Determination of regions required for nuclear import and export of beta-catenin : Implication for distinct molecular interactions involved in bi-directional nuclear pore passage

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Summary

The nucleocytoplasmic transport of macromolecules occurs through the nuclear pore complex (NPC). Most transport events through the NPC are mediated by transport receptor molecules. The localization of β-catenin to the nucleus is a crucial step in the transduction of the Wnt/Wingless signal. Upon activation of the Wnt pathway, β-catenin accumulates in the nucleus. Previous studies indicated that β-catenin could translocate on its own through the nuclear pores without the aid of small GTPase Ran, and shuttles between the cytoplasm and the nucleus. The export of β-catenin also occurs in a Ran-independent manner. Unlike receptor-mediated transport, β-catenin transport appears to use a rather unconventional mechanism, possibly through the direct interaction with NPC components. In this study, I have analyzed the sequence requirement of β-catenin for its Ran-independent import and export in living mammalian cells and in the in vitro transport assay using semi-intact cells. I confirmed that a β-catenin fragment containing both Armadillo repeats 10-12 and the C-terminus possesses most strong nuclear import and export activity. Further dissection of this fragment showed that C-terminus of β-catenin is most important for its import and export. Moreover, I found that region required for β-catenin import and export overlaps, but they are not identical. Competition studies using different transport receptors under different conditions indicated that interaction(s) required for β-catenin import and export differ at NPC.

Introduction

Trafficking of macromolecules across the nuclear envelope plays a key role in coordination of cytoplasmic and nuclear events. The exchange of macromolecules occurs at nuclear pore complex (NPC), one of the largest macromolecular assemblies that exist in eukaryotic cells. The NPC is embedded in the two lipid bilayers of nuclear envelope (NE), with an estimated mass of about 125 MDa consisting from 30 – 50 different proteins in vertebrate cells. Characteristic feature of NPC is to mediate transport of molecules in two different directions, into and out of the nucleus. Molecules smaller than 40 – 60 kDa (or 9nm in diameter) can diffuse through NPC, while bi-directional transport across NPC of larger molecules occurs by active or facilitated mechanisms (for reviews, see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Bednenko et al., 2003).

Recent progresses in the field have revealed a presence of a number of different transport pathways in eukaryotic cells. The biggest class is the transport pathways mediated by the family of importin β like transport factors called importins or exportins (also called karyopherins) (for reviews, see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Weis, 2002). Importins are nuclear import factors that recognize specific import signals present within the import cargoes, and carry the bound cargo from the cytoplasm into the nucleus. Exportins recognize specific export signals present in cargoes and carry the bound cargoes from the nucleus out to the cytoplasm. The small GTPase Ran assures the directional transport of the cargoes mediated by importins and exportins by regulating the loading and unloading of cargoes to transport factors (Izaurralde et al., 1997; Richards et al., 1997). Binding of RanGTP to importins reduces the affinity for their import cargoes whereas binding to exportins stabilizes the affinity for their export cargoes. RanGTP is concentrated in the nucleus, while majority of cytoplasmic Ran is considered to be in GDP-bound form, due to the predominant nuclear

localization of Ran guanine nucleotide exchange factor RCC1 (RanGEF) (Ohtsubo et al., 1989) and cytoplasmic localization of the Ran GTPase-activating protein (RanGAP) (Mahajan et al., 1997; Matunis et al., 1996). This steep gradient of RanGTP and RanGDP across the nuclear envelope allows import complexes to form in the cytoplasm and dissociate in the nucleus, whereas export complexes form in the nucleus and dissociate in the cytoplasm, allowing the unidirectional transport for each cargo (for reviews, see Görlich and Kutay, 1999; Kuersten et al., 2001).

The second class of transport is mediated by nuclear transport factor 2 (NTF2)/p10, which mediate nuclear import of GDP-bound form of Ran from cytoplasm into the nucleus (Ribbeck et al., 1998; Smith et al., 1998). At every transport cycle of importin and exportin mediated transport events, RanGTP exits the nucleus in importin or exportin-bound form. GTP-bound form of Ran is converted into GDP-bound form in the cytoplasm. NTF2 replenishes nuclear Ran by binding specifically to RanGDP and carries the bound Ran back into the nucleus. Vectoriality for this transport is imparted by nucleotide exchange on Ran in the nucleus, which leads to the dissociation of RanGTP from NTF2.

