

An essential role for hGle1 nucleocytoplasmic shuttling in mRNA export

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Gle1 is required for mRNA export in yeast and human cells. Here, we report that two human Gle1 (hGle1) isoforms are expressed in HeLa cells (hGle1A and B). The two encoded proteins are identical except for their COOH-terminal regions. hGle1A ends with a unique four-amino acid segment, whereas hGle1B has a COOH-terminal 43-amino acid span. Only hGle1B, the more abundant isoform, localizes to the nuclear envelope (NE) and pore complex. To test whether hGle1 is a dynamic shuttling transport factor, we microinjected HeLa cells with recombinant hGle1 and conducted photobleaching studies of live HeLa cells expressing EGFP-hGle1. Both strategies show

that hGle1 shuttles between the nucleus and cytoplasm. An internal 39-amino acid domain is necessary and sufficient for mediating nucleocytoplasmic transport. Using a cell-permeable peptide strategy, we document a role for hGle1 shuttling in mRNA export. An hGle1 shuttling domain (SD) peptide impairs the export of both total poly(A)⁺ RNA and the specific dihydrofolate reductase mRNA. Coincidentally, SD peptide-treated cells show decreased endogenous hGle1 localization at the NE and reduced nucleocytoplasmic shuttling of microinjected, recombinant hGle1. These findings pinpoint the first functional motif in hGle1 and link hGle1 to the dynamic mRNA export mechanism.

Introduction

mRNAs undergo a number of processing and maturation events between their transcription by polymerases and their ultimate translation by ribosomes (Maniatis and Reed, 2002). In eukaryotic cells, a key step involves export to the cytoplasm through nuclear pore complexes (NPCs)* embedded in the nuclear envelope (NE) (Nakielny and Dreyfuss, 1999; Wenté, 2000). The export of mRNAs is a stepwise process (Maniatis and Reed, 2002) beginning with the processing of pre-mRNAs into heterogeneous nuclear ribonucleoprotein (hnRNP) complexes that are recognized by transport receptors in the nucleoplasm. The transporters subsequently target the mRNA-bound hnRNP complex (mRNP) to the NPC and mediate translocation through the NPC for release on the

cytoplasmic side. The transporters and the hnRNPs are subsequently reimported into the nucleus and, thus, are considered dynamic shuttling factors.

The molecular paradigms for this pathway were initially established from studies of viral RNA export (Cullen, 2000; Hammarskjöld, 2001). For the HIV-1 pathway, a nuclear export sequence (NES) in the RNA-bound viral Rev protein is recognized by Crm1 (Fischer et al., 1999), a member of a conserved family of transport receptors called karyopherins (also designated exportins/importins/transportins) (Gorlich and Kutay, 1999). Through interaction with specific adaptor proteins, Crm1 also mediates the export of cellular RNAs, such as 5S RNA and U1 snRNA (Fischer et al., 1995), and the 60S ribosomal subunit (Ho et al., 2000). A different karyopherin (exportin-t/Los1) mediates the export of tRNA (Gorlich and Kutay, 1999).

Karyopherins may play multiple roles in the export of mRNPs (Yi et al., 2002), some being required for the export of cellular mRNAs from early response genes (Gallouzi and Steitz, 2001; Gallouzi et al., 2001). Distinct sets of importins are needed for the reimport of shuttling hnRNPs (Michael et al., 1995, 1997). However, studies in yeast, mammalian, and *Xenopus* oocyte systems have illustrated a central role for the novel Mex67/TAP/NXF1 protein (Segref et al., 1997; Gruter et al., 1998; Katahira et al., 1999). In higher eukaryotes, splicing and TAP/NXF1-mediated nuclear export are be-

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*Abbreviations used in this paper: AP, antennapedia peptide; DHFR, dihydrofolate reductase; FLIP, fluorescence loss in photobleaching; GR, glucocorticoid receptor; hGle1, human Gle1; hnRNP, heterogeneous nuclear ribonucleoprotein; IIF, indirect immunofluorescence; LR, leucine rich; mRNP, mRNA-bound hnRNP complex; NE, nuclear envelope; NES, nuclear export sequence; NPC, nuclear pore complex; scGle1, *Saccharomyces cerevisiae* Gle1; SD, shuttling domain; TR, Texas red.

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lieved to be coupled (Maniatis and Reed, 2002). TAP/NXF1, together with its cofactor p15/NXT1 (Fribourg, et al., 2001), promotes the export of both spliced and intronless transcripts through interactions with both mRNPs and NPC proteins (Maniatis and Reed, 2002). Thus, TAP/NXF1 may act as a shuttling bridge between the mRNP complex and the NPC (Zenklusen and Stutz, 2001). Although TAP/NXF1 was shown to cooperate with karyopherins (Shamsher et al., 2002), TAP/NXF1 itself does not bind Ran, and RanGTP hydrolysis is not required for TAP/NXF1-mediated mRNA export (Bachi et al., 2000).

Several other factors are also required for mRNA export, including the DEAD-box RNA helicase Dbp5 and two NPC-associated factors Gle1 and Gle2/Rae1 (Zenklusen and Stutz, 2001; Reed and Hurt, 2002). Dbp5 may remodel hnRNP complexes at the NPC and mediate the release of nonshuttling hnRNPs in the nucleus and shuttling hnRNPs in the cytoplasm. This could serve a critical role in mRNP transport directionality (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999; Zhao et al., 2002). Work from several groups strongly supports the hypothesis that Gle1 plays an essential role in mRNA export (Del Priore et al., 1996; Murphy and Wentte, 1996; Noble and Guthrie, 1996; Watkins et al., 1998). Human Gle1 (hGle1) and *Saccharomyces cerevisiae* Gle1 (scGle1) have no consensus RNA binding sites, and no functional protein motifs have been definitely identified (Reed and Hurt, 2002). Moreover, no hGle1-interacting proteins have been identified. Genetic connections implicate scGle1 as a modulator of scDbp5 activity (Hodge et al., 1999), and scGle1 also interacts with Nup42 (Murphy and Wentte, 1996; Stutz et al., 1997; Strahm et al., 1999), Dbp5, and a nonessential factor, Gfd1 (Hodge et al., 1999; Strahm et al., 1999). Recently, our studies in yeast have shown that soluble inositol hexakisphosphate production is required for efficient scGle1 function (York et al., 1999). Overall, how Gle1 mediates nuclear transport is far from being resolved.

A long-standing question regarding Gle1 function is whether it is a dynamic shuttling factor or a stable component of the NPC (a nucleoporin). Others have suggested that scGle1 is a static nucleoporin (Rout et al., 2000). However, the mammalian Gle1 protein does not cofractionate with NPCs (Cronshaw et al., 2002). Here, we have focused on the human protein. We demonstrate that two hGle1 protein variants (hGle1A and B) are encoded from a single gene. The hGle1B isoform associates with the NE/NPC in HeLa cells and is the predominant variant. We subsequently show that both proteins can shuttle between the nucleus and cytoplasm and that this shuttling activity requires the presence of a novel 39–amino acid domain. Moreover, using a cell-permeable peptide strategy, we found that hGle1 shuttling is essential for poly(A)⁺ RNA export.

Results

Human Gle1 cDNAs encode two isoforms in HeLa cells

Previous studies have shown endogenous hGle1 localization at the NPCs/NE and in the cytoplasm and nucleus (Watkins et al., 1998; Fig. 1 A). Here we show that ectopic expression

of the hGle1 cDNA previously reported by Watkins et al. (1998), in fusion with EGFP, did not produce a protein that localized predominately to the NE of transfected cells (Fig. 1 A). We speculated that this isoform (hereafter designated hGle1A, a 659–amino acid polypeptide; accession no. AAC25561) lacked a critical region required for NPC/NE association. Recently, a human testis cDNA clone was deposited in the human EST database (accession no. AAH30012). The predicted protein product is a 698–amino acid polypeptide (designated hGle1B) identical to hGle1A except at the COOH terminus (Fig. 1, B and D). Based on genomic sequence information (Locus ID 2733), the *hGle1* gene is comprised of 16 exons. Within exon 14, a 5' cryptic splice donor site could account for the expression of two transcript variants, with two corresponding different 3' untranslated regions (Nissim-Rafinia and Kerem, 2002). The predicted products result in the hGle1A version with a unique four–amino acid segment and hGle1B with a different 43–amino acid span at the COOH terminus (Fig. 1 D).

