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Novel Properties of the Nucleolar Targeting Signal of Human Angiogenin

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The polypeptide ligand angiogenin, a potent inducer of angiogenesis, localizes in the nucleus/nucleolus subsequent to endocytosis by relevant cell types. This study examines the kinetic properties of the nucleolar targeting signal (NTS) of angiogenin (IMRRRGL^{35}) at the single cell level. We show that the NTS is sufficient to target green fluorescent protein (GFP), but not β-galactosidase, to the nucleolus of rat hepatoma cells. Mutation of Arg^{33} to Ala within the NTS abolishes targeting activity. Nuclear/nucleolar import conferred by the NTS of angiogenin is reduced by cytosolic factors as well as ATP and is independent of importins and Ran. The NTS also confers the ability to bind to nuclear/nucleolar components which is inhibited by ATP hydrolysis; nonhydrolysable GTP analogs prevent nuclear accumulation in the absence of an intact nuclear envelope through an apparent cytoplasmic retention mechanism. Since the lectin wheat germ agglutinin does not inhibit transport, we postulate a mechanism for angiogenin nuclear/nucleolar import involving passive diffusion of angiogenin through the nuclear pore and NTS-mediated nuclear/nucleolar retention, and with cytoplasmic retention modulating the process. This pathway is clearly distinct from that of conventional signal-mediated nuclear protein import.

Key Words: **angiogenic factors; nuclear protein import; nucleolus; targeting signal.**

Angiogenin, a 14-kDa basic protein found in adenocarcinoma cell-conditioned medium and normal blood plasma, is a potent inducer of angiogenesis (neovascularization) (1–3). It is a member of the pancreatic ribonuclease (RNase) superfamily, with a unique RNase activity that is essential for its angiogenic activity. In addition to the catalytic site involving residues H^{13}, K^{40}, and H^{114} (single letter amino acid code), another critically important region of angiogenin is the "cell binding site", which encompasses residues 60–68 as well as D^{109} and is necessary for angiogenesis. Angiogenin is able to be endocytosed by relevant cell types, subsequent to which it translocates from the cell surface to the nucleus, where it accumulates in the nucleolus (2), the subcellular site of rRNA synthesis and ribosome assembly. A number of other polypeptide ligands (4–10), including members of the fibroblast growth factor (FGF) family (4, 5), have been reported to localize within the nucleus and/or nucleolus subsequent to receptor-mediated endocytosis. The precise function of many of these within the nucleus/nucleolus are largely unknown (6, 7, 10), but in the case of angiogenin, the use of mutant derivatives has established that its cell binding but not catalytic site, is critical for internalization and subsequent nuclear translocation. The implication is that RNase activity within the nucleolus on the part of angiogenin may be critically important for function, with the nuclear/nucleolar translocation of angiogenic molecules such as angiogenin being central to the mechanism of angiogenesis (1, 3).

Despite its physiological importance, relatively little is known concerning the general mechanisms of nucleolar protein uptake or the sequences that mediate it. Further, the kinetics of nucleolar localisation are completely unknown, whilst the reconstitution of nucleolar localisation has not been achieved *in vitro*, with the exception of the serine protease granzyme B (11; see Ref. 9). Although short modular nucleolar targeting signals (NTSs) able to target heterologous proteins to the nucleolus have been identified in several viral proteins (12–14) as well as in the heat shock/chaperone proteins HSC70/HSP70, which conditionally localise to the nucleolus as part of the cellular response to stress (14, 15), many nucleolar-localising proteins appear to

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have very complex NTSs, involving sequences conferring binding to proteins localised within the nucleolus (e.g., 16, 17).

The present study examines the nuclear/nucleolar import of green fluorescent protein (GFP) and β-galactosidase fusion proteins carrying the NTS (IMRRRGL[35]) of angiogenin at the single cell level, comparing results to those for a GFP fusion protein carrying the NTS $R^{33}A$ mutation. The results indicate that the angiogenin NTS has novel properties, with the nucleolar import pathway conferred clearly distinct from that for conventional nuclear localization sequence (NLS)-mediated nuclear protein import.

MATERIALS AND METHODS

Chemicals and reagents. The detergent 3[(3-cholamidopropyl)-dimethylamino]-1-propane-sulfonate (CHAPS) was from Boehringer-Mannheim and AMP-PNP (adenylyl imidodiphosphate) from Calbiochem. Other reagents were from the sources previously described (18–21).

Cell line. Cells of the HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7288C) line were cultured as described previously (18, 19).

Plasmid constructs. Synthetic oligonucleotides containing *Nhe*I ends were ligated into the *Nhe*I-digested plasmid vector pTrcHisA (Novagen) to generate the expression plasmids pHisAng-NTS-GFP and pHis-Ang-NTSmutGFP. The encoded hexa-histidine tagged fusion proteins comprise the angiogenin NTS (SIMRRRGLTS[37]) and NTS R33A mutant sequences, respectively, fused N-terminal to GFP amino acids 4–243. The plasmid expressing the Ang-NTS-β-galactosidase fusion protein was derived by oligonucleotide insertion into the *Sma*I site of the plasmid vector pPR2 as previously (8, 19). The Ang-NTS-β-gal fusion protein contains the angiogenin NTS fused N-terminal to β-galactosidase amino acids 9–1023. As an NLS-containing control, the β-galactosidase fusion protein T-ag-NLS-β-Gal (19), containing the NLS of SV40 large tumour antigen (T-ag), was used. His-tagged GFP lacking NLS/NTS sequences was employed as a further control.

