Journal of Cellular Biochemistry 95:598–610 (2005)

Interaction of the Nuclear Localizing Cytolytic Granule Serine Protease Granzyme B With Importin α or β: Modulation by the Serpin Inhibitor PI-9

Elizabeth J. Blink,[1] Zhou Jiansheng,[1] Wei Hu,[1] Sophina. T. Calanni,[1] Joseph A. Trapani,[3] Phillip I. Bird,[2] and David A. Jans[1,2,4]*

[1]Nuclear Signalling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra City, Australia
[2]Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia
[3]Cancer Immunology Unit, Peter MacCallum Cancer Institute, Melbourne, Australia
[4]ARC Center of Excellence for Biotechnology and Development

Abstract Conditional on perforin-dependent delivery to the nucleus of target cells, the cytolytic granule serine protease granzyme B (GrB) plays a central role in eliciting the nuclear events of apoptosis, as shown by the fact that reducing GrB nuclear entry prevents nuclear apoptosis. Apart from a requirement for cytosolic factors and lack of dependence on the guanine-nucleotide-binding protein Ran, little is known regarding the nuclear import pathway of GrB. In this study we use quantitative yeast two-hybrid and direct binding assays to show that GrB can be recognized independently by either of the nuclear import receptor family members importin (IMP) α and β1, but that these proteins either alone or in combination cannot replace exogenous cytosol to reconstitute GrB nuclear import in vitro. Whereas antibodies to IMPα inhibit transport, indicating that IMPα is required for GrB nuclear import, those to IMPβ enhance transport, implying that IMPβ inhibits GrB nuclear import; consistent with this, the addition of recombinant IMPβ but not IMPα reduces maximal nuclear accumulation in the presence of cytosol. Intriguingly, complexation of GrB with its specific serpin inhibitor PI-9 was found to prevent recognition by IMPβ but not by IMPα, and eliminate the apparent requirement for IMPα for nuclear import. We conclude that GrB nuclear import exhibits complex regulation by IMPs; that heterodimerization with PI-9 can modulate the interaction has implications for protection against apoptosis. J. Cell. Biochem. 95: 598–610, 2005.

Key words: cytolytic granule-mediated apoptosis; granzymes; perforin; serpin PI-9; nuclear protein import

The flux of proteins and other molecules between nucleus and cytoplasm through the nuclear envelope-localized nuclear pore complexes (NPCs) is tightly controlled, with a nuclear localization signal (NLS) essential for the nuclear entry of most proteins >50 kDa [Jans et al., 2000a]. Conventionally, NLS-containing proteins such as the simian virus SV40 large tumor antigen (T-ag) [Hübner et al., 1997], the tumor suppressing retinoblastoma protein (Rb) [Efthymiadis et al., 1997], and inducible transcription factors (TFs) [Sekimoto et al., 1997; Briggs et al., 1998] are recognized by the cytosolic heterodimer of the importin α (IMPα) and β (IMPβ) subunits [Görlich et al., 1995; Imamoto et al., 1995; Rexach and Blobel, 1995]. Other proteins such as constitutively nuclear TFs of the AP-1 [Forwood et al., 2001b] and SRY/SOX [Preiss et al., 2001; Forwood et al., 2001a; Harley et al., 2003] families are recognized directly by IMPβ (IMPβ1), whilst IMPα alone has been reported to be able to transport molecules through the NPC independent of IMPβ1 in certain experimental systems [Hübner et al., 1999; Miyamoto et al., 2002]. A key factor synergising with IMPs to mediate

Grant sponsor: Australian Research Council Discovery Grant; Grant number: DP0208857; Grant sponsor: National Health and Medical Research Council (Australia) fellowship grant; Grant numbers: 143790, 334013; Grant sponsor: National Health and Medical Research Council (Australia) project grant; Grant number: 143710.

*Correspondence to: David A. Jans, Nuclear Signalling Laboratory, Department of Biochemistry and Molecular Biology, PO Box 13D, Monash University 3800, Australia. E-mail: David.Jans@med.monash.edu.au

Received 21 October 2004; Accepted 6 December 2004
DOI 10.1002/jcb.20415

nuclear import is the monomeric guanine-nucleotide-binding protein Ran, which in activated GTP-bound state binds IMPβ to effect release within the nucleus [Moore and Blobel, 1993; Izaurralde et al., 1997]. Ran-independent, IMPβ1-dependent nuclear import of the cyclin B1–Cdc2 complex has been reported [Takizawa et al., 1999], however, as well as import pathways independent of both IMPs and Ran for β-catenin [Yokoya et al., 1999], protein kinase C [Schmalz et al., 1998], and the human immunodeficiency virus HIV-1 Tat transactivator [Efthymiadis et al., 1998], and Vpr regulatory [Jenkins et al., 1998; Jans et al., 2000b] proteins.

Recognition by IMPs does not always lead to nuclear import, as has been shown in a number of instances [Jaekel and Görlich, 1998; Jaekel et al., 1999; Lam et al., 1999; Shakulov et al., 2000; Forwood and Jans, 2002], implying that IMPs have roles in the cell additional to those in facilitating nuclear transport; in this case, the formation of "non-productive" transport complexes can inhibit nuclear import. Additional to roles in positively or negatively regulating nuclear transport, a chaperone role for IMPs in preventing the aggregation in the cytoplasm of proteins with highly charged domains such as NLSs has recently been proposed [Jaekel et al., 2002].

The cytolytic granule serine protease granzyme B (GrB) plays an important role in the nucleus during cytolytic granule-mediated apoptosis by cleaving a number of nuclear proteins. This is central to bringing about the nuclear changes characteristic of apoptosis [Andrade et al., 1998; Trapani et al., 1998a,b; Blink et al., 1999; Jans et al., 1999]; blocking GrB nuclear import through different approaches prevents nuclear apoptosis [Trapani et al., 1998a; Jans et al., 1999]. In intact target cells, GrB nuclear delivery is dependent on the membrane-acting granule component perforin [Jans et al., 1996, 1998, 1999; Trapani et al., 1998a; Browne et al., 1999], whilst nuclear/nucleolar accumulation of cytoplasmic GrB in the absence of perforin has been observed in microinjected cells [Blink et al., 1999], and reconstituted in vitro [Jans et al., 1996, 1998]. The specificity of granzyme nuclear import in the apoptotic process is implied by the fact that the apoptotic caspase CPP32 does not accumulate in the nucleus either in vitro [Jans et al., 1998], or in vivo during apoptosis [Krajewski et al., 1997; Liu et al., 1997]; the non-granzyme serine protease chymotrypsin also does not accumulate in the nucleus in vitro [Trapani et al., 1996]. The presence of sequences within GrB (e.g., **RKAKRTR**^{102} and **KSKAKRTR**^{98} from human and mouse, respectively) which resemble the T-ag NLS (P**KKKRK**V^{132}) and the requirement for cytosol to mediate GrB nuclear import in in vitro nuclear transport assays has led to the hypothesis that soluble factors such as IMPs may recognize GrB and mediate its import into the nucleus [Jans et al., 1996]. GrB nuclear import, however, has been shown to be independent of ATP hydrolysis, and not inhibited by non-hydrolysable GTP analogs, implying lack of dependence on Ran, and suggesting that GrB nuclear import occurs through a novel pathway [Jans et al., 1996, 1998; Trapani et al., 1996].

The GrB specific inhibitor PI-9 is a novel intracellular serpin able to bind irreversibly to GrB and inhibit its proteolytic activity [Sun et al., 1996; Bird et al., 1998]. It is expressed in cytolytic granule-containing lymphocytes and natural killer cells presumably as a mechanism to protect them from GrB; when expressed in transfected breast cancer cells, PI-9 can protect against apoptosis induced by GrB and perforin [Bird et al., 1998]. Recent studies indicate that PI-9 itself can localize in the nucleus, apparently through a pathway ostensibly similar to that of GrB in that nuclear import requires cytosolic factors, but not ATP hydrolysis [Bird et al., 2001]. Importantly, PI-9 also has an active nuclear export mechanism mediated by the exportin Crm1p [Bird et al., 2001; Rodriguez et al., 2003]. Thus, it is not inconceivable that PI-9's protective role against apoptosis may involve export of GrB from the nucleus, where it is required for nuclear apoptosis [Trapani et al., 1998a; Jans et al., 1999].