To determine if HeLa cells express these two mRNA isoforms, we performed semiquantitative PCR studies on total HeLa cDNA (Fig. 1 C). Using a forward (F) primer common to both variants and isoform-specific reverse primers (A and B, respectively; Fig. 1 B), we found that HeLa cells expressed both the short (hGle1A) and long (hGle1B) isoforms and that hGle1B mRNA was ~1,000-fold more abundant than hGle1A. By Northern blotting, the preferential expression of the hGle1B mRNA over hGle1A was also detected in all human tissues analyzed (unpublished data). Finally, ectopic expression experiments in HeLa cells revealed that EGFP–hGle1B localization was similar to endogenous hGle1 (Fig. 1 A). These results argue that hGle1B is the prevalent isoform in human cells.

Identifying an hGle1 domain with nuclear export activity

We speculated that if hGle1 is dynamic and transiently associates with NPCs while entering and exiting the nucleus, it should contain domains/sequences that harbor nucleocytoplasmic transport activities. The region of hGle1 spanning amino acid residues 444–606 was specifically targeted for internal in-frame deletion based on our previous studies of scGle1 (Murphy and Wentte, 1996; Watkins et al., 1998). The COOH-terminal regions of scGle1 and hGle1A/B are highly similar, and the corresponding region in scGle1 contains a putative leucine-rich (LR) NES-like motif that is essential for scGle1 function in mRNA export (Fig. 1 D; see Discussion). As a tool to delineate the region(s) of hGle1 that may harbor nuclear export activity, we generated a panel of ectopically expressed FLAG-tagged hGle1A deletion proteins and analyzed their respective intracellular distributions independent of the endogenous hGle1 by indirect immunofluorescence (IIF) using anti-FLAG antibodies. To assay for export activity, we placed the SV40 large T antigen NLS in frame at the COOH terminus of FLAG–hGle1A. Based on the hypothesis that hGle1 harbors nuclear export activity, it should balance the activity of the heterologous NLS and result in cytoplasmic FLAG–hGle1A–NLS localization. Results from these experiments are summarized in Fig. 2. Although the majority of the FLAG–hGle1A–NLS

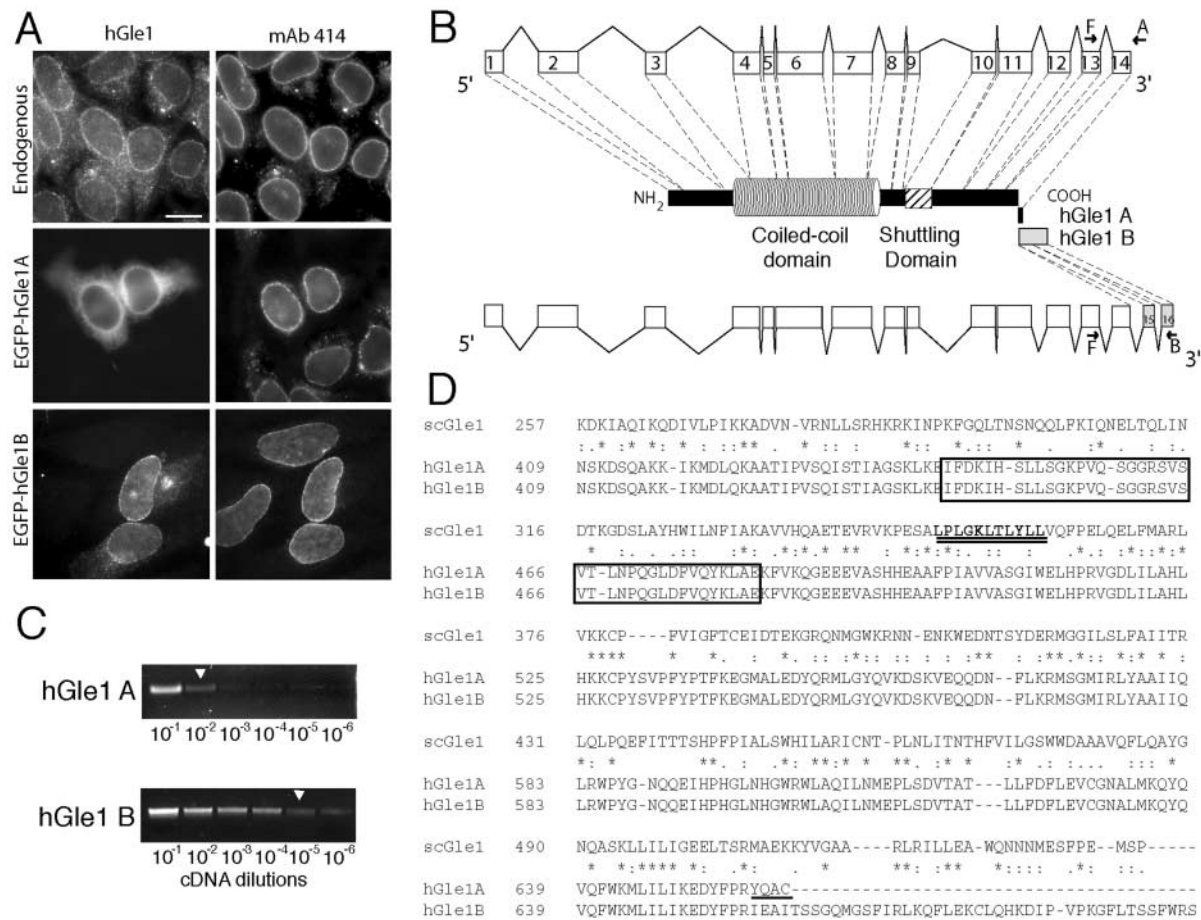


Figure 1. ***hGle1* encodes two mRNA isoforms in HeLa cells.** (A) Intracellular localization of endogenous hGle1 (top; using anti-hGle1 antibodies; Watkins et al., 1998), ectopically expressed EGFP-hGle1A (middle), and EGFP-hGle1B (bottom) in fixed HeLa cells (Fix-Triton; see Materials and methods). Double IIF with mAb414 was used to detect NPC/NE localization. Bar, 10 μ m. (B) Schematic representation of *hGle1*. Exons are boxed, numbered 5' to 3', with nos. 15 and 16 specific to hGle1B (gray boxes). Primers used to specifically amplify hGle1A and hGle1B in RT-PCR experiments are F (common forward primer), A (hGle1A 3' UTR-specific reverse primer), and B (hGle1B-specific reverse primer). (C) Semiquantitative RT-PCR analysis of hGle1A and hGle1B mRNA levels in HeLa cells. Serial dilutions of total HeLa cell cDNA were tested using primer combinations F/A and F/B for hGle1A and hGle1B, respectively. Amplification products (202 bp and 180 bp, respectively) were separated on agarose gel and stained with ethidium bromide. Similar intensities at cDNA dilutions of 10⁻² for hGle1A and 10⁻⁵ for hGle1B (arrowheads) suggest that hGle1B mRNA is 10³-fold more abundant. (D) Sequence comparison (ClustalW) of the COOH-terminal regions of scGle1, hGle1A, and hGle1B (Pearson and Lipman, 1988). Boxed region 444–483 of hGle1A/B harbors shuttling activity. The putative LR-NES in scGle1 is double underlined. The unique four amino acids of hGle1A are underlined. *, identical residues; :, or ., conserved residues.

was detected in the nucleus, FLAG-hGle1A-NLS was also clearly present in the cytoplasm (Fig. 2, lower left). Strikingly, the FLAG-hGle1A $_{\Delta 444-606}$ -NLS protein was localized exclusively in the nucleus. Various NH₂- and COOH-terminal deletions of the 444–606 region were tested to map the export activity. Deletions from the NH₂-terminal side eliminated cytoplasmic localization ($\Delta 444-511$), whereas deletions from the COOH-terminal side up to residue 483 retained the export activity ($\Delta 484-606$) (Fig. 2). Thus, a 39-amino acid region from residues 444–483 was necessary to confer cytoplasmic localization of FLAG-hGle1A-NLS.