Protein expression and purification. His-tagged GFP and GFP fusion proteins were expressed in *Escherichia coli.* One-litre cultures were inoculated with starter culture, incubated for 2 h at 28°C, induced with 0.1 mM isopropylthio-β-D-galactopyranoside (IPTG), and grown for 5 h before harvesting by centrifugation. The cells were lysed by three cycles of freeze thawing, followed by 3 × 60 s sonication. The supernatant was incubated with Ni-NTA agarose in a 15-ml conical tube for 1 h at 4°C on a rocker platform. The resin was pelleted by slow centrifugation, and the supernatant removed and the resin washed with sonication buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0) containing 10 to 50 mM imidazole. The proteins were eluted from the Ni-NTA column with 5 ml of elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 200 mM imidazole, pH 8.0) (22).

β-galactosidase fusion proteins were induced with IPTG, purified by affinity chromatography, and labelled using the sulfhydryl labelling reagent 5-iodacetamido-fluorescein as previously described (18–21).

Nuclear/nucleolar import kinetics. Nuclear import kinetics at the single cell level were measured *in vitro* using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy (CLSM) as previously (18–20). Conventional NLS-dependent nuclear protein import can be reconstituted in this system, conditional upon the exogenous addition of cytosol (reticulocyte lysate, Promega) and an ATP regenerating system (18, 19). Image analysis of CLSM files using the NIH Image public domain software, and curve fitting, were performed as described (18, 21).

In experiments where the ATP dependence of transport was tested, apyrase pretreatment was used to hydrolyse endogenous ATP in both cytosol (10 min at room temperature with 800 units/ml) and perforated cells (15 min at 37°C with 0.2 U/ml) (18), and transport assays performed in the absence of the ATP regenerating system (18–20) which was otherwise used. The dependence of transport on guanine nucleotide-binding proteins was tested using the nonhydrolysable GTP analog GTPγS (final concentration of 300 μM) (23, 24). The specific involvement of Ran was assessed by using recombinant Ran reconstituted with guanine nucleotides in the transport assay as previously (25). Ran was expressed in bacteria as a glutathione-*S*-transferase (GST) fusion protein and cleaved with thrombin to remove the GST moiety, prior to reconstitution with either GDP or GTPγS (26); RanGDP and RanGTPγS were used at a final concentration of 4 μM in the transport assay (26). Inhibition by the lectin wheat germ agglutinin (WGA) at 240 μg/ml (26) was tested as previously (27).

RESULTS

The Angiogenin NTS Is Capable of Targeting GFP but Not β-Galactosidase to the Nucleus/Nucleolus

The fusion proteins Ang-NTS-GFP and Ang-NTS-β-Gal, containing the angiogenin NTS (amino acids 31–35) fused N-terminal to the GFP and β-galactosidase coding sequences, respectively, were expressed in bacteria (see Materials and Methods), and the nuclear import kinetics measured *in vitro* using mechanically perforated HTC cells (17–20). Results were compared to those for GFP and β-galactosidase. Initial experiments indicated a lack of nuclear/nucleolar accumulation unless exogenous cytosol and ATP were both omitted from the *in vitro* assay (Table I, and see below). In the absence of cytosol and ATP, the Ang-NTS-GFP fusion protein exhibited nuclear/nucleolar accumulation, accumulating maximally to levels about 2- to 3.5-fold those in the cytoplasm respectively (Table I, Fig. 1). GFP itself (27 kDa) showed nuclear/nucleolar entry but not accumulation (Table I, Fig. 1), consistent with it being smaller than the molecular weight cut-off for passive diffusion between the nucleus and the cytoplasm (see Ref. 29). In contrast to Ang-NTS-GFP, Ang-NTS-β-Gal was completely excluded from the nucleus (Table I, Fig. 1), indicating that the large β-galactosidase protein (476 kDa), unlike GFP, was not able to be targeted to the nucleus/nucleolus by the angiogenin NTS. This implied that the NTS of angiogenin (14 kDa) can mediate the accumulation of small but not large carrier molecules; the lack of nucleolar accumulation of β-galactosidase fusion proteins containing the NTSs of HIV-1 Tat (18) and the mouse upstream binding factor mUBF (30) has been previously observed.

The nuclear/nucleolar import kinetics were also determined of Ang-NTSmut-GFP, containing the R33A substitution within the angiogenin NTS. Ang-NTSmut-GFP exhibited reduced nuclear accumulation compared to that of Ang-NTS-GFP, with an absence of nucleolar accumulation (Table I, Fig. 1). The results were thus in keeping with the findings from other systems (1) that

TABLE I

Nuclear/Nucleolar Import Kinetics of Ang-NTS-GFP Compared to Those of Ang-NTSmut-GFP, Ang-NTS-β-Gal, and Control Molecules