The third class of transport is mediated by transport receptor family called TAP/NXF in metazoan, or Mex67 in yeast, which function together with a small subunit called p15/NXT in metazoan, or Mtr2 in yeast. These transport receptors were shown to mediate nuclear export of mRNA (Grüter et al., 1998; Katahira et al., 1999; Hurt et al., 2000). Unlike two transport pathways described above, what determines the directional transport of substrates for this transport pathway is not yet dissolved. Until now, direct involvement of Ran in this transport pathway has not been described.

In spite of having significant differences in sequence homology, all classes of transport receptor molecules share common features. Importins, exportins, NTF2, and TAP/p15 all possess the ability to shuttle between the cytoplasm and nucleus through nuclear pore complexes on its own without using

any energy consuming process. All these receptors were demonstrated to bind to specific motifs of nuclear pore complex components called FXFG motifs (for a review, see Ryan and Wente, 2000). Point mutations studies based on crystal structures of receptor molecules have shown that interaction of transport receptors with FXFG motifs plays a crucial roles in NPC passage (Bayliss et al., 2000; Bayliss et al., 2002a, b; Fribourg et al., 2001). Lines of evidence show that these receptors translocate through NPCs solely by direct interaction with NPC components. Based on energetic studies of transport, NPC translocation steps of all transport pathways are considered to operate via similar mechanism categorized as a facilitated transport. However, precise mechanism of NPC translocation is still largely unknown.

β-catenin (Yokoya et al., 1999), Vpr2 (Jenkins et al., 1998), RCC1 (Michael and Macara, 2000), and importin α (Miyamoto et al., 2002) are receptor-free transport. These substrates were shown to translocate through NPC in a Ran-independent manner without using energy consuming process. Among these substrates, nuclear export of β-catenin has been extensively studied. β-catenin is a structural component of adherens junctions, where it binds to the cytoplasmic domains of cadherin. β-catenin is also a transcriptional coactivator in the Wnt signaling transduction. Nucleocytoplasmic concentration of β-catenin thus affect various biological phenomena, including cell differentiation, cell proliferation, cell-cell interactions, cell-matrix interaction, and tumorgenesis (for reviews, see Moon et al., 2002). Several different groups have proposed different mechanisms for nuclear export of β-catenin, including involvement of nuclear export factor CRM1 along with adenomatous polyposis coli (APC) protein (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000), or, Ran-independent receptor-free export (Wiechens and Fagotto, 2001; Yokoya et al, 1999). Since β-catenin binds to many different proteins both in the cytoplasm and the nucleus, in which its binding

partners differ in different cells or under different cellular conditions, different conclusions would be drawn out for mechanism of import and export of β-catenin depending on cellular system that has been examined.

Studies using digitonin-permeabilized cell-free import and export assay, in conjunction with cytoplasmic and nuclear injection, allow me to examine nucleocytoplasmic shuttling activities of β-catenin without considering the effect of its binding partners. In this study, I systematically analyzed the sequence requirement of β-catenin for its import and export using microinjection techniques, together with nuclear import and export assay in digitonin-permeabilized cells. I found that ARM repeats 10–12 and C-terminus tail of β-catenin is a minimum region necessary for both import and export that occurs in a receptor-free manner. Deletion of C-terminus tail from this fragment abolished nuclear import and export activity, while C-terminus tail alone retained weak nuclear import and export activity, indicating that C-terminus tail of β-catenin plays an essential role for both its import and export. Most my data showed that sequence requirement for β-catenin import and export overlaps well.

In order to assess whether import and export of β-catenin proceed through the same molecular interaction at NPC, I performed competition studies using different transport receptors under different conditions. Surprisingly, I found that import and export of β-catenin could be differentially blocked, depending on concentration of receptor proteins and condition examined. My present data present the first evidence that a molecule that possesses overlapping sequence requirement for its import and export transit through NPC into and out of the nucleus using different molecular interaction.

Results

Arm repeats 10-12 and C-terminus of β-catenin is exclusively required for its own nuclear migration.

β-catenin possess an ability to constitutively translocate through nuclear pore complexes (NPCs) in a Ran-independent manner without requiring soluble factors or energy sources like cargo-free importin β. Based on crystal structure, β-catenin can be subdivided into three major domains, the N terminus (amino acids 1-140, indicated as "N" in this study), 12 armadillo (ARM) repeats (amino acids 141-664, ARM is indicated as "R" in this study), and the C terminus (amino acids 665-781, indicated as "C" in this study) (Huber et al., 1997). Comparison of crystal structure of cargo-free importin β and β-catenin revealed the remarkable conformational resemblance between HEAT repeats 4, 5, 6, 7 and 8 of importin β, and ARM repeats 9, 10, 11 and 12 of β-catenin (Lee et al., 2000). HEAT repeats 4, 5, 6, 7 and 8 of importin β cover the region necessary and sufficient for its NPC translocation. These facts led me to focus on the latter 1/3 of β-catenin for its nuclear migration activity.