The 39-amino acid region of hGle1 has intrinsic nucleocytoplasmic shuttling activity

To further examine the transport properties of the 39-amino acid hGle1 region defined above, we conducted a series of microinjection experiments with bacterially expressed and purified GST fusion proteins (Fig. 3). Binucle-

ate HeLa cells were used for nuclear microinjection such that the export and nucleocytoplasmic shuttling activities of GST fusion proteins could be coincidentally examined. First, a GST fusion with only the 39-amino acid putative domain (hGle1 $_{444-483}$ (SD)) was tested by coinjection with Texas red (TR)-labeled 70-kD dextran into one nucleus of a binucleate HeLa cell (Fig. 3 A). The TR-dextran identified injected cells and the subcellular injection site (arrows). The cells were subsequently incubated at either 37°C (Fig. 3 A, left) or 4°C (right), fixed, and processed for IIF microscopy with anti-GST antibodies. At 37°C, 86.5% ($\pm 2.8\%$; $n = 112$) of the microinjected cells showed GST-hGle1 $_{444-483}$ (SD) at the injection site, in the cytoplasm, and in the second nucleus (Fig. 3 A, arrowhead). In contrast, at 4°C, GST-hGle1 $_{444-483}$ (SD) remained at the site of injection in 87% ($\pm 4.3\%$; $n = 81$) of the injected cells. These results indicated that GST-hGle1 $_{444-483}$ (SD) was actively exported from the injected nucleus. In time course experi-

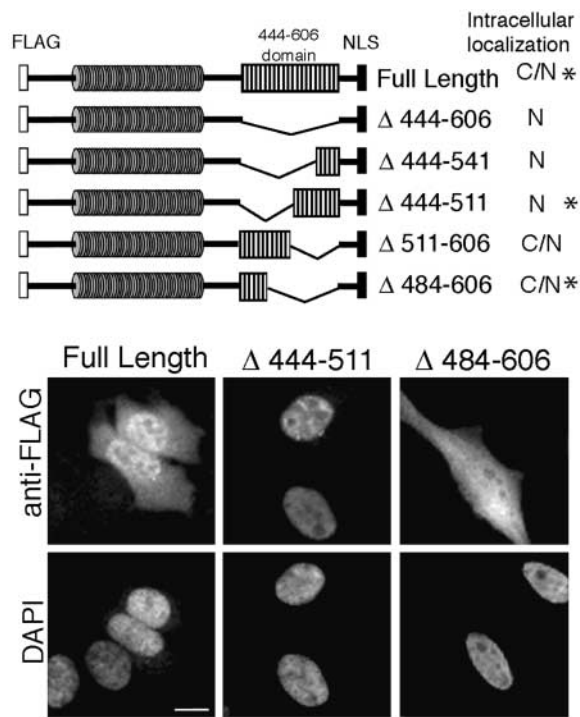


Figure 2. Delineating the hGle1 domain with nuclear export activity. (Top) Schematic representation and summary of the intracellular localizations for FLAG-hGle1A-NLS deletion constructs. C, cytoplasmic localization; N, nuclear localization; *, representative localization below. (Bottom) IIF localization of representative FLAG-hGle1-NLS proteins after transient expression in HeLa cells. Anti-FLAG monoclonal antibodies (top row) and staining for nuclear DNA with DAPI (bottom row) are shown. The cells in each figure are representative of the localization seen in the majority (~70%; $n = 500$) of the cells across the total population of transfectants. Bar, 10 μm .

ments, export was detected at full levels within 2 h after injection (unpublished data). Thus, residues 444–483 of hGle1 were sufficient to mediate nuclear export. In addition, the presence of GST-hGle1_{444–483} (SD) in the second nucleus of binucleate cells suggested that this region also had nuclear import activity. These experiments support the hypothesis that hGle1 harbors a nucleocytoplasmic transport domain, for both import and export. Hence, we have designated this 39-amino acid span the hGle1 nucleocytoplasmic shuttling domain (SD).

To analyze the nucleocytoplasmic shuttling of full-length hGle1, GST-hGle1B and GST-hGle1A (unpublished data) were microinjected into the cytoplasm or nucleus of HeLa cells. After incubation at 37°C, protein localization was determined by IIF. Fig. 3 B shows that GST-hGle1B was exported from the injected nucleus and imported into the second nucleus (left panel, arrowhead) in 84% ($\pm 2.1\%$; $n = 56$) of the injected cells. Nuclear import activity of GST-hGle1B was also detected after cytoplasmic injection of the protein (asterisk). The transport activities appeared to be energy dependent, as shown by the lack of redistribution when incubated at 4°C (Fig. 3 B, right).

To independently test whether hGle1 shuttles, we analyzed the dynamics of EGFP-hGle1B in live HeLa cells (Fig. 3 C). This was based on our observation that ectopically ex-

pressed EGFP-hGle1B localizes like endogenous hGle1 (Fig. 1 A). Using the technique of fluorescence loss in photobleaching (FLIP), we analyzed whether nuclear EGFP-hGle1B could exchange with the cytoplasmic pool of EGFP-hGle1B. By repeatedly bleaching an area of the cytoplasm, the decrease in nuclear EGFP fluorescence over time was monitored. As controls, the same bleach protocol was conducted on EGFP-human coilin (a nonshuttling protein) and EGFP-hNup98 (a mobile nucleoporin) transfected cells (Griffis et al., 2002). Our results show that EGFP-hGle1B nuclear fluorescence was lost by repetitive cytoplasmic bleaching, similar to EGFP-hNup98. In contrast, <9% of nuclear EGFP-coilin fluorescence was lost in the same time frame. Thus, these experiments corroborate the microinjection results and demonstrate that hGle1B harbors nucleocytoplasmic shuttling activity.

To test whether the 39-amino acid region from residues 444–483 was required for hGle1 shuttling, we attempted to analyze the dynamics of EGFP-hGle1B_{ΔSD} in live HeLa cells. Unfortunately, ectopic expression of EGFP-hGle1B_{ΔSD} was toxic to the cells (unpublished data). Thus, GST-hGle1B_{ΔSD} was purified and microinjected into one nucleus of binucleate HeLa cells (Fig. 3 D, left). After incubation at 37°C for 5 h, GST-hGle1B_{ΔSD} was detected only in the injected nucleus in 87.8% ($\pm 6.7\%$; $n = 52$) of injected cells. GST-hGle1B_{ΔSD} was also microinjected into the cytoplasm of HeLa cells and detected in the nucleus in 89.4% ($\pm 2.5\%$; $n = 55$) of cases (Fig. 3 D, right, asterisk). However, over the same time frame after injection, the nuclear signal was not nearly as intense as with GST-hGle1B (Fig. 3, B and D, asterisk). We concluded that GST-hGle1B_{ΔSD} did not have nuclear export activity, and any remaining domain(s) had weak nuclear localization activity. The SD may provide the primary nuclear import pathway (see below). Similar experiments were conducted using GST-hGle1A and GST-hGle1A_{ΔSD} and showed identical results (not depicted).

The SD of hGle1 is required for hGle1-dependent mRNA export

Our previous studies have shown that hGle1 is required for the export of poly(A)⁺ RNA in HeLa cells (Watkins et al., 1998). We speculated that hGle1 nucleocytoplasmic shuttling may be important for its transport function and that this role may be mediated by the interaction of the SD with another factor in the poly(A)⁺ RNA export pathway. To investigate this hypothesis, we used a cell-permeable peptide strategy that is based on inhibiting protein-protein interactions (Pritchard et al., 1999). We reasoned that by competing for the factor(s) required for hGle1 transport, we would abrogate the shuttling of endogenous hGle1. We would then be able to study the effect of a “static” hGle1 on total poly(A)⁺ RNA distribution within the cell by in situ hybridization. A similar strategy was used by Gallouzi and Steitz (2001) in a study of mRNA export pathways.

