to these drugs. This effect was related to an increase in the amounts of RNA produced by several genes regulated by Pap-1, the S. pombe homolog of AP-1 (21). It is not yet known whether this effect is the consequence of altered translation of a regulatory desorption/ionization time-of-flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCI, proteasome-COP9 signalosome-initiation factor 3; PCR, polymerase chain reaction; Puf, Pumilio FBF; PVDF, polyvinylidine difluoride; LDAO, N,N-dimethylodecylamine N-oxide; I.P., immunoprecipitation; I.B., immunoblot.

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§ The abbreviations used are: eIF, eukaryotic translation initiation factor; CSN, COP9 signalosome; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCI, proteasome-COP9 signalosome-initiation factor 3; PCR, polymerase chain reaction; Puf, Pumilio FBF; PVDF, polyvinylidine difluoride; LDAO, N,N-dimethylodecylamine N-oxide; I.P., immunoprecipitation; I.B., immunoblot.
factor or of a direct transcriptional action of Int-6. The loss of Int-6 in S. pombe also causes a weak deleterious effect on chromosome segregation by a modification of the microtubule assembly/dissassembly (23). This effect is highly reinforced by mutations in the ras1 gene. A similar effect was observed with the S. pombe homolog of eIF3-p66, Moe1, which interacts with Int-6. Interestingly, when Moe1 is absent, the level of Int-6 decreases and its subcellular localization is modified, the protein being then present within the nucleus. The inverse situation occurs when the int-6 gene is disrupted. This suggests that the interaction between Moe1 and Int-6 is important for the presence of both proteins in the cytoplasm. The effect on chromosome segregation might be an explanation of the transforming activity of the putative truncated Int-6 forms resulting from mouse mammary tumor virus integration (23). All of these data show that Int-6 exerts complex regulatory roles in the cell and that much remains to be understood about the function of this protein.

With the exception of Saccharomyces cerevisiae, the conservation of Int-6 among various species of eukaryotes (S. pombe (17, 21–23), A. thaliana (16), Drosophila melanogaster (15), Caenorhabditis elegans (19), Xenopus laevis (19), Mus musculus (1) and Homo sapiens (2)) is high. By comparing these sequences, we noticed a good conservation of a consensus phosphorylation site on tyrosine at position 144 in the human sequence. Experiments were therefore undertaken to determine whether Int-6 can be phosphorylated on tyrosine. Under the conditions tested, we did not observe such a phosphorylation, but it appeared that Int-6 coprecipitated a protein showing an apparent molecular mass of 69 kDa, which was very clearly revealed by anti-phosphotyrosine antibodies. This protein has been identified by mass spectrometry, and we show in this report that it associates with the eIF3 translation initiation factor.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Jurkat cells and MT4 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37 °C in a 5% CO2-humidified atmosphere. BHK21 and COS7 cells were cultured under the same conditions except that the medium was Dulbecco modified Eagle’s medium instead of RPMI. COS7 cells were transfected by the calcium-phosphate precipitation method in 100-mm diameter Petri dishes. Treatment of cells with 10 mM H2O2 was performed by the addition of 114 μl of a 3% H2O2 solution in 10 ml of culture medium. After incubation for 15 min, cells were collected by centrifugation and washed three times with phosphate-buffered saline. The pellet of cells was stored at −80 °C after freezing in a liquid nitrogen bath.

Immunoprecipitations and Immunoblots—Cells corresponding to 106 cells/ml were resuspended in 500 μl of buffer A (50 mM Hepes, pH 8.0, 300 mM NaCl, 5 mM MgCl2, 1 mM diithioretil, 10% glycerol, protease inhibitor mixture (complete EDTA free (Roche Molecular Biochemicals)). Lysate was cleared by centrifugation and subjected to nuclease digestion for 90 min (benzozone; Sigma). The whole cell extract was loaded at a flow rate of 0.3 ml/min on a Superose 6 HR10/30 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% (v/v) glycerol, 1 mM diithioretil. Fractions of 0.5 ml each were collected and concentrated with 25 μl of StrataClean resin (Stratagene). Gels for immunoblots were done with one-fifth of each fraction.