The present study investigates the basis of the cytosolic dependence of GrB nuclear import by testing for recognition of GrB by the conventional NLS-binding IMPα and IMPβ subunits, as well as the influence of PI-9 on this interaction. We show that GrB can be recognized independently by either IMP, but although IMPα appears to bind with higher efficiency and be required for nuclear import, IMPβ inhibits transport. That complexation with PI-9 can modulate the interactions is consistent with the possibility that PI-9 may have a role in the regulation of GrB subcellular localization [see Bird et al., 2001].

MATERIALS AND METHODS

Yeast Two-Hybrid Analysis

Yeast transformation/cotransformation of strain PJ69-4A was carried out as previously [Browne et al., 2000; Johansson et al., 2001; Henderson et al., 2002]. A plasmid encoding proteolytically inactive GrB (Ser^{183} to Ala substitution) in plasmid pAS2.1 (GAL4 DNA-binding domain—DBD-vector) [Browne et al., 2000] was used in conjunction with plasmids encoding human IMPα isoforms in the pGAD424 (GAL4 activation domain—AD-vector), obtained from Takemi Enomoto—[Seki et al., 1997]. The IMPβ1 amino acids 262–847 (GAL4 DBD vector) construct has been described previously [Johansson et al., 2001]; it encodes an IMPβ form unable to bind Ran, and hence unable to be dissociated from its import substrates [Herold et al., 1998]. For some experiments, the NLS-binding protein BRAP2 inserted into the pAS2.1 vector [Li et al., 1998], provided by Shang Li, was used. Quantitative analysis of α-galactosidase (Mel1) activity, under control of a GAL4-responsive promoter, as an indication of interaction between GrB and IMPs was performed by analyzing five independently derived clones, after overnight growth in minimal medium, to an OD at 600 nm of 0.5–1.0 at 30°C in YPD (yeast extract/peptone/dextrose) medium. 2.5 ml of culture were then routinely taken, centrifuged, resuspended in 1 ml of 0.1M sodium phosphate buffer (pH 6.0), the OD at 600 nm reassessed to ensure all cultures were identical in terms of cell numbers, and then 0.25 ml aliquots freeze-thawed three times. Seventy-five microliter of cell extract was analyzed in triplicate in a final volume of 200 μl containing 3.125 mM of the non-fluorescent substrate 4-methylumbelliferyl α-D-galactopyranoside (Glycosynth, Cheshire, UK) in microtiterplates. Production of the fluorescent product 4-methylumbelliferone (4-MB; Sigma-Aldrich Co., St. Louis, MI) was followed over time at 30°C using a CytoFluor II Fluorescent Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA) with excitation at 360 nm and emission at 460 nm. After 60–90 min, 100 μl of 0.25M Na-borate (pH 9.8) was added to stop the reaction and amplify the fluorescent signal due to 4-MB. 4-MB was estimated using a standard curve (0–8 nmol) of 4-MB (Sigma Chem. Co.) pipetted onto each microtiter plate, with correction for emission values at 0 min, and results calculated relative to the clone carrying the GrB-GAL4 DBD plasmid alone.

Protein Expression

Human GrB was prepared by immunoaffinity chromatography, and labeled with fluorescein isothiocyanate, as previously [Trapani et al., 1996, 1998b]. Recombinant PI-9 and the mutant derivative ITR, which contains a threonine to arginine substitution at position 327 and is thereby unable to bind GrB, were prepared as described [Bird et al., 1998]. The mouse IMPα and IMPβ subunits [Imamoto et al., 1995] were prepared as bacterially expressed glutathione-*S*-transferase (GST) fusion proteins as previously [Efthymiadis et al., 1997; Hübner et al., 1997].

Cell Culture

Cells of the HTC rat hepatoma line were cultured at 37°C in a humidified incubator (Forma Scientific) with a 5% CO_2 atmosphere in DMEM medium supplemented with 10% fetal calf serum [Efthymiadis et al., 1997; Briggs et al., 1998].

Importin Binding Studies

The ability of mouse IMPs to bind to GrB was assessed by dot and ligand Western blot approaches [Hübner et al., 1997]. As previously, bound IMPs were visualized using a goat anti-GST-specific antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK), alkaline phosphatase-conjugated rabbit anti-goat IgG secondary antibody (Sigma-Aldrich Co.), and nitro blue tetrazolium/bromo chloroindolyl phosphate (Promega Co., Madison, WI). NLS-containing controls included T-ag- and Rb-β-galactosidase fusion proteins (T-ag-NLS-β-Gal and Rb-Bip-NLS-β-Gal, respectively [Efthymiadis et al., 1997; Hübner et al., 1997]), and the human TF fos [Forwood et al., 2001b]. In the case of Western blotting experiments, parallel electrophoretic gels were silver stained according to Sambrook et al. [1989].

GrB/PI-9 Complexation

GrB and PI-9 (or ITR as a negative control) were complexed by mixing at a molar ratio of 2:1 and incubating for 30 min at 37°C [Bird et al., 1998]; the complex is stable during SDS gel electrophoresis.

Nuclear Transport

Analysis of nuclear import kinetics in vitro at the single cell level using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy (CLSM—Bio-RAD Labs MRC-600, Hercules, CA) was performed as previously [Jans et al., 1996; Trapani et al., 1996; Efthymiadis et al., 1997]. NLS-dependent nuclear protein import can be reconstituted in this system through the addition of cytosolic extract (untreated reticulocyte lysate), an ATP regenerating system (creatine kinase/creatine-phosphate/ATP), and transport substrate [Jans et al., 1996]. In experiments where the ability of 1 μM purified recombinant IMPs [Hu and Jans, 1999] to replace cytosol was tested, the latter was substituted with bovine serum albumin (BSA). Where anti-IMPα and IMPβ antibodies (Santa Cruz Biotechnologies, Inc.) were used to neutralize IMP function, preincubation with cytosol was made for 15 min at RT [Hübner et al., 1999; Forwood et al., 2001a,b]. These antibodies have been used previously in the system to block IMPα- or IMPβ-dependent nuclear protein import [Hübner et al., 1999; Forwood et al., 2001a,b], Western blots of reticulocyte lysate using the anti-IMPβ antibody indicating a single 97 kDa band, and blots using the anti-IMPα antibody indicating 58/60 kDa bands [Wei Hu, 2002, Ph.D. thesis, Australian National University]. Image analysis of CLSM files, using the Macintosh NIH Image 1.60 public domain software, and curve fitting was performed as previously [Jans et al., 1996; Hu and Jans, 1999].