Protein/Conditions	Nuclear/nucleolar import parameter[a]				
	Fn/c_{max}	$t_{1/2}$ (min)	Fnu/c_{max}	$t_{1/2}$ (min)	*n*
Ang-NTS-GFP					
No addition	1.90 ± 0.10	1.06 ± 0.13	3.42 ± 0.20	0.87 ± 0.18	8
No addition[b]	1.92 ± 0.09	0.26 ± 0.04	3.78 ± 0.23	0.26 ± 0.09	2
+ Cytosol	1.53 ± 0.16	0.38 ± 0.09	E[c]		3
+ ATP	1.12 ± 0.06	1.03 ± 0.32	E[c]		3
+ Cytosol + ATP	1.11 ± 0.13	1.01 ± 0.15	E[c]		3
AMP-PNP	1.76 ± 0.35	1.07 ± 0.17	3.40 ± 0.22	0.74 ± 0.20	3
+ GTPγS	1.75 ± 0.12	2.41 ± 1.33	2.57 ± 0.23	3.65 ± 1.81	3
+ WGA	1.66 ± 0.03	0.83 ± 0.14	3.62 ± 0.14	0.33 ± 0.20	2
+ RanGDP	1.75 ± 0.15	1.67 ± 0.33	3.13 ± 0.14	1.47 ± 0.14	2
+ RanGTPγS	1.70 ± 0.00	1.50 ± 0.17	3.11 ± 0.13	1.48 ± 0.35	2
Ang-NTSmut-GFP					
No addition	1.49 ± 0.12	1.43 ± 0.54	E[c]		2
+ Cytosol + ATP	1.16 ± 0.15	1.82 ± 0.63	E[c]		2
+ ATP	1.20 ± 0.14	1.58 ± 0.89	E[c]		2
+ AMP-PNP	1.36 ± 0.11	0.34 ± 0.05	E[c]		2
+ GTPγS	1.45 ± 0.13	0.33 ± 0.15	E[c]		2
Ang-NTS-β-Gal					
No addition	0.39 ± 0.18	ND[c]	ND[c]		3
+ Cytosol + ATP	0.66 ± 0.16	ND[c]	ND[c]		2
GFP					
No addition	1.23 ± 0.12	1.54 ± 0.49	E[c]		2
+ Cytosol	1.22 ± 0.00	1.78 ± 0.01	E[c]		1[x]
+ ATP	1.12 ± 0.00	1.03 ± 0.01	E[c]		1[x]
+ Cytosol + ATP	1.17 ± 0.00	1.18 ± 0.02	E[c]		1[x]
T-ag-NLS-β-Gal					
No addition	1.14 ± 0.06	ND[c]	ND[c]		3
+ ATP	1.04 ± 0.05	ND[c]	ND[c]		3
+ Cytosol	1.16 ± 0.09	ND[c]	ND[c]		3
+ Cytosol + ATP	3.09 ± 0.26	6.74 ± 1.16	ND[c]		2
+ Cytosol + ATP + WGA	0.54 ± 0.03	ND[c]	ND[c]		2
+ Cytosol + ATP + RanGDP	4.40 ± 0.60	9.89 ± 1.71	ND[c]		2
+ Cytosol + ATP + RanGTPγS	1.30 ± 0.17	5.53 ± 1.10	ND[c]		2

[a] Raw data (see Figs. 1B, 2B, 3, and 4B) were fitted for the function $Fn/c(t) = Fn/c_{max*}(1 - e^{-kt})$ or $Fnu/c(t) = Fnu/c_{max*}(1 - e^{-kt})$ (17–20), where Fn/c or Fnu/c_{max} is the maximal level of accumulation at steady state in the nucleus or nucleolus, respectively, and t is time in minutes. An Fn/c or Fnu/c_{max} of 1.0 indicates equilibration between nucleus or nucleolus and cytoplasm, with valves below 1 indicating exclusion from the nucleus or nucleolus.

[b] Apyrase pretreatment was used and the ATP-regenerating system omitted (see Materials and Methods).

[c] ND, not able to be determined; E, equivalent concentration in nuclear and nucleolar compartments (i.e., no nucleolar accumulation).

R33 within the angiogenin NTS is essential for nucleolar targeting. Since the R33 mutation, although not affecting cell binding, effects a loss of angiogenic activity, this was also consistent with nuclear/nucleolar translocation being critical to angiogenin's function (2, 3).

ATP Hydrolysis and Cytosolic Factors Inhibit Both Nuclear and Nucleolar Accumulation

Conventional NLS-mediated transport into the nucleus involves two basic steps, the first of which involves recognition of targeting signal of the transport substrate and subsequent docking at the nuclear pore complex (NPC), while the second is energy-dependent and involves translocation through the pore and into the nucleus (29, 31, 32). The first step is mediated by the "NLS receptor," the heterodimeric complex of the specific NLS-binding protein importin α, and nucleoporin-binding NPC docking protein importin β (29, 33–36). Our results, however, clearly indicated that Ang-NTS-GFP nuclear and nucleolar accumulation was inhibited in the presence of either the ATP regenerating system or exogenously added cytosol (Table I, Fig. 2); as indicated above, nuclear/nucleolar accumulation only occurred in the absence of both ATP and exogenous cytosol (Table I). This clearly implied a lack of involvement of importins/Ran and ATP hydro-