Constructs—The HSPC021 cDNA was obtained from the United Kingdom Human Genome Mapping Project Resource Center (Image clone 2018785) (25). The HSPC021 coding sequence was amplified by PCR with the following primers: 5’-CTCAGCTTCCTTTCGGGCTTCTTCA-3’ and 5’-TGTTCTACCTCATCAAAGGTCTCTGTCCCATC-3’; this PCR product was inserted into the BamHI site of the pSG5 (26) derivative pTL1, giving pTL1-HSPC021. The HSPC021 cDNA was fused at its 5’-end to the FLAG epitope into pSG5. It was generated by amplifying the HSPC021 coding sequence from the HSPC021 cDNA library with the following sense and antisense primers: 5’-CGCAAGATCATGCTTATGTCCTTATCCCGCTGATG-3’ and 5’-TGTGTAGATCTCATCAGGATTACATATGTTTATCCCGCTGATG-3’, respectively. After digestion by BglII, the fragment was cloned into the BglII site of pSG5 (26) derivative pTL1, giving pTL1-HSPC021. pSGF-HSPC021 plasmid contains the HSPC021 cDNA fused at its 5’-end to the FLAG epitope into pSG5. It was generated by amplifying the HSPC021 coding sequence from the HSPC021 cDNA library with the following sense and antisense primers as was described previously: 5’-GGGCTCTGTGGCCCGGCGCGGCGCGGAGTACGATCTGACTAC-3’ and 5’-CTACGCTCTTCTCCTGCTTCTCTCGTACATATT-3’. The corresponding cDNA fragments obtained by PCR amplification using pSGF-HSPC021 as matrix along with appropriate primers, which created sites at both ends. The pTL1-Int-6 plasmid has been described previously (19). The vector used to express Int-6 in bacteria was generated by amplifying the Int-6 cDNA from pTL1-Int6 with Pfu polymerase and primers sharing a stretch of 20 nucleotides homologous to pET-15b at both ends. The sequence of the sense and antisense primers was as follows: 5’-GGCTCTGTGGCCCGGCGCGGCGCGGAGTACGATCTGACTAC-3’ and 5’-CTACGCTCTTCTCCTGCTTCTCTCGTACATATT-3’. The product was cloned by homologous recombination (27) in pET-15b vector (Novagen) digested with NdeI and BamHI. For the HSPC021 bacterial expression vector, the cDNA was amplified by PCR for pSGF-HSPC021 and inserted into the BglII site of pET-15b plasmid expression vector. 1 μg of plasmids pTL1-HSPC021 and pSGF-HSPC021 were added to reaction mixtures of 50 μl, and assays were carried out according to the manufacturer’s standard procedures. When the anti-phosphotyrosine antibodies were used, the membrane was blocked with 5% bovine serum albumin instead of 5% dry milk in the other cases. Revelation of the secondary antibody coupled to peroxidase was performed using ECL or ECLplus (Amersham Pharmacia Biotech). When probed with a secondary antibody coupled to peroxidase, membranes were stained with a STORM860 apparatus (Amersham Pharmacia Biotech).

Mass Spectrometry—Proteins were separated by SDS-PAGE and stained with a zinc stain kit (Bio-Rad), and the band at 69 kDa was excised. After a wash with 50 mM acetonitrile, gel pieces were dried in a vacuum centrifuge and reswollen in 20 μl of 25 mM NH4HCO3 containing 0.5 μg of sequencing grade trypsin (Promega). After incubation for 4 h at 37 °C, a 0.5-μl aliquot was removed for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis. Monoisotopic peptide masses were assigned and used for data base searching. The gel pieces were then extracted with 5% formic acid solution and then with acetonitrile. The extracts were combined with the original digest, and the sample was evaporated to dryness. The sample was dissolved in 0.1% formic acid and desalted using a ZipTip Q-TOF hybrid mass spectrometer (Micromas) in order to obtain sequence information.