To test this hypothesis, a peptide corresponding to the SD was synthesized as a COOH-terminal fusion to a 16-amino acid portion of helix 3 from the homeodomain of the *Drosophila* transcription factor antennapedia (Prochiantz, 1999), designated AP-hGle1-SD (Fig. 4 A). The an-

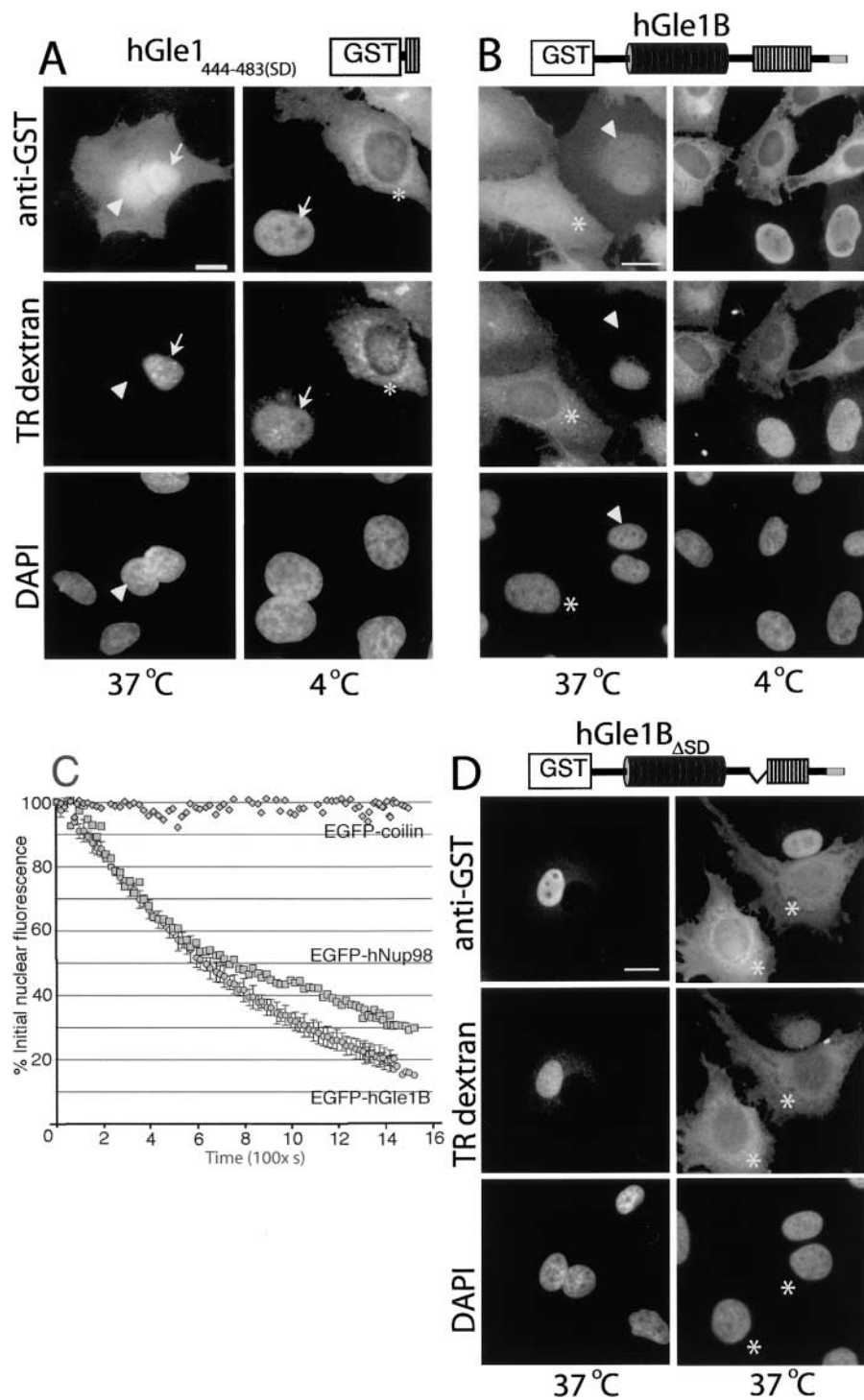


Figure 3. Analysis of GST-hGle1 dynamics in HeLa cells by microinjection.

(A) The hGle1 39-amino acid region from 444–483 has intrinsic nucleocytoplasmic shuttling activity. HeLa cells were comicroinjected with purified proteins and TR-labeled 70-kD dextran. Cells were incubated for 5 h at 37°C or 4°C after injection and processed for IIF microscopy using anti-GST antibodies (top). Nuclear DNA was stained with DAPI (bottom). At 37°C (left), microinjection of GST-hGle1_{444-483(SD)} in one nucleus of a binucleate HeLa cell (top, arrow), as indicated by the TR-labeled dextran (middle), results in GST staining in the second nucleus (arrowhead). At 4°C (right), nuclear export and import activity of GST-hGle1_{444-483(SD)} is not detected. (arrow, nuclear injection; asterisk, cytoplasmic injection). (B and D) The SD is necessary for GST-hGle1B export. Full-length GST-hGle1B (B) and GST-hGle1_{ΔSD} (D) were analyzed by microinjection. (B) After injection into one nucleus of a binucleate cell (arrowhead), GST-hGle1B is in both the cytoplasm and the uninjected nucleus after incubation at 37°C (right). Cytoplasmically microinjected GST-hGle1B (asterisk) shows strong nuclear staining. At 4°C (left), injected protein remains at the site of microinjection. (C) FLIP analysis reveals that EGFP-hGle1B shuttles between the nucleus and cytoplasm in HeLa cells. An area of the cytoplasm was repeatedly bleached, and the loss of nuclear fluorescence was monitored over time. The data points plotted for EGFP-hGle1B represent averages ($n = 6$). EGFP-coilin and EGFP-hNup98 data are representative of the loss of fluorescence detected in several time courses and are consistent with data previously reported (Griffis et al., 2002). (D) Microinjection of GST-hGle1B_{ΔSD} protein in the nucleus results in no staining outside the microinjection site after incubation at 37°C (both panels) (or 4°C; not depicted). In the right panel, partial nuclear localization is observed in two cytoplasmically injected cells (asterisks). Bars, 10 μ m.

tennapeptide peptide (AP) sequence mediates the uptake of peptides into cells and confers stability (Derossi et al., 1996, 1998). As a control, an AP fusion peptide was also synthesized with a randomly scrambled sequence of the same amino acid composition as the SD (designated AP-hGle1-scrSD; Fig. 4 A). As seen in Fig. 4 B, incubating cultured HeLa cells (at <10 passages, see Materials and methods) with media containing 5 μ M AP-hGle1-SD peptide had distinct effects. After 4 h in the presence of AP-hGle1-SD peptide, \sim 5% of cells appeared to undergo apoptosis (asterisk). Interestingly, all the remaining inter-

phase cells showed marked accumulation of poly(A)⁺ RNA in the nucleus. In contrast, no cytotoxicity was detected with the AP-hGle1-scrSD incubation, and all the cells showed normal poly(A)⁺ RNA distribution by in situ hybridization (untreated control cells in Fig. 4 C). In culture media with <10% FBS or with AP-hGle1-SD peptide concentrations >5 μ M, the viability of the cells was drastically reduced. However, if the inhibitory peptide was removed after the 4-h incubation, the cells recovered and showed normal poly(A)⁺ RNA distribution after 12 h (not depicted).