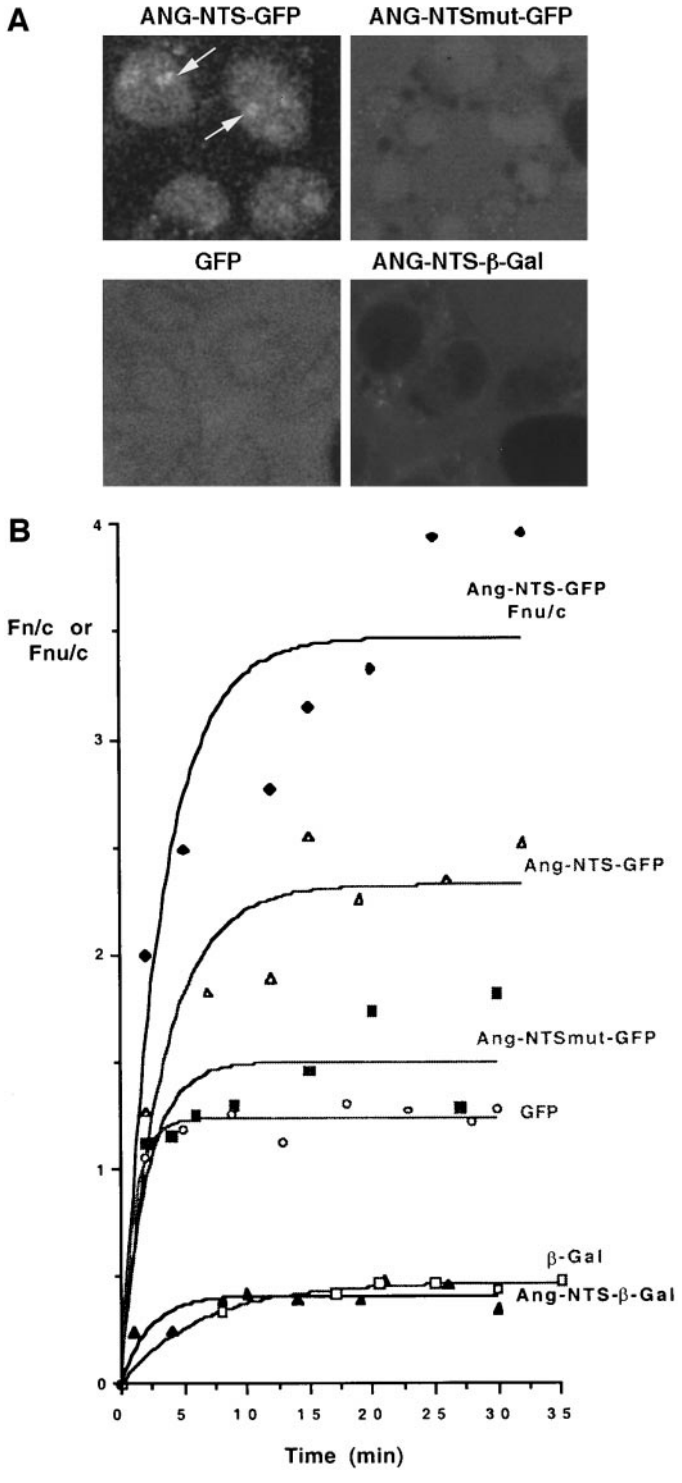

FIG. 1. The angiogenin NTS can target GFP but not β-galactosidase to the nucleus/nucleolus. (A) Visualization of nuclear uptake of angiogenin NTS-containing fusion proteins and GFP using CLSM in mechanically perforated HTC cells after 40 min at room temperature. The arrows indicate nucleoli. (B) Nuclear/nucleolar import kinetics of angiogenin NTS-containing fusion proteins and GFP as measured using quantitative CLSM. Measurements, performed as described in Materials and Methods (8, 11, 18–21), represent a single typical experiment from a series of experiments (see Table I for pooled data), where each point represents the average of 6–10 separate measurements (SEM < 12.8% the value of the mean) for each of nucleolar (Fnu), nuclear (Fn) and cytoplasmic (Fc) fluorescence respectively, with autofluorescence subtracted. Data were fitted for the functions $Fnu/c(t) = Fnu/c_{max*}(1 - e^{-kt})$ or $Fn/c(t) = Fn/c_{max*}(1 - e^{-kt})$, where t is time in minutes, Fnu/c_{max} and Fn/c_{max} are the maximal level of nucleolar and nuclear accumulation, respectively, and k is the first order rate constant (18–21). Pooled data are presented in Table I.

lysis in angiogenin nuclear/nucleolar import, indicating that Ang-NTS-GFP nuclear/nucleolar accumulation occurred through a pathway distinct from that used by conventional NLSs where both cytosolic factors and ATP hydrolysis are absolutely required (18, 24, 29, 37). Consistent with this, direct binding assays (21, 37, 38) to assess importin recognition (importin α, β, or α/β) indicated a lack of binding, in contrast to control molecules (data not shown).

Experiments were performed to assess nucleotide and nucleotide hydrolytic requirements for angiogenin nuclear/nucleolar accumulation or inhibition thereof. The nonhydrolyzable ATP analog AMP-PNP appeared to have no effect on nuclear/nucleolar accumulation of Ang-NTS-GFP (see Table I), implying that ATP hydrolysis was not required for angiogenin NTS-mediated nuclear/nucleolar targeting, but that it was required for its inhibition i.e. ATP-nucleotide binding alone was not enough to inhibit transport. In the presence of the non-hydrolyzable GTP analog GTPγS, which inhibits conventional NLS-dependent nuclear import (18, 23, 24, 37), maximal nucleolar accumulation was significantly reduced ($P < 0.042$) compared to in its absence. The transport rate was also significantly ($P < 0.026$) lower ($t_{1/2}$ of over 3.5 min, compared to less than a minute in its absence) (Table I; Fig. 2). The implication was that GTP hydrolysis was important for nucleolar transport of Ang-NTS-GFP, but since importins did not appear to be involved in the pathway (see above), and cytosolic factors inhibited rather than enhanced transport, Ran involvement was unlikely to represent the basis of this; rather, an as yet unidentified GTPase appeared to be required.