Gel Filtration Column—100 × 106 Jurkat cells were lysed by freeze-thaw in 400 μl of buffer A (50 mM Hepes, pH 8.0, 300 mM NaCl, 5 mM MgCl2, 1 mM diithioretil, 10% glycerol, protease inhibitor mixture (complete EDTA free (Roche Molecular Biochemicals)). Lysate was cleared by centrifugation and subjected to nuclease digestion for 90 min (benzozone; Sigma). The whole cell extract was loaded at a flow rate of 0.3 ml/min on a Superose 6 HR10/30 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% (v/v) glycerol, 1 mM diithioretil. Fractions of 0.5 ml each were collected and concentrated with 25 μl of StrataClean resin (Stratagene). Gels for immunoblots were done with one-fifth of each fraction.

Production of Recombinant Proteins and Far Western Blots—Wild-type HSPC021 and a FLAG-tagged version were synthesized by in vitro transcription/translation using the Transcend RNA and the TNT Coupled Reticulocyte Lysate systems (Promega). 1 μg of plasmids pTL1-HSPC021 and pSGF-HSPC021 were added to reaction mixtures of 50 μl, and assays were carried out according to the manufacturer’s standard procedures. When the anti-phosphotyrosine antibodies were used, the membrane was blocked with 5% bovine serum albumin instead of 5% dry milk in the other cases. Revelation of the secondary antibody coupled to peroxidase was performed using ECL or ECLplus (Amersham Pharmacia Biotech). When probed with a secondary antibody coupled to peroxidase, membranes were stained with a STORM860 apparatus (Amersham Pharmacia Biotech).
To produce Int-6 in bacteria, the E. coli BL21 strain was transformed with the pET-15b-Int-6 plasmid. Bacteria were grown in LB medium at 37 °C to midexponential phase. Induction was carried out at 37 °C with 1 mM isopropyl-1-thio-D-galactopyranoside for 3 h. Cells were centrifuged at 4000 × g; resuspended in 50 mM NaH2PO4, pH 8.5, 300 mM NaCl, 2% LDAO, 10 mM imidazole, 1000 units of benzonase, 0.5 mg/ml lysozyme, Complete EDTA-free inhibitor mixture (Roche Molecular Biochemicals); and sonicated three times for 1 min at 30 watts. Suspension was centrifuged at 10,000 × g for 30 min and applied onto a FLAG-M2 agarose affinity column (Sigma) equilibrated in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Recombinant protein was eluted with 100 μg/ml FLAG peptide in TBS.

Results and Discussion

Coprojection of Int-6 with a Phosphotyrosine Protein—It is known that treatment of cells with phosphatase inhibitors like H2O2 or pervanadate causes a rapid and strong induction of protein phosphorylation on tyrosine. This phenomenon is reversible and has been observed with membrane fractions of Jurkat cell extracts (28). This induction of phosphorylation, which resembles that resulting from TCR/CD3 stimulation, was not observed in the Jurkat variant JCAM, which is deficient in the Lck tyrosine kinase (28). To seek for phosphorylation on tyrosine of Int-6 in T cells, we prepared extracts from either normal or H2O2-treated Jurkat cells. This was also done with the MT4 T-cell line. Immunoblot analysis of these extracts with antibodies recognizing phosphotyrosine proteins showed that H2O2 indeed induced appearance of multiple bands in both cell lines (Fig. 1A, compare lanes 1 and 2). These extracts were used for immunoprecipitation experiments with an antibody directed against the C-terminal 20 amino acids of Int-6 (19). Immunoblot analysis with the antibody to phosphotyrosine of the precipitated proteins did not reveal any band at the position of Int-6. Analysis of the same membrane using an antibody directed against the C-terminal 169 amino acids of Int-6 (19), together with a secondary antibody fluorescently labeled with cyanine 5, showed that equal amounts of Int-6 were precipitated with extracts of both untreated (lanes 1 and 3) or treated with 10 mM H2O2 (lanes 2 and 4) and lysed in Nonidet P-40-desoxycholate buffer. Cell extracts were analyzed by immunoblot with a mix of two monoclonal antibodies directed against phosphotyrosine: 4G10 (ascites supernatant diluted 1:40) and pY99 (purified antibody diluted 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). B, immunoprecipitation using the C-20 antibody to Int-6 was performed with extracts of untreated (lanes 1 and 4) or H2O2-treated (lanes 2 and 5) Jurkat (lanes 1 and 2) and MT4 (lanes 4 and 5) cells. In lanes 3 and 6, the experiment was performed as in lanes 2 and 5 except that the antibody was omitted as a control. The positions of the bands of a molecular weight marker run in parallel are given together with that of the p69 band. n.s., a nonspecific signal due to cross-reaction with the immunoglobulin heavy chain, as well as with protein A released from the beads. C, the same membrane as in B was analyzed with the C-169 antibody to Int-6 and a fluorescently labeled secondary antibody. The image obtained by scanning the membrane using the STORM 860 apparatus is shown. The position of the signal corresponding to Int-6 is indicated.
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with the Jurkat cell extract, but it was not further considered. These observations showed that Int-6 is apparently not phosphorylated on tyrosine by itself but is associated with a phosphotyrosine protein of 69 kDa.