RESULTS

Grb Interacts With IMPα or IMPβ as Shown Using the Yeast Two-Hybrid System

We had previously established that despite being independent of Ran and ATP hydrolysis, GrB nuclear import in vitro requires cytosolic factors [Jans et al., 1996, 1998]. Since GrB contains conventional NLS-like sequences (see above), we decided to assess whether the basis of the cytosolic dependence of GrB nuclear import may relate to GrBs ability to be recognized by IMPs. We initially assessed GrB interaction with IMPs in vivo using the GAL4 yeast two-hybrid (Y2H) system, which has previously been used to examine IMPα interaction with conventional NLS-containing proteins [Seki et al., 1997; Herold et al., 1998; Li et al., 1998; Browne et al., 2000; Johansson et al., 2001; Henderson et al., 2002]. In particular, we took a quantitative approach by measuring expression of the GAL4-controlled-α-galactosidase (Mel1) enzyme to compare interaction strengths. We found that GrB (GAL4 DBD fusion protein) was able to interact with either IMPα or IMPβ (GAL4 AD fusion proteins) as indicated in time course (Fig. 1A), and end point (Fig. 1B) assays where the fluorescent signal indicative of substrate production was amplified using a stop reaction (see "Materials and Methods"). The former indicated that all three IMPα isoforms assayed (IMPα1 [Rch1], -3 [Qip1], and -5 [Srp1]) interacted strongly with GrB, α-galactosidase activity being significantly higher than that shown by yeast carrying the individual GrB or IMPα plasmids alone (Fig. 1A). Specificity of the interaction was implied by the fact that the NLS-binding protein BRAP2, initially isolated as being able to recognize the NLSs of BRCA1 [Li et al., 1998], did not interact with GrB. These results were supported by steady state assays, which indicated c. 3-fold higher induction levels of α-galactosidase activity in GrB-IMPα-expressing yeast clones ($n = 5$) relative to those expressing GrB (GAL4 DBD fusion protein) alone (Fig. 1Bi), and comparable levels in GrB-IMPβ1-expressing yeast clones (Fig. 1Bii). Thus, GrB can be recognized independently by different IMPα isoforms as well as IMPβ1.

GrB is Recognized by the IMPα/β Heterodimer

To confirm the Y2H results, direct binding of IMP to GrB was tested, initially using a dot blot approach [Hübner et al., 1997] with β-galactosidase (β-Gal) fusion proteins as controls. The latter included T-ag-NLS-β-Gal, which contains the conventional T-ag NLS, and Rb-Bip-NLS-β-Gal, containing the bipartite NLS of Rb, both of which are recognized by IMPα/β [Efthymiadis et al., 1997]; β-Gal was employed as a negative control. GrB showed strong recognition by the IMPα/β heterodimer, comparable to that of T-ag-NLS-β-Gal (Fig. 2A), but in stark contrast to the lack of binding observed for β-Gal.

Similar results were obtained using the Western ligand blot technique [Hübner et al., 1997] where granzymes were electrophoresed (silver staining results shown in Fig. 2B) prior to transfer to nitrocellulose, and subsequent blotting with IMPα/β (Fig. 2C). The results

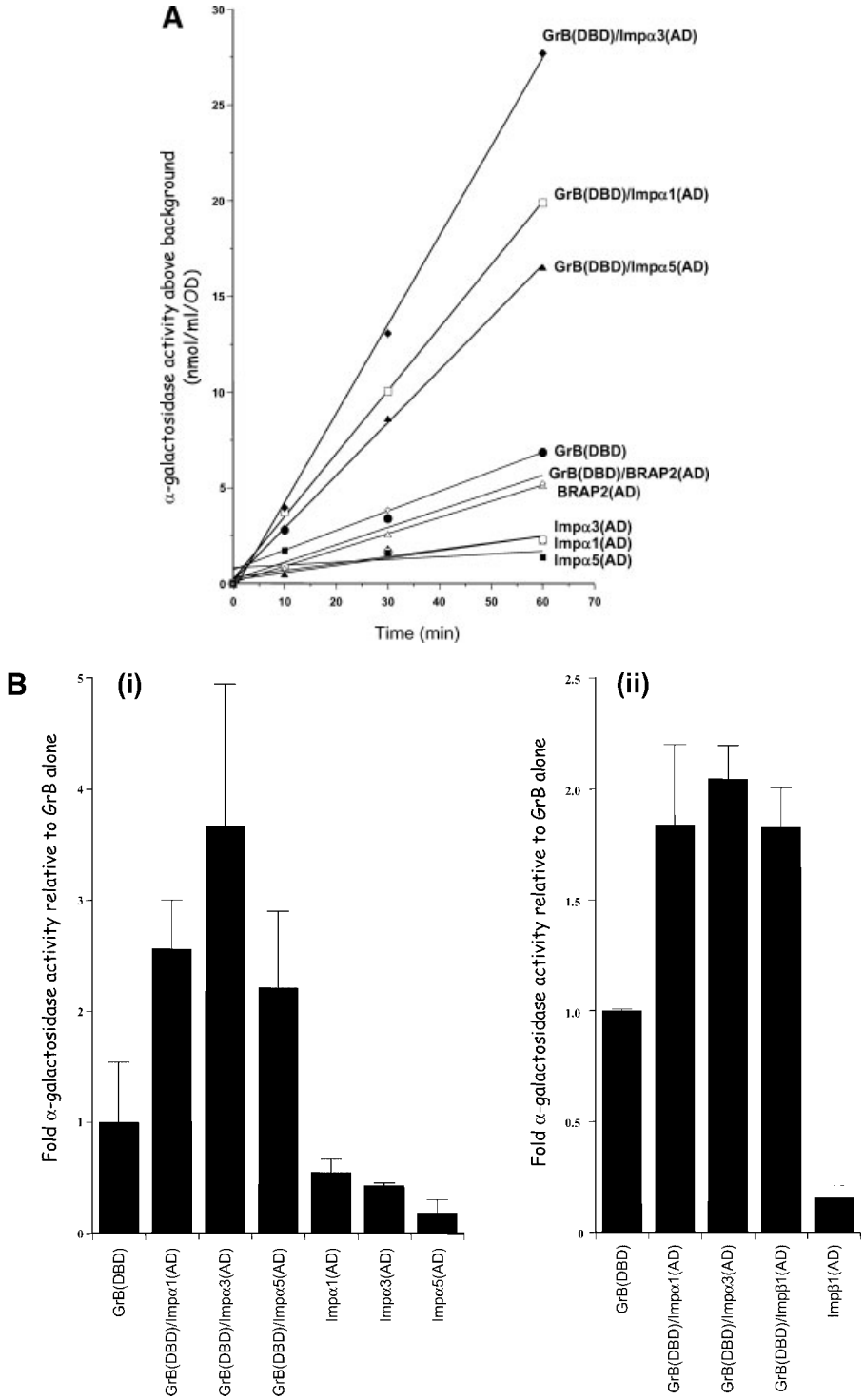

Fig. 1. Recognition of GrB by importins as demonstrated using quantitative yeast two-hybrid analysis. Yeast clones carrying the plasmids indicated (GrB in GAL4 DBD fusion protein vector pAS2.1, and/or IMPs [Seki et al., 1997; Herold et al., 1998] and BRAP2 [Li et al., 1998] in GAL4 AD fusion protein vector pGAD424) as indicated were cultured and assayed for reporter gene (α-galactosidase) expression as described in ''Materials and Methods.'' Results are shown either for a 60 min time course performed in triplicate (where the SEM was not >16% the value of the mean) (**A**); and the mean (SE indicated, n = 5) (**B**) for end point results expressed in terms of fold-induction relative to yeast expressing GrB-GAL4-DBD alone from experiments on two different days (i and ii), where the fluorescent signal was amplified using a stop reaction (see ''Materials and Methods'').