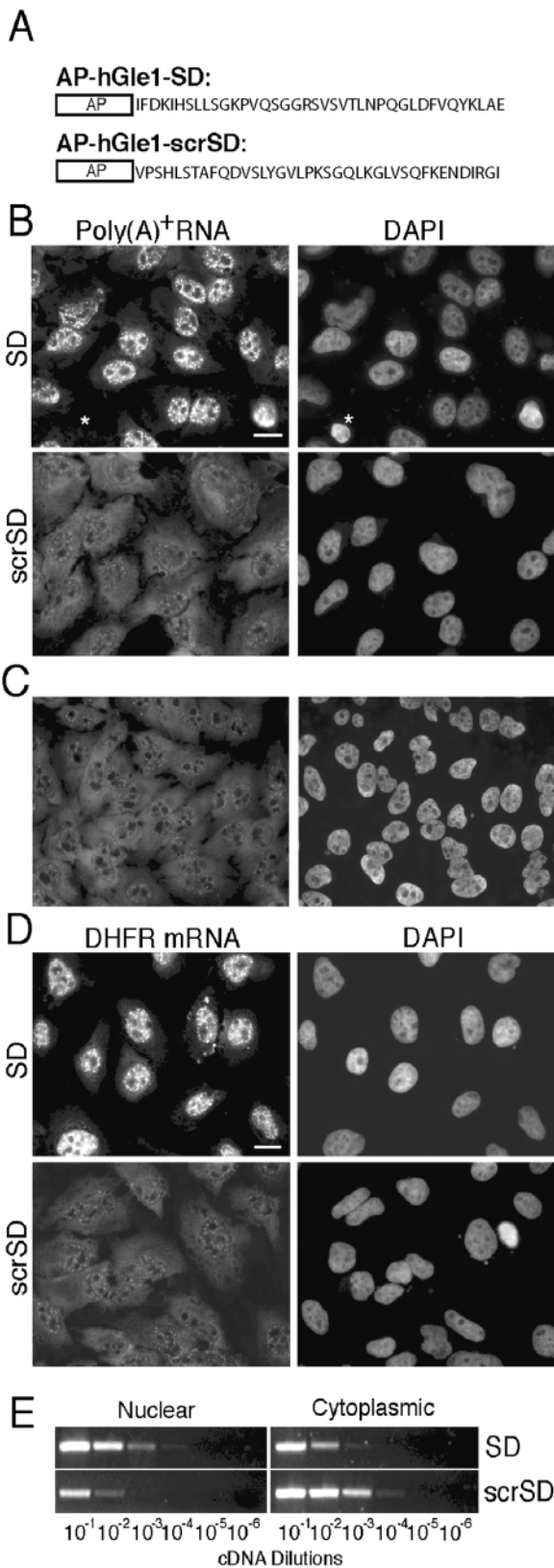


Figure 4. The AP-hGle1-SD peptide results in nuclear poly(A)⁺ RNA accumulation. (A) Schematic representation of the cell-permeable peptides used. AP-hGle1-SD, antennapedia 16-amino acid leader peptide (AP) followed by hGle1 amino acids 444–483 (hGle1-SD) or followed by randomly scrambled sequence of identical SD composition (AP-hGle1-scrSD). (B) In situ hybridization experiments detect nuclear poly(A)⁺ RNA accumulation in AP-hGle1-SD-treated

To ensure that the increase in nuclear poly(A)⁺ RNA levels did not reflect a perturbation in the polyadenylation process, we also analyzed the export of an individual mRNA. In situ hybridization experiments using a dihydrofolate reductase (DHFR) mRNA-specific digoxigenin-labeled antisense oligonucleotide were conducted. Incubation with the AP-hGle1-SD peptide resulted in nuclear accumulation of DHFR mRNA (Fig. 4 D). In contrast, the control AP-hGle1-scrSD peptide had no effect. To confirm the in situ hybridization experiments, we analyzed the relative amounts of DHFR mRNA in cytoplasmic and nuclear fractions isolated from HeLa cells pretreated with the peptides (Fig. 4 E). Using a semiquantitative RT-PCR strategy, the presence of AP-hGle1-SD peptide markedly decreased the levels of DHFR transcript in the cytoplasmic fraction. Moreover, there was a corresponding increase in the nuclear signal relative to the control AP-hGle1-scrSD-treated cells. Together, the in situ hybridization experiments and biochemical fractionation data confirm that AP-hGle1-SD peptide impairs the export of mRNA from the nucleus.

To test whether the decrease in mRNA export resulted from a general perturbation of transport through NPCs, protein import and export activity was analyzed. For nuclear import, cells transiently expressing a glucocorticoid receptor (GR) GFP fusion protein were treated with AP-peptides for 4 h (Fig. 5 A). In the absence of the agonist dexamethasone, a substantial GR-GFP cytoplasmic pool was observed. When dexamethasone was added to induce import, nuclear accumulation of GR-GFP was observed in the presence of either peptide. To determine if protein export from the nucleus was perturbed, the intracellular distribution of karyopherin/importin β 1 was monitored by IIF. We hypothesized that if the general nuclear protein export pathways were affected by AP-hGle1-SD peptide, karyopherin/importin β 1 would accumulate in the nucleus of treated cells. However, the cellular distribution of this shuttling karyopherin was not affected (Fig. 5 B). Thus, protein import and export are not markedly inhibited under conditions where AP-hGle1-SD decreases poly(A)⁺ RNA export. These data suggest that hGle1-SD function is specifically essential for the mRNP export process.

cells. HeLa cells (<10 passages) were incubated for 4 h with 5 μ M of AP-hGle1-SD (SD) or the control AP-hGle1-scrSD peptide (scrSD) in normal growth medium. In situ hybridization using digoxigenin-labeled oligo(dT)₃₀ detected total poly(A)⁺ RNA. Nuclear DNA was visualized by DAPI staining. *, cell undergoing apoptosis. (C) Poly(A)⁺ RNA distribution in untreated HeLa cells shows distribution similar to AP-hGle1-scrSD-treated cells. (D) AP-hGle1-SD impairs nuclear export of DHFR mRNA. Cells treated with AP-peptides were processed for in situ hybridization using a digoxigenin-labeled DNA probe against DHFR mRNA. Bar, 10 μ m. (B–D) Respective hybridization signals represent equivalent exposure times. (E) Semiquantitative analysis of intracellular DHFR mRNA distribution in subcellular fractions of AP-peptide-treated cells. After treatment with peptides, total RNA from nuclear and cytoplasmic fractions was isolated and reverse transcribed with oligo(dT)₁₈, and serial dilutions were made. Subsaturation PCR-based amplifications with DHFR-specific primers were performed (see Materials and methods), and products were separated on agarose gels and stained with ethidium bromide.

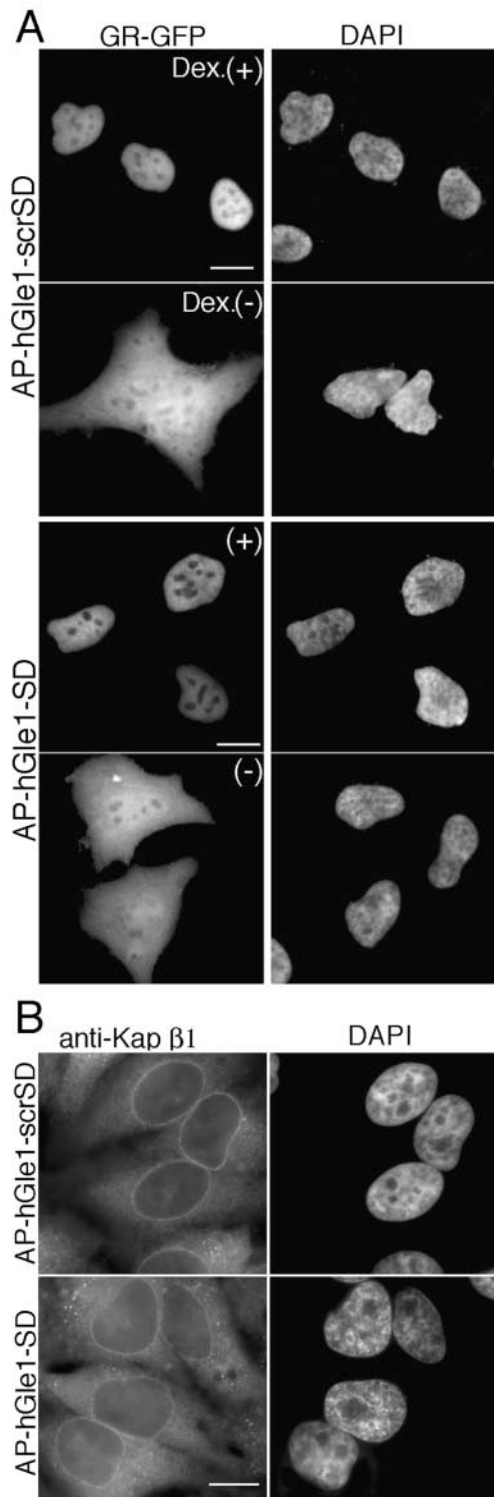


Figure 5. Nuclear protein import and export are not perturbed in HeLa cells treated with AP-peptides. (A) HeLa cells transfected with a GR-GFP expression vector were treated with 5 μ M of AP-peptide. Dexamethasone (Dex+) or an equivalent volume of ethanol (Dex-) was added in the last 30 min before fixation and DAPI staining. Dexamethasone addition specifically induces nuclear import of the cytoplasmic GR-GFP pool in the presence of either peptide. (B) Karyopherin/importin β 1 (Kap β 1) intracellular localization in AP-hGle1-SD- and control peptide (scrSD)-treated cells is not perturbed. Cells were treated with peptides as described above, fixed, and processed for IIF using anti-Kap β 1 antibodies. Bars, 10 μ m.