Identification of p69 by Mass Spectrometry—To characterize p69, immunoprecipitation using the C-20 antibody to Int-6 was performed on a larger scale (see "Experimental Procedures"). Proteins were eluted by incubation in a buffer containing 1% SDS at 37 °C. This moderate temperature was important to avoid release of contaminant proteins associated with the beads (data not shown). After acetone precipitation and SDS-PAGE, the gel was stained, and the band migrating at 69 kDa was excised. Proteins present in the gel piece were digested with trypsin, and peptides were analyzed by mass spectrometry. MALDI-TOF analysis indicated that 12 of 25 peptides characterized by MALDI-TOF corresponded to HSPC021. B protein domains of the rice, nematode, fly, and human HSPC021. The sequences of HSPC021 from Oryza sativa (Os), C. elegans (Ce), D. melanogaster (Dm), and H. sapiens (Hs) were analyzed by the pSscan program on the ISREC Profile Scan Server. The PCI domain (light gray box), the tetratricopeptide repeat (TPR) (gray box), and the Pumilio FBF RNA binding domain repeat (Puf/R) (black box) are represented. The PCI domains are located between positions 407 and 484 in C. elegans and between positions 403 and 465 in O. sativa. The tetratricopeptides are between positions 313 and 346 in D. melanogaster and between positions 284 and 317 in D. melanogaster. The Puf repeat is between positions 512 and 537 in D. melanogaster. The GenBank accession numbers for these sequences are as follows: H. sapiens, AF077207; D. melanogaster, AE003542; C. elegans, U28739; O. sativa, AP001859 (BAA94770).

Data bank searches showed that HSPC021 is well conserved among distant organisms. Homologs are indeed present in rice, Drosophila, and nematode. However, this search did not identify homologs in both S. pombe and S. cerevisiae. The sequence of the different HSPC021s was scanned using the pSscan program on the ISREC server (available on the World Wide Web at www.isrec.isb-sib.ch/software/) to identify particular protein domains. This showed that the rice and nematode proteins contain a proteasome-COP9 signalosome-initiation factor 3 (PCI) domain (Fig. 2B). The program did not identify it in the human and Drosophila sequences, but the region is well conserved (36.4% identity and 39.0% similarity between C. elegans and H. sapiens; 40.3% identity and 37.7% similarity between C. elegans and D. melanogaster). This motif exists in three subunits of eIF3 (p170, p110, and Int-6), in five subunits of the lid of the human 26 S proteasome (PSMD-3, -12, -6, -8, and -13), and in six subunits of the human COP9 signalosome (CSN1, -2, -3, -4, -7, and -8). Scanning of the Drosophila and human proteins identified a tetra tricopeptide repeat (Fig. 2B). This motif, present in one or several copies in various proteins, folds into two antiparallel α-helices and is involved in protein-protein interactions (30). Association of at least three repeats forms a domain to which peptides can bind in an extended conformation (31). Finally, this analysis showed that the Drosophila sequence contains a Pumilio-FBF repeat (Puf repeat). This sequence is present in the RNA binding domain of the Drosophila protein Pumilio that regulates translation of the hunchback mRNA. A complex between the mRNA, Pumilio, Nanos, and brain tumor impedes its translation in the posterior compartment of the Drosophila embryo (32–35). The Pumilio RNA binding domain includes eight Puf repeats plus additional sequences at the N-terminal and C-terminal boundaries (36). The recent determination of the structure of the Puf domain showed that the Puf repeats fold into a motif including three α-helices, which form a rainbow-like arc (37). Interestingly, this structure can be compared with that formed by armadillo repeats of β-catenin, the HEAT repeats of protein phosphatase 2A, and the tetra tricopeptide repeats of protein phosphatase 5 (37). All of these proteins can be considered as members of a family of helical repeat proteins that present a surface for interaction with other molecules, proteins or RNAs. A Puf repeat was not detected by the program in human HSPC021, but this part is very well conserved between Drosophila and humans (64% identity and 36% similarity). This motif is also well conserved between Drosophila and nematode (32% identity and 56% similarity). It is intriguing that Drosophila HSPC021 includes both a tetra tricopeptide and Puf repeat. Considering the similarity between the structures of these motifs, it is possible that they participate to a larger conformation interacting with other proteins. In this regard, it is interesting to note that a Puf repeat also exists in the CSN2 subunit of the human COP9 signalosome and that the CSN1 and CSN3 subunits of this complex include a tetra tricopeptide repeat.