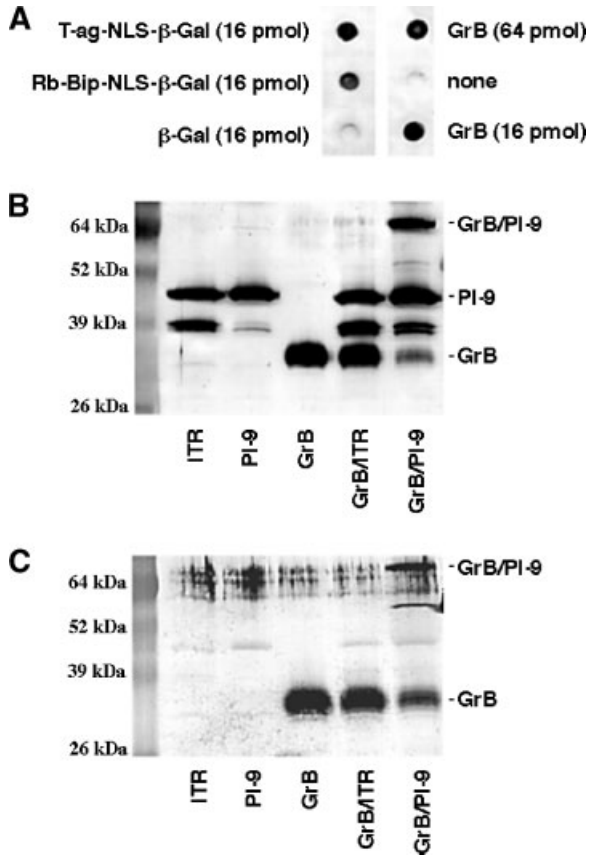

Fig. 2. Recognition of GrB by IMPs and modulation by PI-9 as demonstrated using dot blot (**A**) and Western blot (**B**) analysis. A: GrB (16 or 64 pmol, as indicated) was spotted onto nitrocellulose, together with the positive controls T-ag-NLS-β-Gal and Rb-Bip-NLS-β-Gal, which contain conventional NLSs, and the negative control β-Gal (all 16 pmol). Hybridization with IMPα-GST/IMPβ-GST was then performed as described in ''Materials and Methods,'' with visualization performed using anti-GST and alkaline phosphatase-linked anti-goat antibodies [Hübner et al., 1997]. B and **C**: GrB (16 pmol) was pre-incubated with inhibitors PI-9 and ITR in a 1:2 ratio prior to running on parallel SDS–PAGE gels, one of which was subsequently silver stained (B), and the other (C) transferred to nitrocellulose and hybridized with IMPα/β and bound IMPs detected [Hübner et al., 1997] as for (A). In (B) and (C), the molecular weights of the standards (leftmost lane; Benchmark™ protein ladder from GIBCO Invitrogen Co., Carlsbad, CA) are shown on the left, with the positions of GrB, PI-9, and the GrB/PI-9 complex indicated on the right.

(Fig. 2A,C) confirmed that GrB was recognized by IMPα/β, to an extent completely comparable to that of the NLS-containing-human TFs such as the cAMP-response-element binding protein CREB (data not shown) [Forwood et al., 2001b].

Complexation With PI-9 Reduces Recognition of GrB by IMPs

Like GrB, the specific GrB inhibitor PI-9 appears to be able to localize in the nucleus [Bird et al., 2001], which may relate to its role in protecting against cytolytic granule-mediated nuclear apoptosis. The effect of precomplexation with PI-9 (the c. 70 kDa GrB–PI-9 complex is evident in the right-most lane of Fig. 2B) on the interaction of GrB with IMPs was also assessed using dot (not shown) and Western ligand blot approaches. A mutant PI-9 derivative—ITR [Bird et al., 1998], which is unable to bind GrB (evident in the fifth lane of the gel in Fig. 2B)—was used as a negative control. Binding of the IMPα/β heterodimer to the GrB/PI-9 complex was evident (the rightmost lane of Fig. 2C), but the extent of binding to the complex was reduced compared to that seen for GrB alone. This implied that PI-9 might either mask an NLS within GrB, or alter its conformation such that IMPα/β could no longer bind. Importantly, when incubated with ITR, no reduction in IMPα/β binding to GrB was observed (Fig. 2C), implying that the results for PI-9 masking of GrB recognition by IMPs were specific. Neither PI-9 nor ITR was recognized to a significant extent by IMPα/β (Fig. 2B and not shown).

IMPα and IMPβ Recognize GrB Independently With Lack of Binding of IMPβ to the GrB/PI-9 Complex

There are a number of pathways distinct from the conventional IMPα/β-mediated nuclear import pathway which involves direct recognition of the import cargo by IMPβ1 [Tiganis et al., 1997; Chan et al., 1998; Hübner et al., 1999; Lam et al., 1999; Preiss et al., 2001; Forwood and Jans, 2002; Harley et al., 2003]. To assess if recognition of GrB by the IMPα/β heterodimer was via IMPα or IMPβ, Western ligand blots were carried out as above using either IMPα or IMPβ alone (Fig. 3BC), with IMPα/β as a control (Fig. 3A). Results indicated that GrB could be recognized by either subunit to an extent comparable to binding to control molecules such as CREB [Forwood et al., 2001b], and in contrast to negative controls such as β-Gal (not shown). IMPα exhibited the strongest binding, and was also clearly able to recognize the GrB/PI-9 complex (indicated by the arrow; Fig. 3B), but binding to the GrB/PI-9 complex by IMPβ was negligible (Fig. 3C). The amount of binding of either IMP subunit to GrB was reduced compared to that for IMPα/β (Fig. 3A), the expected result if both subunits were able to bind to GrB. The reduced binding in

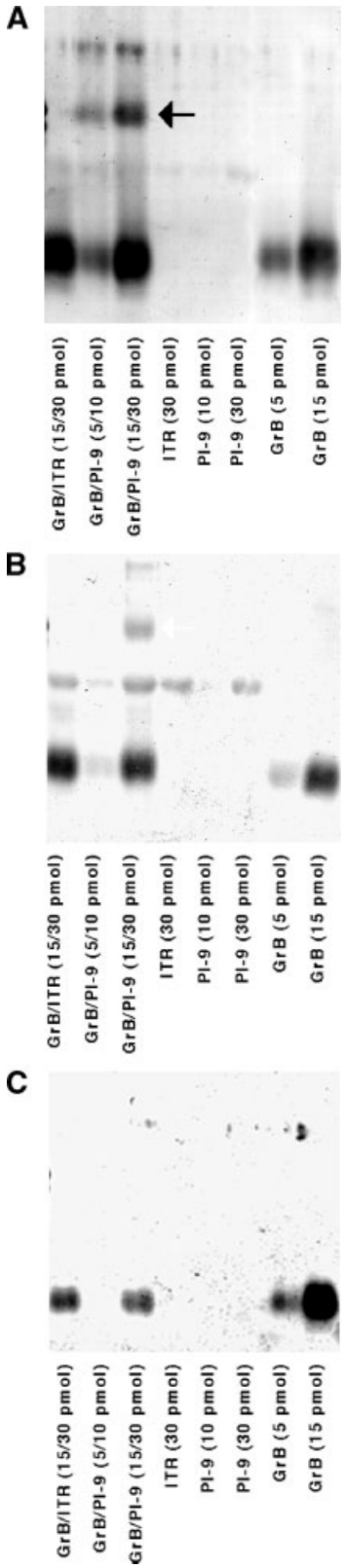

Fig. 3. Independent binding of IMPα and IMPβ to GrB and modulation by PI-9. Western ligand blot analysis was carried out as described in the legend to Figure 2C, for IMPα-GST/IMPβ-GST (**A**), IMPα-GST (**B**), and IMPβ-GST (**C**). The white arrows indicate the position of the GrB/PI-9 complex.

the presence of precomplexed PI-9 (Figs. 2C and 3A) was presumably due to the fact that the GrB/PI-9 complex is not recognized by IMPβ (Fig. 3C).

Nuclear Import of GrB is Dependent on IMPα but Inhibited by IMPβ; Effect of PI-9

The above results suggested that IMPα and IMPβ may play a role in GrB nuclear import, with PI-9 able to modulate this. We accordingly set out to test whether IMPs and PI-9 may affect GrB nuclear import in vitro using several approaches. In the first set of experiments, we employed antibodies specific for IMPα and IMPβ previously shown to inhibit nuclear import of the conventional IMPα/β-recognized NLS-containing T-ag-NLS-β-Gal fusion protein, as well as the IMPβ-recognized SRY and CREB proteins, in the case of the anti-IMPβ antibody [Hübner et al., 1999; Forwood et al., 2001a,b]. We found that antibodies specific for IMPα inhibited GrB maximal nuclear import in the presence of cytosol by about 50% ($P < 0.047$; Fig. 4A, Fig. 4B left panel), whereas those specific for IMPβ enhanced transport significantly ($P < 0.014$; Fig. 4B left panel, see Fig. 5A for pooled data). The rate of GrB nuclear import ($t_{1/2}$ of 0.97 ± 0.12 min) was markedly higher than that for Rb-NLS-β-Gal ($t_{1/2}$ of 5.7 ± 1.1 min), and was not significantly changed in the presence of the antibodies. Results for the control molecules Rb-NLS-β-Gal (Fig. 4A and 4B right panel; see Fig. 5C for pooled data) and T-ag-NLS-β-Gal (not shown) indicated inhibition by both antibodies. Identical experiments performed using antibodies to PI-9 (or PI-6 as a control) indicated no significant effect on GrB nuclear accumulation (Fig. 5D), or on that of control molecules (not shown). The clear implication of these experiments was that IMPα was required for GrB nuclear import, whereas IMPβ inhibited nuclear import.