In order to dismiss the possibility of Ran involvement formally, recombinant Ran was expressed in bacteria as previously (25), reconstituted with either GDP or GTPγS (representing the GTP-bound form) (26), and included in transport assays. The presence of either RanGDP or RanGTPγS did not affect the maximal nuclear/nucleolar accumulation levels or rates of accumulation of Ang-NTS-GFP significantly (Table I; Fig. 3, left and middle panels). This was in stark contrast to the effects seen for the conventional T-ag NLS-containing control molecule T-ag-NLS-β-Gal whereby, RanGTPγS, as previously (25), significantly ($P < 0.0045$) reduced nuclear accumulation, whereas RanGDP markedly increased Fn/c_{max} (Table I; Fig. 3, right panel). It was concluded that a requirement for

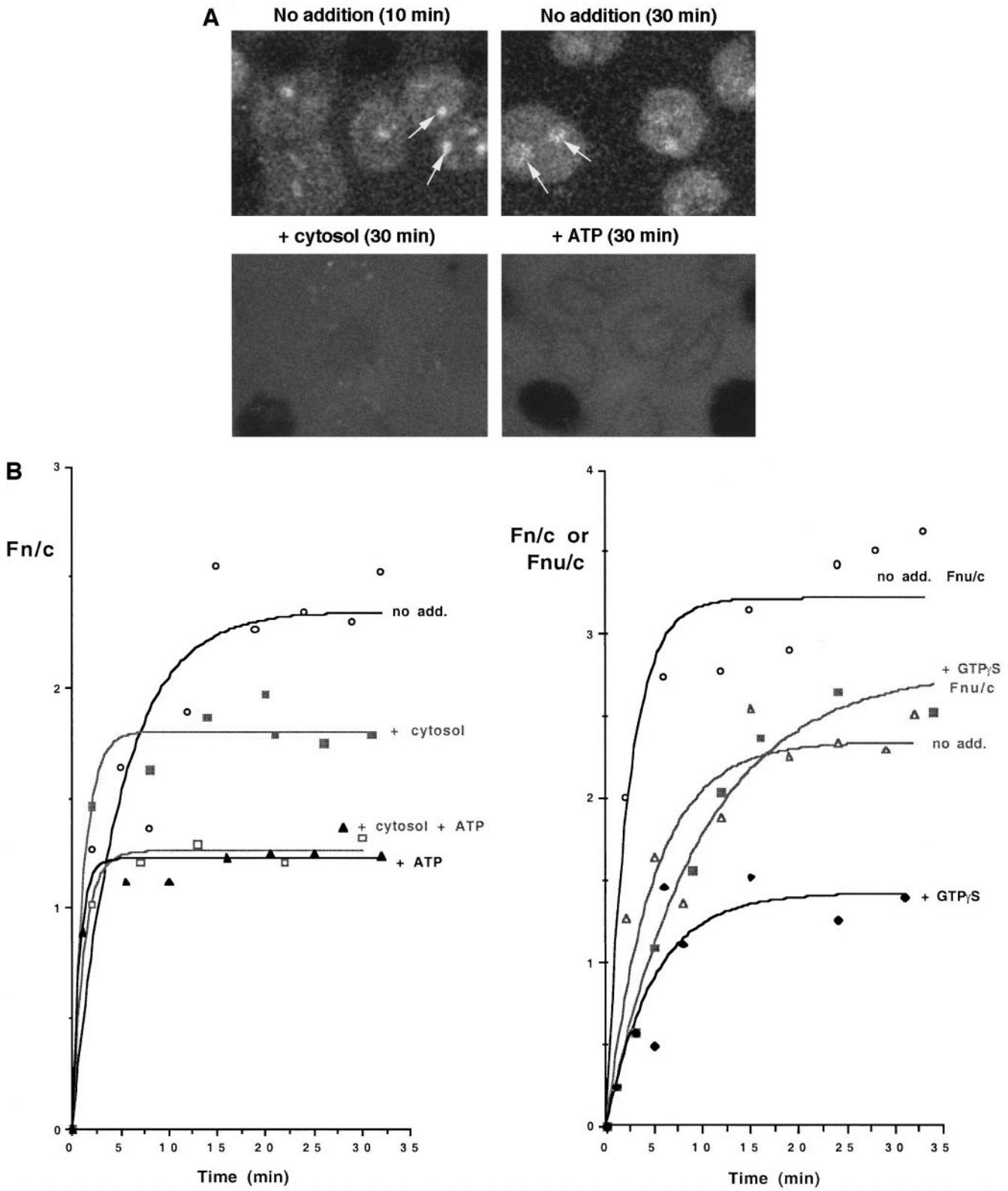

FIG. 2. Nuclear/nucleolar import conferred by the angiogenin NTS is inhibited by exogenous cytosol, ATP, and GTPγS. (A) CLSM visualization of nuclear uptake of Ang-NTS-GFP in the absence and presence of exogenous cytosol, and an ATP regenerating system at the times indicated. The arrows indicate nucleoli. (B) Nuclear/nucleolar import kinetics of Ang-NTS-GFP in the absence and presence of exogenous cytosol, an ATP regenerating system (left panel), and GTPγS (right panel) were determined as described in the legend to Fig. 1B. Pooled data are presented in Table I.