To confirm that p69 indeed corresponded to HSPC021, a rabbit polyclonal antibody directed against the C-terminal 19 amino acids of the protein was generated. As tested with extracts of Jurkat cells, this antibody revealed a unique band at 69 kDa, in agreement with the calculated molecular mass of 66.7 kDa (data not shown and Fig. 3, B and C, lanes 1). Extracts of Jurkat cells treated with H2O2 were immunoprecipitated with antibodies to either Int-6 or HSPC021. Proteins were analyzed by immunoblot with antibodies directed against either HSPC021 or phosphotyrosine. This experiment showed that HSPC021 was precipitated with equal efficiencies by antibodies to Int-6 or to HSPC021 (Fig. 3A, lanes 2 and 3). This suggests that all HSPC021 is bound to Int-6. This also showed that the band recognized by the antibody to HSPC021 is the same as that revealed by the antibody to phosphotyrosine. These results establish that the phosphotyrosine protein precipitated with Int-6 is HSPC021. It was further examined whether the association of Int-6 with HSPC021 depends on the...
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The antibody directed against the C-terminal 20 amino acids of Int-6 reacts only against this specific subunit of eIF3 and precipitates HSPC021 (Fig. 3). To further strengthen the notion that HSPC021 is associated with eIF3 and to establish that its precipitation by the eIF3 antibody was not due to a poor specificity of this goat antiserum, it was also tested whether an antibody raised against the N-terminal 19 amino acids of eIF3-p66 precipitates HSPC021. Immunoblot analysis of an extract of Jurkat cells with this antibody revealed a unique band at the correct molecular weight (Fig. 5C, lane 1). It was verified whether this antibody also worked for immunoprecipitation by analyzing the precipitated proteins by immunoblot with the same antibody. With the exception of the strong nonspecific signal due to the heavy chain of the immunoglobulins, this revealed a unique band at the position of p66 (Fig. 5C, lane 3). However, the intensity of the signal observed was not very strong, indicating that the ability of this antibody to work for immunoprecipitation was limited. The same experiment was done except that the antibody to HSPC021 was used to analyze the precipitated proteins by immunoblot. The band corresponding to HSPC021 was clearly detected (Fig. 5C, lane 6), and replacement of the antibody to eIF3-p66 by the preimmune antibody directed against the C-terminal 20 amino acids of Int-6 treated or H2O2-treated were immunoprecipitated using the antibody to Int-6. As a control, antibody was omitted in the immunoprecipitation reaction (lane 1). The position of the 75-kDa band of a molecular weight marker run in parallel is indicated. B, extracts of untreated or H2O2-treated Jurkat cells were directly analyzed using the antibody to HSPC021 or used for immunoprecipitation with the antibody to Int-6. In lanes 3 and 5, the antibody was omitted in the immunoprecipitation reaction. C, extracts of BHK21 (upper panel) as well as of COS7 cells (lower panel) were directly analyzed by immunoblot using the antibody to HSPC021 (lane 1) or used for immunoprecipitation with the antibody to Int-6 (lane 3). In lane 2, the antibody was omitted.