In this study, to determine the region of β-catenin necessary and sufficient for its NPC passage, I bacterially expressed variety of β-catenin deletions, purified to homogeneity, and used as transport substrates in all experiments. These fragments were tagged with either GFP, FLAG epitope or, GST and GFP in the case for small fragments below the diffusion size. In order to assess whether region involving ARM repeats 10-12 actually possess an important role in nuclear migration, I first examined the nuclear import activity of two fragments, one fragment containing armadillo repeats 10-12 and C-terminus (GST-R10-C-GFP), and the other is the fragment containing N-terminus and armadillo repeats 1-9 (FLAG-N-R9-His_6). As shown in Fig.2b, GST-R10-C-GFP efficiently migrated into the nucleus when injected into the cell cytoplasm like full-length GFP-β-catenin. On the other hand, cytoplasmically injected FLAG-N-R9-His_6 did not migrate into the nucleus (Fig.2c). When examined

in digitonin-permeabilized cells, GST-R10-C-GFP rapidly migrated into the nucleus in the absence of soluble factors or energy sources like full length GFP-β-catenin (Fig.2e). In the similar experiment, FLAG-N-R9-His_6 did not accumulate into the nucleus of digitonin-permeabilized cells (Fig.2f). The nuclear migration of GST-R10-C-GFP was inhibited by wheat germ aggulutinin (WGA), and N-terminal half of importin β (impβ449), which possess region necessary and sufficient for NPC passage (Fig.3A, B). These observations confirm that the observed nuclear migration is not the result of passive diffusion. From these results, I concluded that β-catenin region involving ARM repeats 10-12 and C-terminus contains information necessary and sufficient for its own nuclear migration.

C-terminus portion of β-catenin possesses an essential role in its nuclear migration

I further examined the region within R10-C of β-catenin required for its import activity in more detail by further dissecting this fragment. As shown in Fig.3, β-catenin fragment containing Arm repeats 10-12 (GST-R10-12-GFP) did not migrate into the nucleus neither in the living cells nor in the digitonin-permeabilized cells. On the other hand, β-catenin fragment containing only the C-terminus tail (GST-C-GFP) weakly migrated into the nucleus in living cells and in the digitonin-permeabilized cells. Addition of ARM repeats 11 and 12 to the C-terminus tail (GST-R11-C-GFP) increased the import activity (Fig. 3B), but addition of ARM repeat 9 to the Arm repeats 10-12 (GST-R9-12-GFP) did not confer the import activity (Fig. 3A). Nuclear migration of GST-C-GFP and GST-R11-C-GFP were inhibited by WGA or impβ449, and occurs in the absence of soluble factors and energy sources, indicating that the observed nuclear accumulation of these two fragments occurs in a facilitated manner like full-length β-catenin. Moreover, addition of non-tagged full-length β-catenin competitively inhibited the nuclear import of GST-R10-C-GFP, GST-R11-C-GFP, and GST-C-GFP (Fig.4A).

Inverse experiments show that the addition of β-catenin fragment containing R10-C, R11-C, or C competitively inhibited the nuclear import of full-length GFP-β-catenin (Fig.4Bd, e and f). Addition of β-catenin fragment containing R10-12, which does not show the nuclear import activity by itself, did not affect the nuclear import of β-catenin (Fig.4B, C). These results further confirm that β-catenin fragments comprising from R10-C, R11-C, and C are involved in mediation of nuclear import of full-length β-catenin. However in these experiments, I also noticed that β-catenin fragment containing R10-C inhibit the nuclear import of GFP-β-catenin as efficiently as full-length β-catenin, while inhibitory activities of fragments containing R11-C and C were lower when compared to those of R10-C.

Together, these results show that C-terminus tail of β-catenin possesses an essential role in the nuclear import, but its activity is not strong enough as full-length β-catenin. Arm repeats involving R10-12, together with C-terminus tail is required for complete nuclear migrating activity of β-catenin.

Sequence determination of region required for β-catenin export.