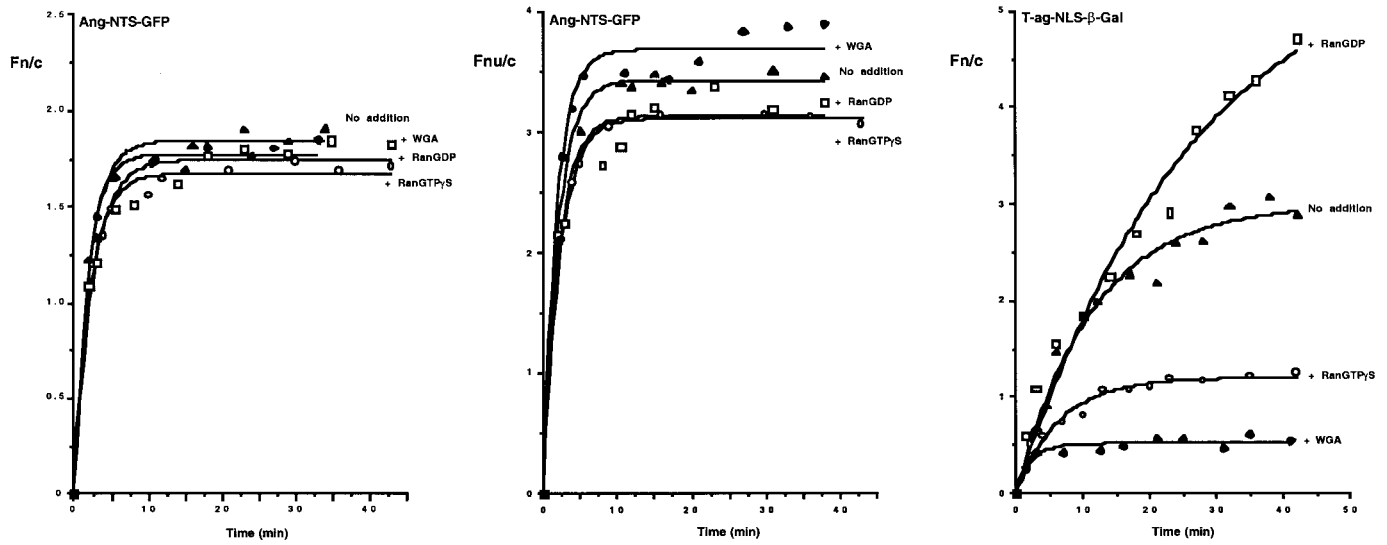

FIG. 3. Nuclear/nucleolar import conferred by the angiogenin NTS is not modulated by Ran or WGA, in contrast to that mediated by the T-ag NLS. Nuclear/nucleolar (left and middle panel, respectively) import kinetics of Ang-NTS-GFP in the absence of exogenous cytosol or ATP, or nuclear import kinetics of the conventional NLS-containing fusion protein T-ag-NLS-β-Gal (right panel) in the presence of exogenous cytosol and an ATP regenerating system, in the absence or presence of WGA or Ran reconstituted with either GDP or GTPγS (25, 26), were determined as described in the legend to Fig. 1B. Pooled data are presented in Table I.

Ran did not constitute the basis of GTPγS inhibition of Ang-NTS-mediated nuclear import (Fig. 2), and indeed, that Ran was most likely not involved at all in the Ang-NTS-conferred nuclear import pathway.

The Ang-NTS Mediates Binding to Nucleolar Components

The lack of involvement of importins and Ran in the Ang-NTS-conferred nuclear import pathway (above), indicated that Ang-NTS-GFP, like angiogenin itself but unlike the large Ang-NTS-β-Gal fusion protein, might enter the nucleus by passive diffusion, and accumulate within it through binding to nuclear/nucleolar components. To investigate this possibility, we tested the ability of the nucleoporin-function inhibiting lectin WGA to inhibit Ang-NTS-mediated nuclear/nucleolar import (27). We found that WGA did not inhibit Ang-NTS-GFP nuclear/nucleolar accumulation markedly (Fig. 3, left and middle panels), in stark contrast to its significant ($P < 0.0006$) inhibition of T-ag-NLS-β-Gal nuclear accumulation (Fig. 3, right panel; Table I). This implied that Ang-NTS-GFP can enter the nucleus by passive diffusion, in contrast to T-ag-NLS-β-Gal.

The detergent Chaps can be used to perforate the nuclear envelope to enable molecules to diffuse freely between cytoplasm and nucleoplasm; accumulation within the nucleus/nucleolus under these conditions can only occur through binding to nuclear/nucleolar components (4). Analysis of Ang-NTS-GFP nuclear/nucleolar import in the presence of Chaps indicated nucleolar accumulation (Fnu/c_{max} of c. 3.4; Fig. 4), clearly indicating that the angiogenin NTS confers binding to nucleolar components. Consistent with this, neither Ang-NTSmut-GFP nor GFP exhibited nucleolar accumulation in the presence of CHAPS (Fig. 4A; data not shown). Exogenous cytosol and ATP both inhibited nucleolar accumulation in the presence of CHAPS in similar fashion to in its absence (Fig. 2; Table I). Experiments in the absence of an intact nuclear envelope (Chaps treatment) in the presence of GTPγS revealed markedly reduced maximal nucleolar accumulation of Ang-NTS-GFP (Fnu/c_{max} of 1.9), implying that GTPase activity was essential for binding to nucleolar components. Intriguingly, nuclear exclusion of Ang-NTS-GFP (Fn/c_{max} c. 0.6) (Fig. 4B) was also observed under these conditions; that nuclear exclusion was observed even though there was no barrier to diffusion between the nuclear and cytoplasmic compartments, implied a cytoplasmic retention mechanism (see Ref. 18), whereby GTPase activity appeared to be required for release from binding to a cytoplasmic retention factor.