H2O2 treatment (i.e. on the phosphorylation on tyrosine of HSPC021). To this end, proteins from Jurkat cells either untreated or H2O2-treated were immunoprecipitated using the antibody to Int-6 and analyzed by immunoblot with the antibody to HSPC021. The association of HSPC021 with Int-6 was not significantly modified by exposure of the cells to H2O2 (Fig. 3B, compare lanes 4 and 6). Direct analysis of the extracts did not show any variation in the amount of HSPC021 in response to the H2O2 treatment (Fig. 3B, lanes 1 and 2). It was further evaluated whether the association of Int-6 with HSPC021 also occurs in cell types other than T-cells. Extracts of BHK21 and COS7 cells were immunoprecipitated using the C-20 antibody to Int-6 and analyzed by immunoblotting with the antibody to HSPC021. This latter protein was found to be present in the immunoprecipitated proteins in both cell types (Fig. 3C, lane 3). Taken together, these data show that HSPC021 is associated with Int-6 in various cell types and that phosphorylation of HSPC021 on tyrosine does not impede or increase this interaction.

HSPC021 Is Associated with Human eIF3—As discussed previously, Int-6 is present in multiple protein complexes. In order to evaluate whether HSPC021 is also part of a larger protein assembly, a Jurkat whole cell extract was fractionated on a gel filtration column. Each fraction was tested by immunoblot for the presence of Int-6, HSPC021 and eIF3. The eIF3 antibody, a kind gift of Dr. Hershey, does not recognize with equal efficiency all of the subunits of the complex (3). Under these conditions, mainly four of them were detected: p170; p116 and p110, which both appeared as a single band; and p44. By considering these bands, eIF3 was observed to elute in five fractions (Fig. 4, lanes 6–10) with an apparent molecular mass of 900 kDa, which is significantly larger than that previously given for purified eIF3 (~600 kDa). Int-6 was clearly observed in the five fractions where the four other subunits of eIF3 were present, but it was also present in fractions corresponding to lower molecular weights in which p170 and p44 were absent. HSPC021 exhibited a pattern very similar to that of Int-6 although slightly less extended toward the lower molecular weights (Fig. 4, lane 12). These data suggested that HSPC021 might be associated with eIF3, although previous systematic analyses did not identify this protein in purified forms of the translation initiation factor. Several experiments were undertaken to test this hypothesis. The antibody directed against eIF3 was used to perform immunoprecipitations using extracts of Jurkat cells. To evaluate whether the association of HSPC021 with eIF3 is weak, this being a possible explanation for the absence of HSPC021 in purified eIF3s, the extracts were prepared in two types of buffer: the Nonidet P-40 lysis buffer and the more stringent radioimmune precipitation buffer. Proteins immunoprecipitated by the antibody to eIF3 were analyzed by immunoblot using the anti-HSPC021 antibody. A strong signal at 69 kDa was observed with both buffers (Fig. 5A, lanes 4 and 6). Immunoblot analysis of purified bacterially expressed HSPC021 indicated that the eIF3 antibody was not able to recognize the band corresponding to this protein (data not shown). Thus, the precipitation of HSPC021 by the eIF3 antibody is probably due to a tight association between the protein and the complex rather than to a direct binding of the immunoglobulins to HSPC021. The reverse experiment was performed but gave negative results. The exact reason for this is not known, but the most likely explanation is that the binding of the immunoglobulins to HSPC021 hinders association of the protein with eIF3. We then asked whether phosphorylated HSPC021 also binds to eIF3. Proteins of an extract of Jurkat cells exposed to H2O2 were precipitated with the antibody to eIF3 and analyzed by immunoblot with the antibodies directed against phosphorytosine. This experiment clearly detected the band at 69 kDa (Fig. 5B, lane 2). This band was not very intense, but the efficiency of the induction of phosphorylation on tyrosine was not very high in this particular extract (data not shown). We conclude from this result that phosphorylation of HSPC021 on tyrosine does not impede its association with eIF3.