Yokoya et al. (1999) previously showed that β-catenin possesses ability to rapidly exit the nucleus in mammalian cultured cells. In the microinjection experiments using Xenopus oocytes, β-catenin was reported to exit the nucleus in a Ran-independent manner. In the digitonin-permeabilized cells, β-catenin exits the nucleus without requiring the exogenous addition of soluble factors or energy sources (Wiechens and Fagotto 2001, see also Fig.5B). These results indicate that export of β-catenin may not require families of exportins, but it exits the nucleus on its own in the same way it enters the nucleus. Such behavior of β-catenin represents a typical case that this molecule possesses ability to transit through the NPC in a facilitated manner. Therefore, I was interested to know whether β-catenin

fragments that enter the nucleus also exit the nucleus in the same way.

To examine the export of β-catenin fragments in living cells, HeLa cells were fused by polyethylenglycol (PEG), and the recombinant β-catenin fragments tagged with GST and GFP were co-injected with BSA-Cy3 in the of the nucleus of the homokaryon. As shown in Fig.5A, after incubation for 30 min at 37℃, β-catenin fragments comprising from R10-C and C terminus, which possess import activity, were exported from nucleus to the cytoplasm and then reimported into all of the nucleus in homokaryons. β-catenin fragments comprising from R10-12, which did not possess import activity, remained in the injected nucleus showing that this fragment has no export activity in living cells. However in the similar experiments, β-catenin fragments comprising from R11-C, which possess ability to migrate into the nucleus, did not show nuclear export activity in living cells.

I next examined the export activity of β-catenin fragments in the digitonin-permeabilized cells. For this, β-catenin fragments possessing ability to migrate into the nucleus were first incubated with digitonin-permeabilized cells, and then the cells were subjected to 2nd incubation to examine export activity. As shown in Fig.5B, fluorescent intensity of the nucleus significantly reduced after 2nd incubation performed in the absence of soluble factors or energy sources, while strong nuclear fluorescence remained when 2nd incubation was performed in the presence of WGA. These results show that full-length β-catenin rapidly exits the nucleus in the absence of soluble factors, which is consistent with previous report indicating that this protein exits the nucleus on its own. Export inhibition by WGA shows the integrity of nuclear envelope during the 2nd incubation for export assay. In the similar experiments, I found β-catenin fragments comprising from R10-C and C terminus exit the nucleus in the absence of soluble factors or energy sources (Fig.5B). It is notable that export activity of C terminus was weak compared to those of full-length β-catenin and β-catenin fragment R10-C.

On the other hand, as in living cells, β-catenin fragments comprising from R11-C did not exit the nucleus at all.

These results show that β-catenin fragment comprising from R10-C, which was exclusively necessary for nuclear import, has strong nuclear export activity. β-catenin fragment comprising from C terminus, which plays an essential role in its nuclear import, also appears to possess an essential role in its nuclear export, although its activity is not strong enough as full-length β-catenin or R10-C as in the case of its nuclear import activity. However, β-catenin fragment comprising from R11-C, which clearly possesses nuclear import activity both in living cells and in digitonin-permeabilized cells, did not exit the nucleus at all both in living cells and in digitonin-permeabilized cells.

Competition studies show molecular interaction required for β-catenin import and export are not identical.

A translocation process through NPC has been shown to be reversible in many cases (Kose et al., 1997, Nalienly and Dreyfuss 1998, Nachury and Weiss, 1999). Therefore, a behavior of R11-C was rather unexpected, since substrate possessing an ability to migrate into the nucleus in a facilitated manner would also be expected to exit the nucleus in the same way. If R11-C binds to some structure or component(s) inside the nucleus, it may not exit the nucleus due to the nuclear retention. However, this issue is difficult to address directly. For the alternative approach to obtain information regarding whether import and export of β-catenin proceed through the same molecular interaction(s), I examined the inhibitory effect on β-catenin import and export in the presence of various concentrations of molecules that can translocate through the NPC on its own.

In the initial experiments, I was surprised to find that β-catenin is unable to inhibit its own nuclear

export up to 10μM concentration, a maximum concentration which was possible to add in our assay (Fig.6). This was intriguing since import of β-catenin can be significantly inhibited at 3μM concentration of β-catenin. β-catenin does not accumulate into the nucleus of Hela cells against the concentration gradient when examined under confocal microscopy without fixation (data not shown). Therefore, intra and extra nuclear concentration of β-catenin should be always identical during this import and export competition assay. The addition of same amount of competitor molecules that can transit through NPC in both directions should affect import and export of β-catenin similarly if molecular interaction required for import and export are identical. Inability for β-catenin to inhibit its own export up to 10μM concentration indicate that binding sites necessary for β-catenin export did not reach the saturation in our assay at this concentration of β-catenin. Import inhibition shows that addition of 3μM concentration of β-catenin reach the saturation of binding sites required for its import under the same condition.