In contrast to ATP, the nonhydrolyzable ATP analog AMP-PNP did not inhibit nuclear entry nor nucleolar accumulation of Ang-NTS-GFP in the presence of CHAPS (Fig. 4B), implying that ATP-hydrolysis, but not ATP-binding, inhibited intranucleolar binding conferred by the angiogenin NTS. In the presence of ATP, nuclear exclusion was not observed (i.e., Fn/c_{max}/Fnu/c_{max} was not <1; see Fig. 4B) indicating the absence of cytoplasmic retention; rather, ATP hydrolysis appeared to inhibit accumulation in the nucleus/nucleolus in the absence of an intact nuclear envelope

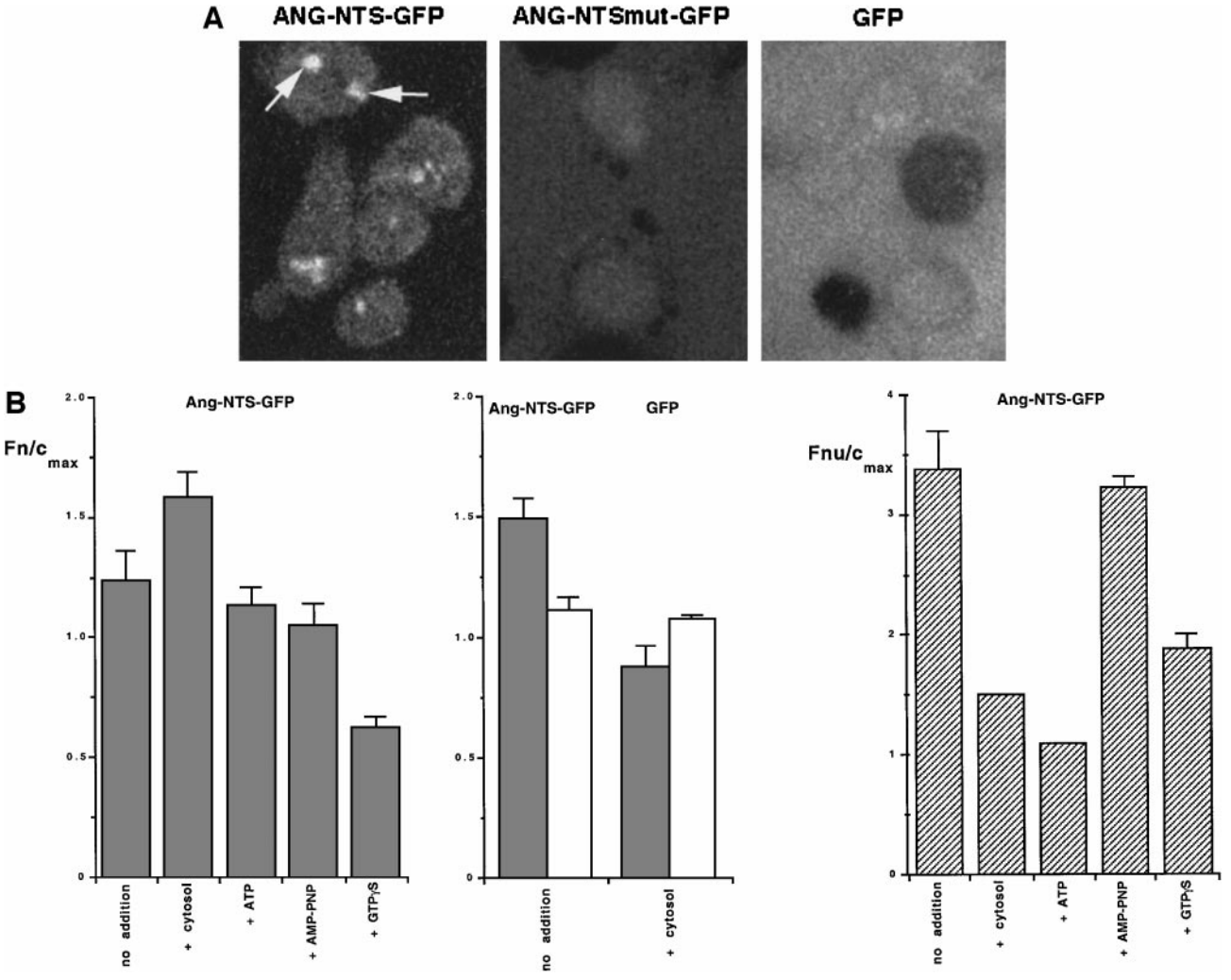

FIG. 4. Nuclear/nucleolar accumulation conferred by the angiogenin NTS in the absence of an intact nuclear envelope. Cells were treated with the nuclear envelope-permeabilizing detergent CHAPS (18) in all cases. (A) Visualization of nuclear/nucleolar uptake of angiogenin NTS-containing GFP fusion proteins was performed using CLSM after 5 min at room temperature. The arrows indicate nucleoli. (B) Nuclear/nucleolar accumulation in the absence and presence of exogenous cytosol, ATP, and the nonhydrolyzable nucleotide analogs indicated was quantitated as described in the legend to Fig. 1B. Results are shown for the mean ± SEM ($n > 3$) for Fnu/c_{max} and Fn/c_{max}.

by preventing/reducing nuclear/nucleolar retention. Thus, angiogenin NTS-conferred nucleolar binding, and presumably thereby nucleolar accumulation even in the presence of an intact nuclear envelope, appears to be modulated by cytoplasmic retention, with GTP and ATP hydrolytic activities regulating the process at the cytoplasmic and nuclear/nucleolar ends of the process respectively.