Inhibition of hGle1-SD activity by AP-hGle1-SD peptide perturbs endogenous hGle1 localization at NPCs

In parallel with the study of poly(A)⁺ RNA distribution, we analyzed the localization of endogenous hGle1 by IIF microscopy in cells treated with AP-peptides. Different permeabilization and fixation conditions were used to discriminate between cytoplasmically accessible hGle1 and nuclear hGle1 (Fig. 6). In untreated wild-type cells processed by fixation and complete Triton X-100 permeabilization (Fix-Triton), a pool of hGle1 is localized at the NPC/NE, and a fraction of the hGle1 is cytoplasmically accessible (Watkins et al., 1998; Fig. 1 A). In cells treated with the control AP-hGle1-scrSD peptide and processed by Fix-Triton, endogenous hGle1 was localized in a typical wild-type pattern, and cells exhibited normal poly(A)⁺ RNA distribution (Fig. 6 A, top). However, when cells were treated with the AP-hGle1-SD peptide, distinct hGle1 staining at the NPC/NE was less apparent in cells with impaired poly(A)⁺ RNA export (Fig. 6 A, bottom).

To further analyze the perturbations in hGle1 localization induced by the AP-hGle1-SD peptide, cells were treated with digitonin alone to selectively permeabilize the plasma membrane before fixation (Digitonin-Fix; Fig. 6 B). Under these conditions, very little anti-hGle1 staining was observed in cells affected by the AP-hGle1-SD peptide (Fig. 6 B, asterisks). This contrasted with the strong signal detected in AP-hGle1-scrSD cells and in an unaffected cell (Fig. 6 B, arrowheads). The lack of anti-lamin B staining in similarly treated Digitonin-Fix cells served as a control for NE integrity. To visualize the intranuclear distribution of hGle1, cells were permeabilized with digitonin, fixed, and then incubated with Triton X-100 (Digitonin-Fix-Triton). By this method, cells treated with the control AP-hGle1-scrSD showed uneven and low levels of nucleoplasmic hGle1 (Fig. 6 C). The AP-hGle1-SD cells seemed to show a slight increase in nucleoplasmic levels; however, the intranuclear staining pattern was not significantly changed. Western blotting confirmed that the hGle1 protein size and levels were not altered in the presence of the AP-hGle1-SD peptide (not depicted).

To more directly address the effect of AP-hGle1-SD on hGle1 nucleocytoplasmic shuttling, we analyzed the distribution of microinjected GST-hGle1A in cells treated with the AP-hGle1-SD peptide. Compared with the control AP-hGle1-scrSD cells, the presence of the AP-hGle1-SD peptide resulted in less efficient import of cytoplasmically injected GST-hGle1A into the nucleus ($90 \pm 2.5\%$; $n = 49$ microinjected HeLa cells) (Fig. 7 A, left). Similarly, the AP-hGle1-SD peptide impaired the export of nuclear-injected GST-hGle1A ($88.5 \pm 3\%$; $n = 43$ injected cells) (Fig. 7 A, right).

To confirm that the hGle1 mislocalization was not due to a general perturbation of the NPC or NE structure, the distribution of nucleoporins recognized by mAb414 (including p62; Davis and Blobel, 1986) was examined. Cells treated with AP-hGle1-SD peptide and accumulating nuclear poly(A)⁺ RNA showed mAb414 staining at the NPC/NE (Fig. 7 B, bottom) that was similar to the control (Fig. 7 B, top) and untreated cells (Fig. 1 A). Thus, the treatment with AP-hGle1-SD peptide and inhibition of poly(A)⁺ RNA had no adverse effect on global NPC structure/composition.

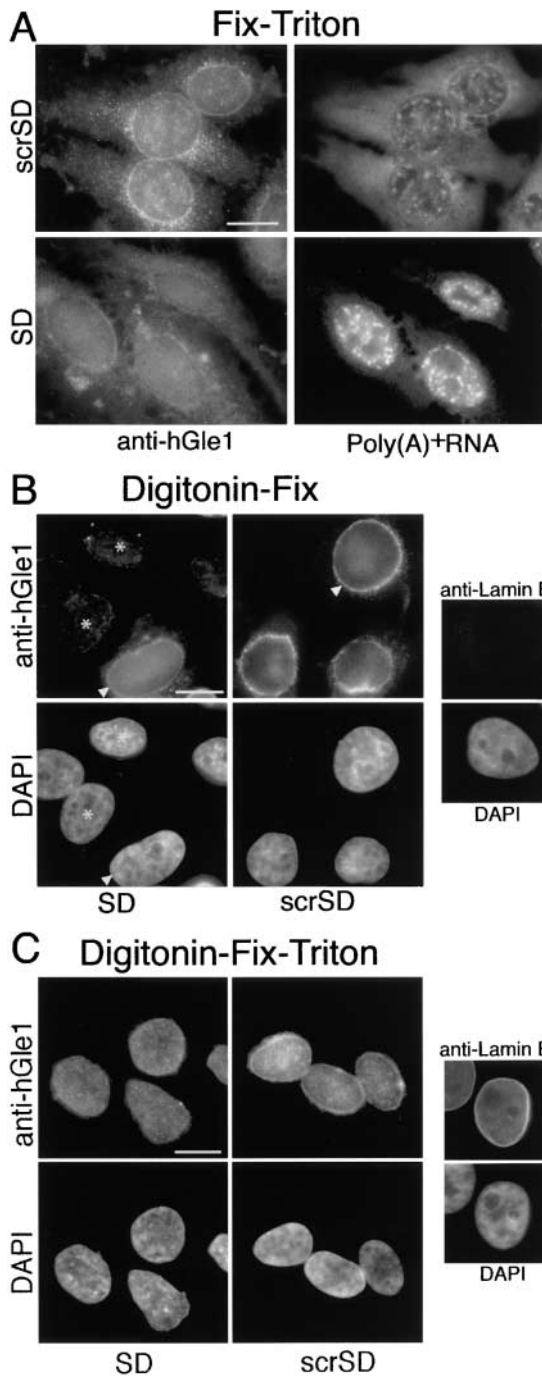


Figure 6. AP-hGle1-SD peptide results in mislocalization of endogenous hGle1 localization when poly(A)⁺ RNA export is inhibited. HeLa cells were incubated with AP-hGle1-SD (SD) or control peptide (scrSD), processed for IIF using affinity-purified anti-hGle1 antibodies (A–C), and in situ hybridized with oligo (dT)₃₀ (A) or stained with DAPI (B and C). Hybridized probe and bound hGle1 antibodies were simultaneously detected using rhodamine-labeled anti-Dig Fab antibodies and FITC-labeled anti-rabbit antibodies, respectively. Images showing in situ hybridization results reflect equivalent exposure times. (A) Fix-Triton permeabilization detects total hGle1 pool and shows changes in anti-hGle1 localization in AP-hGle1-SD-treated cells. (B) Digitonin-Fix permeabilization detects only the cytoplasmically accessible pool. AP-hGle1-SD results in decreased levels of cytoplasmically accessible hGle1 (asterisk) compared with unaffected cells (arrowheads, at passage >150; see Materials and methods) or cells treated with control peptide. The absence of lamin B staining using anti-lamin B antibodies (far right)

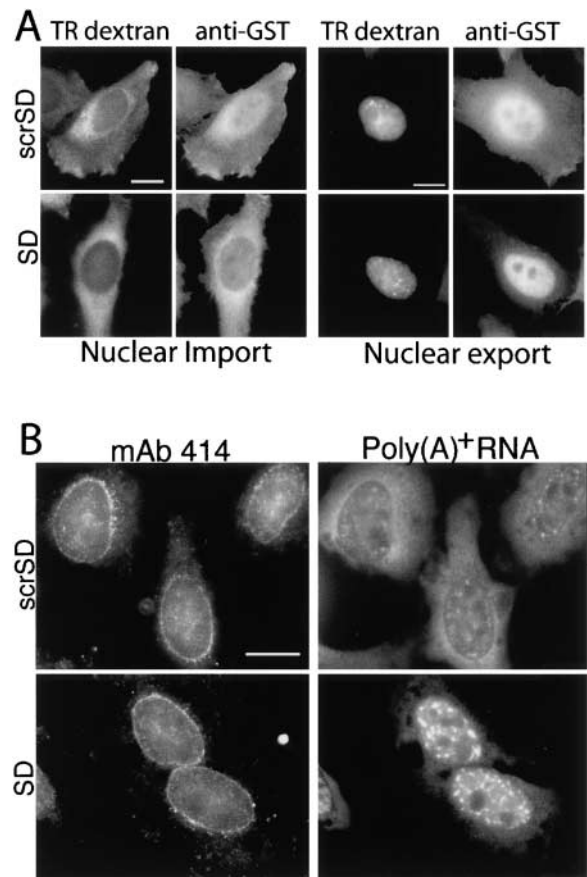


Figure 7. The AP-hGle1-SD peptide inhibits the shuttling of GST-hGle1. (A) HeLa cells were pretreated with AP-peptides (3 h) and microinjected with GST-hGle1A in the cytoplasm (left) or nucleus (right) of the cells. After recovery (2 h), the intracellular distribution of GST-hGle1A was analyzed by IIF using anti-GST antibodies. Compared with cells treated with the scrSD control peptide, less GST-hGle1 is detected outside the microinjection site in the majority of cells analyzed. (B) NPC/NE association of nucleoporins recognized by mAb414 is not perturbed by AP-hGle1-SD treatment. HeLa cells incubated with AP-hGle1-SD (SD) or control peptide (scrSD) were processed for in situ hybridization with oligo(dT)₃₀ and coincident IIF with mAb414. The mAb414 staining is similar in AP-hGle1-SD and scrSD cells despite the poly(A)⁺ RNA export defect in the AP-hGle1-SD cells. Bars, 10 μ m.