Antibody additions to cytosol were also made to assess the nuclear import of precomplexed GrB/PI-9, with results differing from those observed in the absence of PI-9. Complexation with PI-9 per se did not appear to inhibit GrB nuclear import, but rather slightly enhanced

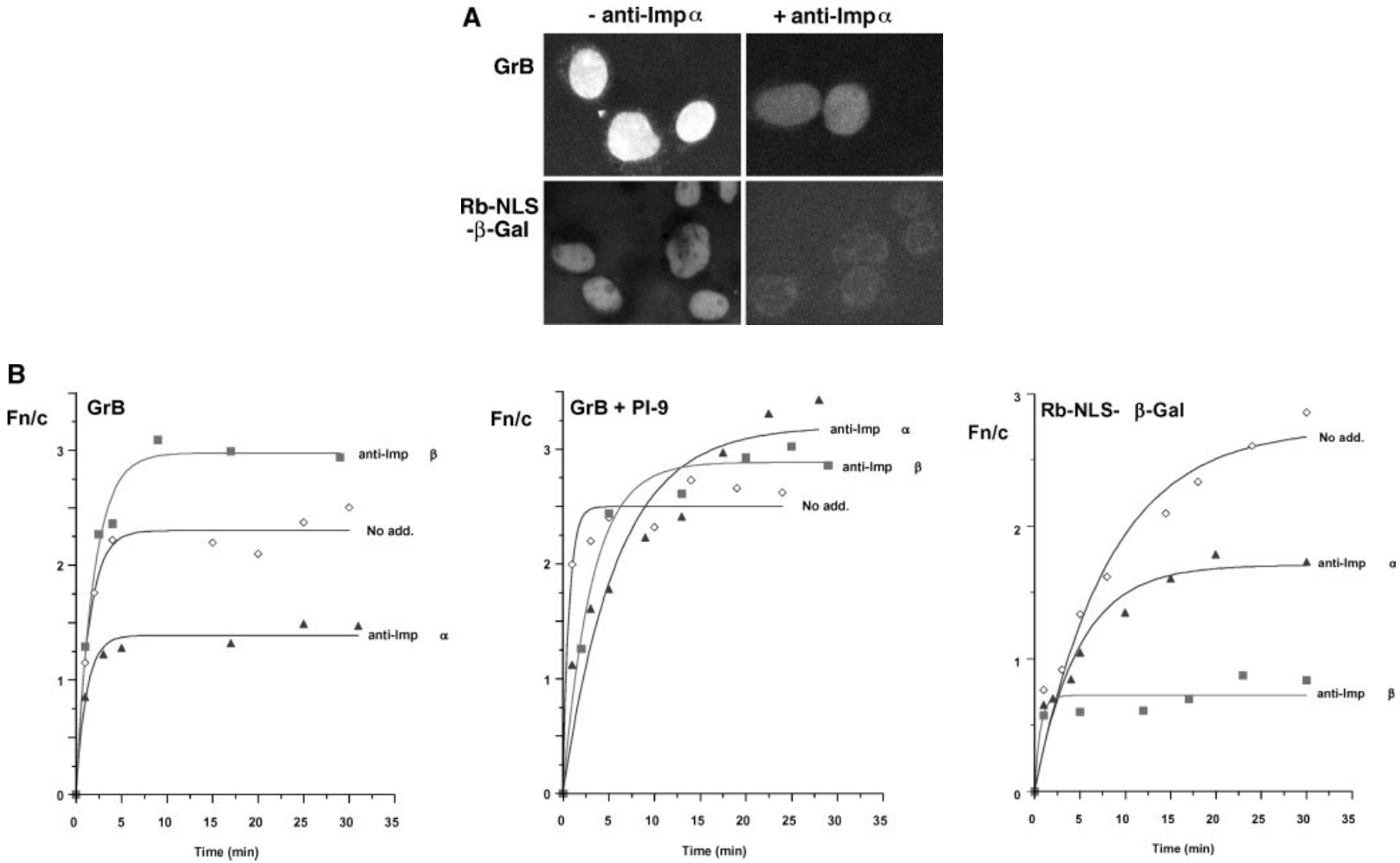
A
- anti-Impα
+ anti-Impα
GrB
Rb-NLS
-β-Gal
B
Fn/c
GrB
anti-Imp β
No add.
anti-Imp α
Time (min)
Fn/c
GrB + PI-9
anti-Imp α
anti-Imp β
No add.
Time (min)
Fn/c
Rb-NLS- β-Gal
No add.
anti-Imp α
anti-Imp β
Time (min)

Fig. 4.

nuclear accumulation, whereby not only the maximal nuclear accumulation (Fig. 4B, central panel; see Fig. 5B for pooled data), but also the rate of nuclear import ($t_{1/2}$ of 0.45 ± 0.11 in the presence of PI-9) was increased. The effect of antibody additions on the nuclear import of PI-9-complexed GrB transport was minimal (Figs. 4B and 5B). No reduction (but rather a slight increase) in the maximal level of nuclear accumulation by anti-IMPα antibodies was observed, implying that the GrB/PI-9 complex does not absolutely require IMPα for nuclear import. In addition, no significant increase in Fn/c_{max} was evident in response to the addition of anti-IMPβ antibodies, which was attributable to the fact that PI-9 prevents IMPβ binding to GrB (see Fig. 3B), and thereby IMPβ-mediated inhibition of GrB nuclear import. In considering all of these experiments, it should be remembered that the GrB-PI-9 complexation was not complete (see Fig. 2B) [Trapani et al., 1998a], so that the quantitative differences observed in the presence of PI-9 compared to in its absence almost certainly represent an underestimation of the overall effects.

The above experiments were broadened by testing the effect on GrB nuclear import of adding recombinant IMPs to cytosol (Fig. 6). Consistent with the above results, the addition of IMPβ was found to inhibit nuclear accumulation by about 50% (Fig. 6B, left panel), whereas IMPα and the IMPα/β heterodimer did not have a marked effect. To test whether IMPs alone could replace cytosol in terms of mediating GrB nuclear import, analogous experiments were performed in the absence of cytosol (Fig. 6B, right panel). IMPα, IMPβ, and the IMPα/β enhanced GrB nuclear import weakly above that in their absence; this was maximally, however, only about 50% the level of accumulation observed in the presence of exogenous cytosol. Thus, although IMPα appears to be required for GrB nuclear import, it alone, or in combination with IMPβ cannot replace the cytosolic requirement for GrB nuclear import, implying that additional cytosolic factors are required.