The antibody directed against the C-terminal 20 amino acids of Int-6 treated or H2O2-treated were immunoprecipitated using the antibody to H2O2 and analyzed by immunoblot using the antibody to HSPC021. The antibody was directed against the C-terminal 20 amino acids of Int-6 was used for immunoblot with the antibody to HSPC021 (upper panel) as well as antibodies to phosphotyrosine as described in the legend to Fig. 1 (lower panel). As a control, antibody was omitted in the immunoprecipitation reaction (lane 1). The position of the 75-kDa band of a molecular weight marker run in parallel is indicated.
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Fig. 4. Coelution of eIF3, HSPC021, and Int-6 on a gel filtration column. A whole cell extract of Jurkat cells was loaded on a Superose 6 gel filtration column. Fractions were analyzed by immunoblot with antibodies to eIF3 (upper panel), HSPC021 (middle panel), and Int-6 (lower panel). Lane 1 corresponds to the extract loaded onto the column. The elution volume corresponding to the different fractions in ml is indicated at the top. The column was calibrated with protein standards. Their position of elution is indicated above the immunoblots.

Fig. 5. Association of HSPC021 with eIF3. A, Jurkat cell extracts were prepared in either Nonidet P-40 (lane 1) or radioimmune precipitation (lane 2) buffers. These extracts were used for immunoprecipitation using the antibody directed against eIF3 (lanes 4 and 6), and precipitated proteins were analyzed by immunoblot with the antibody to HSPC021. In lanes 3 and 5, normal goat serum was used in the immunoprecipitation reaction as a control. The positions of the 83- and 62-kDa bands of molecular mass markers are represented. B, an extract of H2O2-treated Jurkat cells was used for immunoprecipitation using the antibody to eIF3, and immunoblotting was done with the antibodies directed against phosphotyrosine. In lane 1, the antibody was omitted. C, an extract of Jurkat cells was used for immunoprecipitation using the antibody to eIF3-p66. Immunoblotting was done with the antibodies directed against either p66 (lanes 1–5) or HSPC021 (lanes 4–6). In lanes 1 and 4, the extract was directly loaded. In lanes 2 and 5, the antisera was replaced by preimmunized serum as control. The position of the signal corresponding to either p66 or HSPC021 (p69) is indicated. D, constructs expressing HSPC021 either with an N-terminal FLAG tag (lanes 1 and 2) or without (lanes 3 and 4) were used for in vitro transcription/translation using the TNT system (Promega). Proteins were labeled by the addition in the reaction of biotinylated lysines. The products were diluted in Nonidet P-40 buffer, and immunoprecipitations were carried out using the antibody to eIF3. After SDS-PAGE and transfer to PVDF, the membrane was probed with streptavidin coupled to peroxidase. In lanes 1 and 3, normal goat serum was used in the immunoprecipitation reaction.

To further strengthen the notion that this protein is associated with the translation initiation factor, it was tested whether newly synthesized HSPC021 could incorporate into eIF3. Both wild type and a N-terminal FLAG-tagged form of HSPC021 were produced by in vitro coupled transcription/translation. During synthesis, proteins were labeled by incorporation of biotinylated lysines. Proteins were immunoprecipitated using the antibody to eIF3 and analyzed by immunoblot with streptavidin coupled to peroxidase. For both proteins, the signal at 69 kDa was clearly detected (Fig. 5D, lanes 2 and 4).

Similar experiments were also performed by transfecting COS7 cells with vectors expressing FLAG-tagged forms of HSPC021 corresponding to either the entire coding sequence or both N-terminal and C-terminal deletion mutants (Fig. 6A). All proteins were expressed at the same level (Fig. 6B). Extracts of transfected cells were precipitated with the antibody to eIF3, and immunoblot was performed with the M2 monoclonal antibody directed against the FLAG epitope. A band at 69 kDa was clearly observed in the extracts corresponding to expression of the entire HSPC021 protein (Fig. 6C, lanes 2 and 6), but for each mutant no signal was observed (Fig. 6C, lanes 3, 4, 7, and 8). This experiment showed that HSPC021 incorporates into endogenous eIF3 and that this event is hindered by alterations of both N-terminal and C-terminal ends of the protein. Either loss of important domains or alteration of the overall structure of the protein might explain this lack of incorporation into eIF3. That the putative Puf repeat at the end of the protein is necessary (mutant 1–536) is likely to explain why binding of the antibody directed against the last 19 amino acids of HSPC021 impedes its association with eIF3 (see above).