Overall, we concluded that competing for the hGle1-SD-interacting factor(s) resulted in coincident nuclear accumulation of mRNA, decreased the endogenous hGle1 fraction associated with the cytoplasmic NPC face, and reduced the nucleocytoplasmic shuttling of GST-hGle1 protein.

Discussion

In this report, we have addressed the question of whether hGle1 is a stable component of the NPC or if instead it behaves as a shuttling transport factor. Our results support the conclusion that although the major hGle1 isoform (hGle1B)

confirms NE integrity under these conditions. (C) Detection of nuclear hGle1 was accomplished by combined Digitonin-Fix-Triton permeabilization. Lamin B staining confirms access of antibodies to intranuclear proteins. Bars, 10 μ m.

in HeLa cells has a steady-state localization at the NPC, its role in transport hinges on nucleocytoplasmic dynamics. These conclusions are based on several pieces of evidence. First, we identified a 39–amino acid domain in hGle1 that has intrinsic nucleocytoplasmic shuttling activity. Second, photobleaching (FLIP) of EGFP–hGle1B in live HeLa cells shows that hGle1 shuttles. Third, we demonstrated that an hGle1 39–amino acid domain is required for hGle1 nucleocytoplasmic shuttling. Fourth, by disrupting the function of the SD using a cell-permeable peptide strategy, we showed that endogenous hGle1 association with NPCs is transient. Finally, we observed that interfering with hGle1 shuttling and NPC localization is coincident with a significant decrease in poly(A)⁺ RNA export. Overall, this pinpoints the first functional motif in hGle1, demonstrates that hGle1 is a shuttling mRNA export factor, and closely links hGle1 function to the dynamic mRNA export pathway.

We also report that two hGle1 isoforms are expressed in HeLa cells, hGle1A and hGle1B. Both are identical except for their very COOH-terminal regions. Two lines of evidence suggest that the main hGle1 protein in HeLa cells is hGle1B. First, in contrast to EGFP–hGle1A, EGFP–hGle1B intracellular localization is highly similar to endogenous hGle1. Second, semiquantitative RT-PCR experiments show that hGle1B is the more abundant mRNA isoform in HeLa cells. We speculate that the unique 43–amino acid region at hGle1B's COOH terminus is required for NPC/NE association. Interestingly, this region of hGle1B shares similarities with scGle1 (Fig. 1 D), which is also predominately localized at NPCs (Murphy and Wentz, 1996; Rout et al., 2000). Our future work will address whether each hGle1 isoform plays distinct *in vivo* roles.

Based on recent proteomic analysis of the mammalian NPC (Cronshaw et al., 2002) and these studies, we conclude that hGle1 is not a stable component of the NPC and that hGle1 is not required for overall maintenance of NPC structure and function. In cells treated with AP–hGle1-SD peptide, no perturbations in the localization of nucleoporins recognized by mAb414, in GFP–GR protein import, or in karyopherin/importin β 1 nuclear export were detected. In light of recent elegant reports on hNup153 (Nakielnny et al., 1999; Daigle et al., 2001), hNup98 (Griffis et al., 2002), Npap60/Nup50 (Lindsay et al., 2002), and scNup2 (Dilworth et al., 2001), it is becoming evident that a subpopulation of factors with steady-state NPC localization have unexpected dynamic properties. We propose that hGle1B transiently associates with NPCs during its function as a shuttling mRNA export mediator.

Results from our photobleaching experiments and cell-permeable peptide studies strongly support the conclusion that endogenous hGle1 has a bona fide SD and dynamically enters and exits the nucleus. In the presence of competing AP–hGle1-SD peptide, the association of endogenous hGle1 with the NPC/NE was substantially reduced, and the shuttling of recombinant hGle1 was severely impaired. Coincidentally, AP–hGle1-SD peptide induced nuclear poly(A)⁺ RNA accumulation, suggesting that hGle1 is involved in a molecular cascade that leads to the export of mRNP complexes out of the nucleus. It is possible that AP–hGle1-SD peptide may compete for hGle1's nucleoporin

binding site(s) or for an adaptor protein that bridges the interaction of hGle1 with nucleoporins at NPCs. The latter would be similar to the interaction networks of karyopherin/importin β 1 for α (Gorlich et al., 1995a,b) or Crm1 for Rev (Fornerod et al., 1997). Alternatively, the SD peptide may affect more than Gle1. If the unidentified SD-binding factor has additional interacting partners, their interactions could be equally perturbed. Thus, isolating an hGle1-SD-interacting protein is of great interest. As a dynamic NPC-associated protein, we speculate that hGle1 either actively participates in the export process or facilitates the nuclear import of an unidentified factor that would mediate mRNA export.

Interestingly, the amino acid sequence of the hGle1 SD is not similar to any of the previously characterized nucleocytoplasmic SDs (Fig. 8). A direct alignment of the hGle1 SD with the M9 of hnRNP A1 suggests weak similarity, which could result in a potential interaction with the M9-binding karyopherin β 2A/transportin-1 (Siomi et al., 1997). However, the key consensus residues implicated in transportin-1 binding are not present in hGle1 (Pollard et al., 1996). We tested for an hGle1-SD and transportin-1 interaction and found that bacterially expressed GST–transportin-1 does not directly bind immobilized hGle1-SD peptide (unpublished data). This suggests that transportin-1 is not directly involved in hGle1 shuttling. Further deletions within the hGle1 SD and selective point mutations based on interspecies homology analysis have not yet been successful in separating the nuclear import and export activities (unpublished data).

Although structurally similar to hGle1, the yeast homologue scGle1 does not harbor a similar 39–amino acid peptide sequence. Rather, the same region of scGle1 contains a putative LR-NES-like motif (Murphy and Wentz, 1996; Watkins et al., 1998; Fig. 1 D). This motif in scGle1 is required for scGle1 function, suggesting that it may use the Rev–Crm1 export pathway to exit the nucleus (Murphy and Wentz, 1996). Although the scGle1 putative LR-NES by itself mediates export in heterologous transport assays, an in-

Nucleocytoplasmic shuttling sequences

hGle1 SD	IFDKIHSLLSGKPVQSGGRSVSVTLNPOGLDFVQYKLAE
hnRNP A1	SQSSNF ^{***} GMPKGGAF–GGRSSG–PYGGGGQYFAKPRNQGGY
hnRNP K	GFSADETWDSAIDTWSPEWQMAV
HuR	RRFGGPVHHQAQRFRFSPMGVDHMSGLSGVNVV

Alignment of hGle1 SD with hnRNP A1 M9 transport sequence

hnRNP A1	SQSSNF ^{***} GMPKGGAF–GGRSSG–PYGGGGQYFAKPRNQGGY
hGle1 SD	IFDKIHSLLSGKPVQSGGRSVSVTLNPOGLDFVQYKLAE--

Figure 8. **Comparison of the hGle1 SD with nucleocytoplasmic shuttling sequences.** The amino acid sequence of the hGle1 SD does not share significant similarities with other nucleocytoplasmic transport signals (Michael et al., 1995, 1997; Fan and Steitz, 1998). There are limited similarities with the hnRNP A1 M9 transport signal (boxed), but the hGle1 SD lacks the critical GPM triplet (asterisks) required for efficient transport activity of M9 (Bogerd et al., 1999).

teraction between scGle1 and scCrm1 has not been established (Watkins et al., 1998; Stutz and Rosbash, 1998). It is also unclear whether endogenous scGle1 shuttles. Taken together, the Gle1 mRNA export mechanisms in yeast and human cells may have subtle differences. Further studies in yeast and human cells may reveal how two highly homologous proteins can function by potentially different mechanisms and ultimately lead to a similar outcome, the export of mature mRNPs from the nucleus.