DISCUSSION

The present study builds on our demonstration [Jans et al., 1996, 1998] that GrB accumulates in the nucleus through a novel pathway that is independent of ATP hydrolysis or Ran, but dependent on cytosolic factor(s). Since Ran had been established not to be involved in the pathway, it had been assumed that IMPs are probably not the basis of the cytosolic dependence of GrB nuclear import. The results here, however, clearly indicate that GrB can be recognized independently by the NLS receptor subunits IMPα and IMPβ as shown using in vivo and in vitro assays as well as the IMPα/β heterodimer primarily through IMPα. Further, antibodies to IMPα inhibit GrB nuclear import, whilst those to IMPβ enhance import. These findings imply that the cytosolic dependence of GrB nuclear import may in part be due to a requirement for IMPα, and that, additionally, IMPβ may have an inhibitory role in the pathway. The latter is also supported by the fact that the addition of IMPβ to cytosol reduces GrB nuclear accumulation (Fig. 6), presumably by competing for IMPα. Conventional NLS-dependent nuclear protein import relies on the IMPα/β heterodimer together with Ran to mediate transport substrate recognition, docking at the NPC, energy-dependent translocation through the NPC, and ultimately release within the nucleus, so that it seems clear that GrB does not follow this pathway to the nucleus [see Jans et al., 1996]. Although IMPα would appear to be required, the fact that IMPα alone cannot replace cytosol to effect GrB nuclear accumulation in vitro (Fig. 6) indicates that other factor(s)

Fig. 4. (*Overleaf*) Effect on nuclear import of GrB, with or without precomplexed PI-9, or an NLS-containing control molecule, of antibodies to IMPs. **A**: Visualization of nuclear import of fluorescently labeled GrB or the NLS-containing control Rb-NLS-β-Gal using CLSM in mechanically perforated HTC cells in the presence of an ATP regenerating system and exogenous cytosol after 15 min, in the absence or presence of anti-IMPα antibodies. **B**: Nuclear import kinetics of GrB in the absence (left panel) or presence (middle panel) of precomplexed PI-9 (''Materials and Methods''), and Rb-NLS-β-Gal (right panel) in vitro, as determined using quantitative CLSM in mechanically perforated HTC cells in the presence of an ATP regenerating system and exogenous cytosol, in the absence or presence of the indicated antibodies. Measurements, performed as described in ''Materials and Methods'' [Efthymiadis et al., 1997; Hübner et al., 1997; Trapani et al., 1998a,b; Bird et al., 2001], represent a single typical experiment (pooled data shown in Fig. 5), where each point represents the average of 4–8 separate measurements (SEM <11.2% the value of the mean) for each of nuclear (Fn) and cytoplasmic (Fc) fluorescence, respectively, with autofluorescence subtracted. Data were fitted for the function $Fn/c(t) = Fn/c_{max} \times (1 - e^{-kt})$, where t is time in minutes, Fn/c_{max} is the maximal level of nuclear accumulation, and k is the first order rate constant [Efthymiadis et al., 1997; Lam et al., 1999]. No add., no addition.

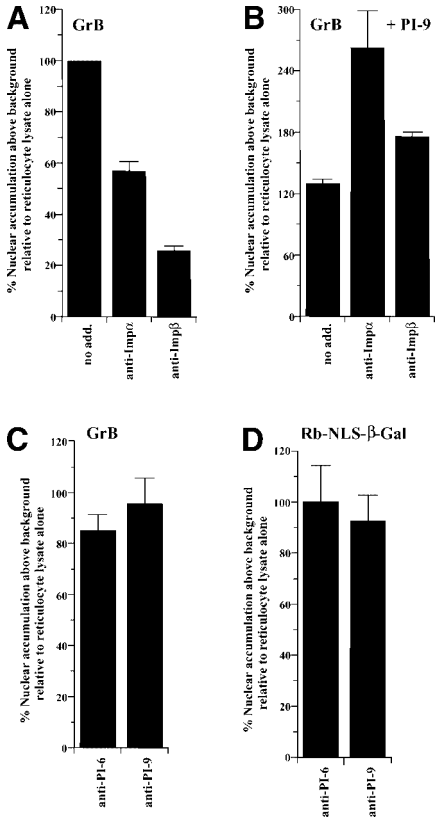

Fig. 5. Effect on nuclear import of GrB, with or without precomplexed PI-9, and an NLS-containing control molecule, of antibodies to IMPs and PI-9. Nuclear import kinetic measurements were performed, and data fitted as described in the legend to Figure 4B. Results are shown for the mean values ± SEM ($n > 2$) for the level of maximal nuclear accumulation (Fn/c_{max}). No add., no addition.

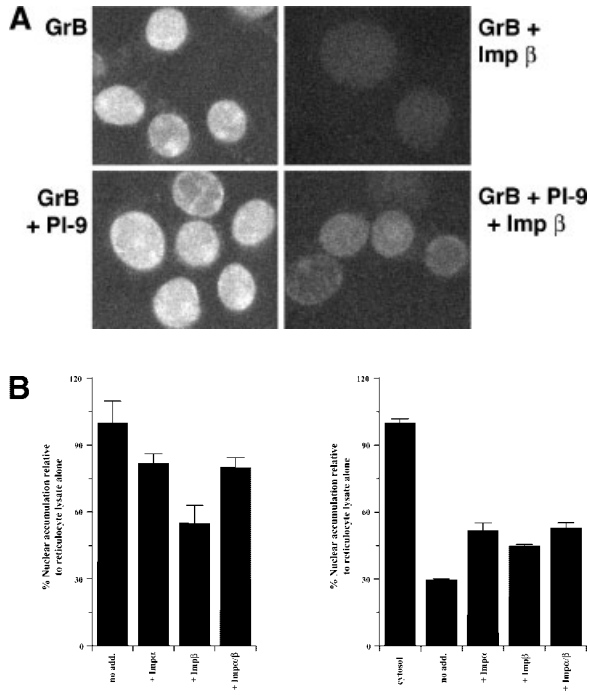

Fig. 6. Effect on nuclear import of GrB of the addition of IMPs in the absence or presence of exogenous cytosol as indicated. **A**: Visualization of nuclear import of fluorescently labeled GrB in the presence of an ATP regenerating system and exogenous cytosol after 15 min, in the absence or presence of recombinant IMPβ without (top panels) or with (bottom panels) precomplexation with PI-9. **B**: Nuclear import kinetic measurements were performed, and data fitted as described in the legend to Figure 4B. Results are shown for the mean values ± SEM ($n > 2$) for the level of maximal nuclear accumulation (Fn/c_{max}). No add., no addition.

are required, probably to mediate translocation through the NPC [Jans et al., 1996]. Interestingly, an IMPβ-independent pathway mediated by IMPα, which like IMPβ is known to bind to specific nucleoporins at the NPC [Belanger et al., 1994; Aitchison et al., 1996], has been described for IMPα from the plant *Arabidopsis thalania* [Hübner et al., 1999]. The latter, in fact, appears to be able to mediate translocation through the NPC independent of IMPβ, possibly through NLS-dependent microtubule association [see Smith and Raikhel, 1999; Lam et al., 2002] and/or direct association with nucleoporins [Belanger et al., 1994; Aitchison et al., 1996; Smith and Raikhel, 1999; Matsuura et al., 2003], while IMPβ- and Ran-independent nuclear translocation of IMPα from mouse and yeast has more recently been reported [Miyamoto et al., 2002]. Finally, that IMP-dependent nuclear protein import can be Ran-independent has been shown for the cyclin B1–Cdc2 complex [Takizawa et al., 1999]. Clearly, the breadth of the diversity of IMP- and Ran-dependent and -independent pathways appears not yet to be exhausted; exactly how these observations relate to the nuclear import pathway for GrB nuclear import is unclear at this stage.