Taken together, these results establish that HSPC021 specifically binds eIF3. Previous works with purified eIF3 did not identify this protein. The exact reason for this discrepancy is not known. A survey of the numerous expressed sequence tags corresponding to HSPC021 indicates that it is ubiquitously expressed; hence, the reason for its absence in purified eIF3 is probably not the consequence of a lack of expression. The most likely explanation is that the purification processes caused removal of this subunit. In agreement with this possibility is the fact that the size of the complex observed in the gel filtration column appears higher than that reported for purified eIF3. A strong support to the notion of HSPC021 being tightly associated with eIF3 comes also from results published during completion of this work on the A. thaliana form. Indeed, it has been reported that eIF3 purified from this organism includes 12 subunits, one of them corresponding to the A. thaliana homolog of HSPC021 (38). In agreement with the data published by Burks et al., the results presented in this paper show that HSPC021 strongly binds the eIF3 translation initiation factor in mammals. The data previously obtained in vitro with the purified form of eIF3 indicate that HSPC021 is not essential to the activity of this translation factor. This protein is likely to have a regulatory role, which remains to be understood.

Protein-Protein Interaction of HSPC021 with Int-6—Assuming that HSPC021 binds eIF3, we wondered whether its association with Int-6 was direct or depended on other proteins of the translation initiation factor. This was first evaluated by coexpressing Int-6 with the various FLAG-tagged forms of HSPC021 in COS7 cells. Int-6 was found to coprecipitate with the entire HSPC021 but also with the four mutants (Fig. 7A).
Both proteins bound to HSPC021 (Fig. 7A, lanes 1 and 5). Since the HSPC021 mutants did not incorporate into eIF3 (Fig. 6C), this result is in favor of a direct protein-protein interaction between HSPC021 and Int-6. To further establish this point, Far Western experiments were performed with bacterially expressed proteins. FLAG-tagged HSPC021 was produced in E. coli and purified by affinity onto an anti-FLAG M2 gel. The purified FLAG-HSPC021 protein was migrated through a SDS protein gel and transferred to PVDF membranes. These latter were incubated with purified or C terminus-truncated Int-6. These proteins were further detected by probing with an antibody directed against the N-terminal 19 amino acids of Int-6. The amount of bovine serum albumin or HSPC021 used in the gel is indicated in ng. The position of the band corresponding to the entire FLAG-tagged HSPC021 is indicated (FH).

This was clearly dependent upon expression of the different HSPC021 forms, since no signal at the position of Int-6 was detected when cells were transfected only with the Int-6 expression vector (Fig. 7A, lanes 1 and 5). Since the HSPC021 mutants did not incorporate into eIF3 (Fig. 6C), this result is in favor of a direct protein-protein interaction between HSPC021 and Int-6. To further establish this point, Far Western experiments were performed with bacterially expressed proteins. FLAG-tagged HSPC021 was produced in E. coli and purified by affinity onto an anti-FLAG M2 gel. The purified FLAG-HSPC021 protein was migrated through a SDS protein gel and transferred to PVDF membranes. These latter were incubated with purified or C terminus-truncated Int-6. These proteins were further detected by probing with an antibody directed against the N-terminal 19 amino acids of Int-6. The amount of bovine serum albumin or HSPC021 used in the gel is indicated in ng. The position of the band corresponding to the entire FLAG-tagged HSPC021 is indicated (FH).

In conclusion, the data presented in this report show that HSPC021 is a eIF3-associated protein that can be phosphorylated on tyrosine and that directly interacts with Int-6. Interestingly, eIF3-p170 has been shown recently to bind to the intracellular domain of the receptor tyrosine kinase TrkA (39). This suggests that the activity of eIF3 might be modulated by tyrosine kinases. Future studies should help to understand how post-translational modifications of the eIF3 subunits affect translation. In particular, it will be important to determine whether these events affect this process globally or allow modulation of translation of specific mRNA. The complexity of eIF3 suggests that it can integrate many regulatory events. This might be an exciting challenge.
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