Our work in this report strongly suggests that hGle1 dynamics are key to the poly(A)⁺ RNA export mechanism. It will be of great interest to identify which factor(s) interacts with hGle1A and B and, in particular, the hGle1 SD. We predict that this information will be valuable in ascertaining the molecular cascade leading to hGle1-mediated mRNP export.

Materials and methods

Tissue culture cell experiments

HeLa cells were cultured in complete medium (DME; Invitrogen) supplemented with 10% FBS (Invitrogen Corp.), 10 mM sodium pyruvate, and 100 U/ml Pen/Strep at 37°C in 5% CO₂. Cells were microinjected with the Eppendorf InjectMAN micromanipulator using ~2 mg/ml of recombinant protein mixed with 1 mg/ml of TR-labeled 70-kD dextran (lysine fixable; Molecular Probes) diluted in 10 mM phosphate buffer, pH 7.6, 75 mM KCl. Transfections were done using SuperfectTM (QIAGEN) following the manufacturer's recommendations. 12–14 h later, transfected cells were fixed and processed for IIF or direct EGFP fluorescence. Live cell imaging was conducted as described by Griffis et al. (2002), except that imaging media contained 10% FCS.

Cell-permeable peptide assay and nuclear import assay

Peptides were synthesized by FAST-moc chemistry and purified by reverse-phase HPLC, and the sequence was confirmed by mass spectrometry. Each peptide harbors the antennapedia sequence at its NH₂ terminus (RQIKIWFQNRRMKWKK) (Fig. 4 A). For assays, peptides were first diluted in complete DME and added cells (CCL-2 cells; lot no. 2462426; passage <10 after receipt from American Type Culture Collection) and grown to 70–80% confluency. After 4 h incubation, cells were processed for *in situ* hybridization and/or IIF. We noted a decrease in the susceptibility of HeLa cells after passage 150 from reception; 10% of the population appeared to become resistant to the effects of AP-hGle1-SD peptide, likely due to diminished peptide uptake. This trend increases with passage number, ultimately resulting in AP-hGle1-SD-resistant HeLa cell populations. Nuclear import activity in AP-peptide-treated cells was determined after transfection with pK7.GR.GFP expression vector (Carey et al., 1996) and using dexamethasone treatment (10 μg/ml; Sigma-Aldrich).

IIF

HeLa cells were processed by three methods, Fix-Triton, Digitonin-Fix, and Digitonin-Fix-Triton, as described by Watkins et al. (1998). The primary antibodies used were affinity-purified rabbit anti-GST (1:200), affinity-purified rabbit anti-hGle1 (1:200; Watkins et al., 1998), rabbit anti-lamin B (1:50; anti-peptide NC-6; gift of N. Chaudhary and G. Blobel, The Rockefeller University, New York, NY), mAb414 (1:10; Davis and Blobel, 1986), rabbit anti-FLAG (1:200), and mouse anti-importin β (anti-p97; 1:1,000; Affinity BioReagents, Inc.). FITC-conjugated donkey anti-rabbit (1:200) or FITC-conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Cells were examined using an Olympus BX50 microscope. Digital images were recorded with a Photometric Coolsnap HQ camera (Roper Scientific) using MetaVue 6.0 software and digitally manipulated in Adobe Photoshop 7.0[®].

In situ hybridization

Cells were processed as described by Watkins et al. (1998). 3'-digoxigenin-labeled oligo(dT)₃₀ probe was synthesized (Wente and Blobel, 1993) and used at 7 μg/ml. The 3'-digoxigenin-labeled DHFR probe complementary to nucleotides 530–569 of DHFR mRNA (Gallouzi and Steitz, 2001) was synthesized by Integrated DNA Technologies Inc. and used at 20 μg/ml. After hybridization, cells were blocked for 1 h with 6% nonim-

mune sheep serum (Jackson ImmunoResearch Laboratories), and hybridized probes were detected with 1:200 sheep anti-digoxigenin Fab-rhodamine (Roche Applied Science). For *in situ*/IIF double experiments, polyclonal anti-hGle1 antibodies (1:200) or mAb414 (1:10) were used in combination with 1:200 FITC-conjugated sheep anti-rabbit or sheep anti-mouse (Sigma-Aldrich), respectively.

Cytoplasm/nuclear RNA isolation and RT-PCR

HeLa cells (5 × 10⁶) treated or untreated with AP-peptides were scraped off culture plates and suspended in ice cold RNase-free PBS. Nuclear and cytoplasmic RNA fractions were isolated after the NP-40 lysis procedure (Greenberg and Ziff, 1984), except that the lysis buffer was supplemented with 1 mM DTT and 1 U/μL of RNasinTM (Promega). Cell lysis was monitored by bright field microscopy, and the integrity of the isolated nuclei was confirmed by nucleoplasmic exclusion of the 70-kD TR-labeled dextran (not depicted). Total RNA from each fraction was isolated using TrizolTM reagent (Invitrogen). Genomic DNA contamination of the purified cytoplasmic and nuclear RNA was estimated by PCR before reverse transcription using the same conditions as below (not depicted). Each RNA fraction was subsequently reverse transcribed using Superscript IITM and random hexamers (Invitrogen) as per the manufacturer's recommendations. Serial dilutions of the resulting cDNAs were made, and 10% of each was used in PCR studies. For DHFR gene amplification (28 cycles), specific primers (forward, 5'-CAGAACATGGGCATCGGCAAGAAGC-3'; reverse, 5'-AAACAGAACTGCCACCACTATCCA-3') were used. 50% of each reaction was separated by electrophoresis through a 1.7% agarose gel stained with ethidium bromide. Amplification of hGle1A and hGle1B was conducted using the above conditions and a common forward primer, F (5'-ATACCAGTTCAGTCTTGGAGATGC-3'), with the respective A-specific reverse primer (5'-TCAACGAGAGGGACTGGAC-3') or B-specific reverse primer (5'-AGGAGCGCCAGAAGGAGG-3').

Plasmid constructs

Cloning of hGle1 cDNA isoforms. IMAGE clone no. 22734 was used to generate full-length hGle1A NH₂-terminally tagged with EGFP (pSW1409; CLONTECH Laboratories, Inc.) or FLAG (pSW1102). Based on partial EST sequence information, hGle1B-specific primers were designed and used to generate a 2.3-kbp fragment by using the HerculaseTM polymerase mix (Stratagene) and PCR. hGle1B cDNA was subsequently cloned into pEGFP-C1 (pSW1482; CLONTECH Laboratories, Inc.).

FLAG-hGle1_Δ-NLS constructs. Oligonucleotides encoding the SV40 large T antigen NLS were annealed and cloned at the 3' end of FLAG-hGle1A, yielding FLAG-hGle1A-NLS (pSW1116). Digestion of pSW1116 with BglII generated FLAG-hGle1_{Δ444-606}-NLS (pSW1127). Deletions of the hGle1 444–606 region were made by PCR and cloned back into pSW1127, generating pSW1190 (FLAG-hGle1_{Δ444-541}-NLS), pSW1206 (FLAG-hGle1_{Δ444-511}-NLS), pSW1416 (FLAG-hGle1_{Δ511-606}-NLS), and pSW1415 (FLAG-hGle1_{Δ484-606}-NLS).

GST expression constructs. Full-length hGle1A and B were cloned in frame with GST in pGex-3X series (AP Biotech) (pSW728 and pSW1487, respectively). These were used to generate pGex-hGle1A_{Δ444-483} (pSW1407) and pGex-hGle1B_{Δ444-483} (pSW1496). An hGle1 DNA fragment coding for region 444–483 was amplified by PCR from IMAGE clone no. 22734 and cloned into pGex 5X-2 (AP Biotech), yielding pGex-hGle1₄₄₄₋₄₈₃ (SD) (pSW1412).

Purification of recombinant proteins

GST fusion proteins were expressed in BL21 cells and purified according to the manufacturer's instructions (AP Biotech), except cells were lysed by French press (12,000 PSI). After affinity chromatography, fusion proteins were eluted with 15 mM reduced glutathione (Sigma-Aldrich) in 150 mM NaCl, 50 mM Tris, pH 8.5. Eluates were dialyzed against microinjection buffer (10 mM phosphate buffer, pH 7.6, 75 mM KCl) and stored at –70°C.

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