The results here indicating that IMPβ can act as an inhibitor of GrB nuclear import (and IMPα as a possible inhibitor of GrB–PI-9 complex nuclear import—see Fig. 5B) may relate to reports [Jaekel and Görlich, 1998; Jaekel et al., 1999] that the recognition of nuclear import substrates such as ribosomal proteins rpL23a and histone HI by IMPα/β can be "non-productive" in terms of not leading to nuclear import [see also Lam et al., 1999]. Thus, high affinity complexation of IMPs with a potential nuclear import substrate may not lead to

nuclear translocation, but rather to the inhibition of nuclear import, as observed here for IMPβ and GrB. Analogously, IMPα inhibits IMPβ-specific binding to transport substrates such as PTHrP, GAL4, and the telomere binding factor TRF1 [Chan et al., 1998; Lam et al., 1999; Forwood and Jans, 2002]. The physiological significance of this should not be underestimated in the whole-cell context of competing transport receptors of the IMP family and nuclear targeting signals [Jaekel and Görlich, 1998; Jaekel et al., 1999; Jans et al., 2000a], although exactly how this may relate to the regulation of GrB nuclear import and its role in nuclear apoptosis requires further experimentation. Of relevance in this context are recent observations that IMPs may play chaperone-type roles in the cytoplasm, additional to or as a prelude to their role in nuclear transport, to bind and keep disaggregated proteins containing highly charged sequences (e.g., NLSs) [Jaekel et al., 2002]. Clearly, the cellular roles of IMPs, and IMPα in particular, extend beyond those specifically in nuclear protein import [see also Jaekel and Görlich, 1998; Hübner et al., 1999; Jaekel et al., 1999, 2002; Lam et al., 1999; Forwood and Jans, 2002; Miyamoto et al., 2002].

That IMPα/β-interactions with GrB can be modulated by binding of the serpin PI-9 to GrB is an intriguing finding, of relevance to PI-9s ability to protect against GrB/perforin-mediated apoptosis [Bird et al., 1998]. Complexation of GrB with PI-9 prevents recognition of GrB by IMPβ, probably through a masking effect or by eliciting a conformational change which prevents the inhibition of GrB import by IMPβ (Fig. 6). Perhaps more interestingly, antibodies to IMPα do not inhibit GrB nuclear import when it is complexed to PI-9 (Fig. 5B), implying that the GrB–PI-9 complex is not able to use the IMPα-dependent nuclear import pathway. Thus, GrB's nuclear import properties are altered when it is complexed with PI-9, which presumably contributes to preventing GrB nuclear import. The fact that PI-9 is a nuclear export signal (NES)-containing protein [Bird et al., 2001; Rodriguez et al., 2003] means that one of PI-9s primary functions may be to effect GrB export from the nucleus and thereby prevent apoptosis elicited at the level of the nucleus. In this respect, it is essentially analogous to the NES-containing cAMP-dependent protein kinase (PK-A) inhibitor PK-I [Wen et al., 1995], which down-regulates cAMP-induced gene activation both by preventing PK-A activity, and by ferrying the PK-A catalytic subunit out of the nucleus. Complexation with PI-9 thus would not only inhibit GrB proteolytic activity in nucleus and cytoplasm, but would also effect GrB export from the nucleus, thus preventing cleavage of GrBs nuclear apoptotic substrates through multiple mechanisms; the cytoplasmic GrB-PI-9 complex may also conceivably prevent GrB nuclear import by competing with uncomplexed GrB for IMPs and other components of the nuclear import machinery. Future work in this laboratory will focus on determining the detailed mechanisms regulating GrB nuclear localization, and direct involvement of PI-9 therein as implicated by the studies here. This should enhance our understanding of the specific role of GrB in cytolytic granule-mediated apoptosis, and the protective function of PI-9 in cytotoxic cells.

ACKNOWLEDGMENTS

Takemi Enomoto and Shang Li are acknowledged for supplying the IMPα and BRAP2 Y2H plasmids, respectively.

REFERENCES

Aitchison JD, Blobel G, Rout MP. 1996. Kap104p: A karyopherin involved in the nuclear transport of messenger RNA binding proteins. Science 274:624–627.

Andrade F, Roy S, Nicholson D, Thornberry N, Rosen A, Casciola-Rosen L. 1998. GrB directly and efficiently cleaves several downstream caspase substrates: Implications for CTL-induced apoptosis. Immunity 8:451–460.

Belanger KD, Kenna MA, Wei S, David LD. 1994. Genetic and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. J Cell Biol 126: 619–630.

Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, Trapani JA, Bird PI. 1998. Selective regulation of apoptosis: The cellular serpin proteinase inhibitor 9 prevents granzyme B-mediated but not Fas-mediated cell death. Mol Cell Biol 18:6387–6398.

Bird CH, Blink EJ, Hirst CE, Buzza MS, Steele PM, Sun J, Jans DA, Bird PI. 2001. Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires a non-conventional nuclear import pathway and the export factor Crm1. Mol Cell Biol 21:5396–5407.

Blink EJ, Trapani JA, Jans DA. 1999. Perforin-dependent nuclear targeting of granzymes: Central role in the nuclear events of granule-exocytosis-mediated apoptosis? Immunol Cell Biol 77:206–215.

Briggs LJ, Stein D, Goltz J, Corrigan VC, Efthymiadis A, Hübner S, Jans DA. 1998. The PK-A site (Ser^{312}) enhances dorsal nuclear import through facilitating nuclear localization sequence/importin interaction. J Biol Chem 273:22745–22752.

Browne KA, Blink E, Sutton VR, Froehlich CR, Jans DA, Trapani JA. 1999. Cytosolic delivery of GrB by bacterial toxins: Evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol 19:8604–8615.

Browne KA, Johnstone RW, Jans DA, Trapani JA. 2000. Filamin (280 kDa actin-binding protein) is a caspase substrate, and is also cleaved by the cytotoxic T lymphocyte protease granzyme B during apoptosis. J Biol Chem 275:39262–39266.

Chan CK, Hübner S, Hu W, Jans DA. 1998. Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: Implications for non-viral DNA delivery. Gene Therapy 5:1204–1212.

Efthymiadis A, Shao H, Hübner S, Jans DA. 1997. Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) in vivo and in vitro. A comparison with the SV40 large T-antigen NLS. J Biol Chem 272:22134–22139.

Efthymiadis A, Briggs LJ, Jans DA. 1998. The HIV-1 Tat nuclear localization sequence confers novel nuclear import properties. J Biol Chem 273:1623–1628.

Forwood JK, Jans DA. 2002. Nuclear import pathway of the telomere elongation supressor TRF1; inhibition by importin α. Biochemistry 41:9333–9348.

Forwood JK, Harley V, Jans DA. 2001a. The C-terminal nuclear localization signal of the SRY HMG domain mediates nuclear import through importin β1. J Biol Chem 276:46575–46582.

Forwood JK, Lam MHC, Jans DA. 2001b. Nuclear import of the CREB and AP-1 transcription factors is dependent on importin β1 and Ran, and independent of importin α. Biochemistry 40:5208–5217.

Görlich D, Vogel F, Mills AD, Hartmann E, Laskey RA. 1995. Distinct functions for the two importin subunits in nuclear protein import. Nature 377:246–248.

Harley VR, Layfield S, Mitchell C, Forwood JK, John AJ, Briggs LJ, McDowall S, Jans DA. 2003. Defective importin-β recognition and nuclear import of the sex-determining factor SRY are associated with XY sex reversing mutations. Proc Natl Acad Sci USA 100:7045–7050.

Henderson MJ, Russell AJ, Hird S, Munoz M, Clancy JL, Lehrbach GM, Calanni ST, Jans DA, Sutherland RL, Watts KW. 2002. EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. J Biol Chem 277:26468–26478.

Herold A, Truant R, Wiegand H, Cullen BR. 1998. Determination of the functional domain organization of the importin α nuclear import factor. J Cell Biol 143:309–318.

Hu W, Jans DA. 1999. Efficiency of importin alpha/beta-mediated nuclear localization sequence recognition and nuclear import. Differential role of NTF2. J Biol Chem 274:15820–15827.

Hübner S, Xiao CY, Jans DA. 1997. The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin. J Biol Chem 272:17191–17195.

Hübner S, Smith HM, Hu W, Chan CK, Rihs HP, Paschal BM, Raikhel NV, Jans DA. 1999. Plant importin alpha binds nuclear localization sequences with high affinity and can mediate nuclear import independent of importin beta. J Biol Chem 274:22610–22617.