DISCUSSION

We examine here for the first time the kinetics of nuclear/nucleolar import conferred by the NTS of the polypeptide ligand angiogenin, establishing its novel properties. The angiogenin NTS clearly does not confer nuclear import through a pathway utilised by conventional NLSs, since importins and Ran are not required. ATP hydrolysis appears to inhibit angiogenin-NTS-conferred nuclear/nucleolar accumulation (whereas the nonhydrolyzable ATP analog AMP-PNP does not), whilst GTP hydrolysis (not Ran-mediated) appears to facilitate nuclear/nucleolar accumulation since the nonhydrolyzable GTP analog GTPγS reduces the maximal level and rate of nuclear/nucleolar accumulation (see Fig. 2B). The NTS confers binding to nuclear/nucleolar components, which also requires GTP hydrolysis (see Fig. 4B), with, intriguingly, nuclear exclusion effected by GTPγS. We hypothesise that the angiogenin NTS mediates binding to an insoluble cytoplasmic factor, with GTP hydrolysis required for release from this cytoplasmic retention; comparable observations have been made for ATP hydrolysis and release from

cytoplasmic retention for HIV-1 Tat (18). It thus seems reasonable to postulate that the GTPase activity necessary for angiogenin nuclear/nucleolar accumulation in the presence of an intact nuclear envelope (Fig. 2) may specifically relate to the release from cytoplasmic retention to allow subsequent passive diffusion into the nucleus/nucleolus and then accumulation through intranuclear/intranucleolar binding. It is clear from the experiments in the presence of CHAPS (Fig. 4) that, in contrast to GTP hydrolysis, ATP hydrolysis specifically modulates (inhibits) nuclear/nucleolar retention rather than cytoplasmic retention, since ATP (but not AMPNP) prevents accumulation in the absence of an intact nuclear envelope, but does not lead to nuclear exclusion. GTP and ATP hydrolytic activities thus appear to be distinct in regulating cytoplasmic and nuclear/nucleolar retention, respectively, mediated by the angiogenin-NTS. That passive diffusion is the mechanism of angiogenin nuclear entry is indicated by the fact that WGA does not inhibit nuclear/nucleolar accumulation, and is consistent with the observation that Ang-NTS-β-Gal (c. 476 kDa) is excluded from the nucleus, in contrast to Ang-NTS-GFP (c. 27 kDa).

The results for angiogenin here imply that the transport of proteins to the nucleolus may be fundamentally different to NLS-dependent protein targeting to the nucleus. Supporting this idea are our studies relating to the nucleolar localising cytolytic granule serine proteases granzymes A and B (20, 39) and the malignancy factor parathyroid hormone related protein (PTHrP) (9); in the former case, cytosolic factors but not GTP or ATP hydrolysis are required, whereas importin β and Ran, possibly in concert with an additional cytosolic factor, appear to target PTHrP to the nucleus/nucleolus independent of importin α. Thus, although the pathway of angiogenin nucleolar import appears to be unique in several ways, none of the proteins above localize in the nucleolus through a conventional NLS-dependent importin α/β-mediated pathway, which may relate to the fact that all three proteins have in common the ability to localize in the nucleus/nucleolus subsequent to endocytotic uptake by the cell. Intriguingly, all of them also have the ability to bind to nuclear/nucleolar components (see 9, 39), which would appear to be critical to nucleolar accumulation, again distinct from conventional NLS-mediated nuclear protein import where intranuclear binding is not integral to the transport process (see 5, 18). Nucleolar accumulation of mUBF (30), Nsr1 (40), and the shuttling protein nucleolin (41) has similarly been reported to involve binding to nucleolar components; in terms of the requirement for GTP hydrolysis for intranucleolar binding, however, angiogenin would appear to be unique in this context.

The results here add to the growing support for the idea that polypeptide ligands can localize in the nucleus/nucleolus through specific targeting signals (6, 7, 10). In addition to binding to and activating cell surface receptors (4–10, 42–44), growth factors/cytokines such as FGF-1, -2 or -3, interleukin-1 and -5, platelet derived growth factor PDGF A and growth hormone, with or without their membrane receptors, appear to play direct signalling roles in the nucleus, stimulating mitogenesis, inhibiting apoptosis, or modulating other cellular functions in the long or short term. For most of these the exact functional role within the nucleus is unclear, but in the case of angiogenin, the mechanistic importance of nuclear/nucleolar angiogenin would appear to relate directly to its RNase activity that is essential for its angiogenic activity. This implies strongly that nucleolar RNase activity is central to the angiogenic process. Future work in this laboratory will seek to establish the precise molecular details of nucleolar targeting of angiogenin, as well as identifying the targets of its RNase activity within the nucleus/nucleolus.

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