Imamoto N, Shimamoto T, Takao T, Tachibana T, Kose S, Matsubae M, Sekimoto T, Shimonishi Y, Yoneda Y. 1995. In vivo evidence for involvement of a 58 kDa component of nuclear pore-targeting complex in nuclear protein import. EMBO J 14:3617–3626.

Izaurralde E, Kutay U, von Kobbe C, Mattaj IW, Görlich D. 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. EMBO J 16:6535–6547.

Jaekel S, Görlich D. 1998. Importin β, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J 17:4491–4502.

Jaekel S, Albig W, Kutay U, Bischoff FR, Schwamborn K, Doenecke D, Görlich D. 1999. The importin β/importin 7 heterodimer is a functional nuclear import receptor for histone H1. EMBO J 18:2411–2423.

Jaekel S, Mingot J-M, Schwarzmaier P, Hartmann E, Görlich D. 2002. Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. EMBO J 21:377–386.

Jans DA, Jans P, Briggs LJ, Sutton V, Trapani JA. 1996. Nuclear transport of GrB (fragmentin-2)-dependence on perforin in vivo and cytosolic factors in vitro. J Biol Chem 271:30781–30789.

Jans DA, Briggs LJ, Jans P, Froelich CJ, Parasivam G, Kumar S, Sutton VR, Trapani JA. 1998. Nuclear targeting of the serine protease granzyme A (fragmentin-1). J Cell Sci 111:2645–2654.

Jans DA, Sutton VR, Jans P, Froelich CJ, Trapani JA. 1999. Bcl-2 blocks perforin-induced nuclear translocation of granzymes, concomitant with protection against the nuclear events of apoptosis. J Biol Chem 274:3953–3961.

Jans DA, Xiao C-Y, Lam MHC. 2000a. Nuclear targeting signal recognition: A key control point in nuclear transport. Bioessays 22:532–544.

Jans DA, Jans P, Juelich T, Briggs LJ, Xiao C-Y, Piller SC. 2000b. Intranuclear binding by the HIV-1 regulatory protein Vpr is dependent on cytosolic factors. Biochem Biophys Res Commun 270:1055–1062.

Jenkins Y, McEntree M, Weis K, Greene W. 1998. Characterization of HIV-1 Vpr nuclear import: Analysis of signals and pathways. J Cell Biol 143:875–885.

Johansson M, Brooks A, Jans DA, Vasudevan SG. 2001. A small region of dengue virus encoded NS3 confers interaction with both the nuclear transport receptor importin-β and the viral RNA Dependent RNA Polymerase NS5. J Gen Virol 82:735–745.

Krajewski S, Gasgoyne RD, Zapata JM, Krajewski M, Kitada S, Chhanabhal M, Horsman D, Berean K, Piro LD, Fugier-Vivier I, Liu YJ, Wang H-C, Reed JC. 1997. Immunolocalization of the ICE/Ced-3-family protease, CPP32 (Caspase-3), in non-Hodgkin's lymphomas, chronic lymphocytic leukemias, and reactive lymph nodes. Blood 89:3817–3825.

Lam MHC, Briggs LJ, Hu W, Martin TJ, Gillespie MT, Jans DA. 1999. Importin β recognizes parathyroid hormone-related protein (PTHrP) with high affinity and mediates its nuclear import in the absence of importin α. J Biol Chem 274:7391–7398.

Lam MHC, Thomas RJ, Loveland KL, Schilders S, Min Gu, Martin TJ, Gillespie MT, Jans DA. 2002. Transport of parathyroid hormone related protein (PTHrP) to the

nucleus is dependent on microtubules. Mol Endocrinol 16:390–401.
Li S, Ku C-Y, Farmer AA, Cong Y-S, Chen C-F, Lee W-H. 1998. Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs. J Biol Chem 273:6183–6189.
Liu X, Zou H, Slaughter C, Wang X. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89: 175–184.
Matsuura Y, Lange A, Harreman MT, Corbett AH, Stewart M. 2003. Structural basis for Nup2p function in cargo release and karyopherin recycling in nuclear import. EMBO J 22:5358–5369.
Miyamoto Y, Hieda M, Harreman MT, Fukumoto M, Saiwaki T, Hodel AE, Corbett AH, Yoneda M. 2002. Importin α can migrate into the nucleus in an importin β- and Ran-independent manner. EMBO J 21:5833–5842.
Moore MS, Blobel G. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature 365:661–663.
Preiss S, Argentaro A, Clayton A, John A, Jans DA, Ogata T, Nagai T, Barroso I, Schafer A, Harley V. 2001. Compound effects of point mutations causing campomelic dysplasia/autosomal sex reversal upon SOX9 structure, nuclear transport, DNA binding and transcriptional activation. J Biol Chem 276:27864–27872.
Rexach M, Blobel G. 1995. Protein import into nuclei: Association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell 83:683–692.
Rodriguez JA, Span SW, Kruyt FA, Giaccone G. 2003. Subcellular localization of Crm1: Identification of a novel leucine-rich nuclear export signal conserved in anti-apoptotic serpins. Biochem J 373:251–259.
Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor: CSH Laboratory Press.
Schmalz D, Hucho F, Buchner K. 1998. Nuclear import of protein kinase C occurs by a mechanism distinct from the mechanism used by proteins with a classical nuclear lcoalization signals. J Cell Sci 111:1823–1830.
Seki T, Tada S, Katada T, Enomoto T. 1997. Cloning of a cDNA encoding a novel importin-alpha homologue, Qip1: Discrimination of Qip1 and Rch1 from hSrp1 by their ability to interact with DNA helicase Q1/RecQL. Biochem Biophys Res Commun 234:48–53.
Sekimoto T, Imamoto N, Nakajima K, Hirano T, Yoneda Y. 1997. Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. EMBO J 16:7067–7077.
Shakulov VR, Vorobjev IA, Rubtsov YP, Chichkova NV, Vartapetian AB. 2000. Interaction of yeast importin alpha with the NLS of prothymosin alpha is insufficient to trigger nuclear uptake of cargos. Biochem Biophys Res Commun 274:548–552.
Smith HMS, Raikhel NV. 1999. Protein targeting to the nuclear pore. What can we learn from plants. Plant Physiol 119:1157–1163.
Sun J, Bird CH, Sutton V, McDonald L, Coughlin PB, De Jong TA, Trapani JA, Bird PI. 1996. A cytosolic GrB inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes. J Biol Chem 271:27802–27809.
Takizawa CG, Weis K, Morgan DO. 1999. Ran-independent nuclear import of cyclin B1-Cdc2 by importin β. Proc Natl Acad Sci USA 96:7938–7943.
Tiganis T, Flint AJ, Adam SA, Tonks NK. 1997. Association of the T-cell protein tyrosine phosphatase with nuclear import factor p97. J Biol Chem 272:21548–21557.
Trapani JA, Browne KA, Smyth MJ, Jans DA. 1996. Localization of granzyme B in the nucleus: A putative role in the mechanism of cytotoxic lymphocyte-mediated apoptosis. J Biol Chem 271:4127–4133.
Trapani JA, Jans DA, Jans P, Smyth MJ, Browne KA, Sutton VR. 1998a. Efficient nuclear targeting and the nuclear consequences of apoptosis induced by GrB and perforin are caspase-dependent, but cell death is caspase-independent. J Biol Chem 273:27934–27938.
Trapani JA, Jans P, Smyth MJ, Froelich CJ, Williams EA, Sutton VR, Jans DA. 1998b. Perforin-dependent nuclear entry of GrB precedes apoptosis and is not a consequence of nuclear envelope dysfunction. Cell Death Differ 5:488–496.
Wen W, Meinkoth JL, Tsien RY, Taylor SS. 1995. Identification of a signal for rapid export of proteins from the nucleus. Cell 82:463–473.
Yokoya F, Imamoto N, Tachibana T, Yoneda Y. 1999. Beta-catenin can be transported into the nucleus in a Ran-unassisted manner. Mol Cell Biol 10:1119–1131.