I next examined how export and import of β-catenin can be competitively inhibited by different molecules that can translocate through NPC on its own. I chose three different molecules as a competitor. One is importin β, a most well characterized import receptor, which show very high affinity with NPC. Second molecule is importin α, which was recently shown to migrate into the nucleus on its own. Importin α consists from 10 armadillo repeats, and sequence expanding from arm repeat 9 to arm repeat 10 has been shown to be important for this import. Third molecule is CAS, a family of importin β which function as an export receptor for importin α. I have confirmed that all these molecules do not bind to β-catenin (data not shown). As shown in Fig.7, importin β inhibited both nuclear import and export of β-catenin at very low concentration. Export inhibition occurred by addition of importin β as low as 0.1μM concentration, while import inhibition required addition of

0.3μM concentration of importin β. I did not see any import nor export inhibition by addition of importin α up to 10μM concentration, indicating that importin α may not share sites of NPC required for β-catenin import and export. On the other hand, CAS inhibited export of β-catenin at 10μM concentration, while this concentration of CAS did not inhibit the β-catenin import. Raising the concentration of CAS up to 20μM slightly showed the inhibition of β-catenin import, indicating that CAS is not incapable to inhibit β-catenin import, but requires higher concentration to inhibit the β-catenin import. β-catenin does not bind to importinβ or CAS, either in the presence or absence of small GTPase Ran. Competition studies performed with importin β and CAS show that these importin β family proteins can inhibit the export of β-catenin at lower concentration than its import, which is an opposite results with the competition studies performed with β-catenin itself .

Finally, I examined whether importin β can inhibit import and export of β-catenin in the presence of small GTPase Ran and ATP. Presence of ATP and Ran affects a behavior of importin β (Kose et al., 1997, 1999a, b), possibly due to the generation of RanGTP as a primary effect. Presence of ATP stimulates export of β-catenin, leading to the apparent weaker nuclear accumulation. However, unlike importin β, this effect of ATP is Ran-independent (data not shown, also discussed below). As shown in Fig.8, I found that import of β-catenin is not inhibited by the addition of importin β in the presence of both Ran and ATP, but export of β-catenin is clearly inhibited under the same condition. These results also provide another evidence that molecular interactions required for β-catenin import and export at nuclear pores are not exactly identical.

Discussion

Yokoya et al. (1999) previously reported that ß-catenin was able to shuttle between the cytoplasm and the nucleus without using soluble factors and energy sources regardless of the absence or presence of Wnt signaling. It is supposed that β-catenin migrates via interactions with the nuclear pore complex (NPC) components like importin β because this molecule was shown to bind directly to FG-repeat containing NPC component (Fagotto et al., 1998). In this study, I showed that C-terminal region comprising from R10-C of β-catenin plays an essential role for both import and export of this protein. R10-C fragment enters and exits the nucleus without using soluble factor like full-length β-catenin, but R10-12, a fragment which excludes C-terminus from R10-C, loses nuclear import and export activity (Fig.3A and Fig.5A). Moreover, C-terminus alone shows weak nucleocytoplasmic shuttling activity. These results show that C-terminus is important domain for import and export of β-catenin.

C-terminus of β-catenin is proposed to play a role as a transcriptional activation domain (Hecht et al., 1999; van de Wetering et al., 1997). Portion involving R10-C of β-catenin is also known to bind several factors, which are important for β-catenin function. These factors include CREB-binding protein (CBP) and the closely related homologue p300 (Takemaru and Moon, 2000; Hecht et al., 2000), ICAT (Inhibition of β-catenin and Tcf) (Tago et al., 2000), Teashirt (Tsh) (Gallet et al., 1999) and Chibby (Takemaru et al., 2003). CREB/p300, ICAT, Tsh and Chibby bind to R11-C, R10-12, C-terminus, and R10-C of β-catenin, respectively. TATA-binding protein (TBP) also binds with three different regions of β-catenin containing C-terminus of β-catenin (Hecht at al., 1999). I propose a new role that the C-terminus portion functions in the nucleocytoplasmic shuttling of β-catenin through nuclear pores, and this region may interact with NPC component.

Transport receptors like importin β pass through NPCs in a facilitated manner without using energy, and such nucleocytoplasmic transport is considered to be reversible. My present data show that region of β-catenin required for its import basically overlaps well with the region required for its export. Therefore, it was rather unexpected to know that β-catenin fragment R11-C did not exit the nucleus because this fragment enters the nucleus (Fig.3 and Fig.5). There are two possibilities to explain this phenomena: 1) R11-C region of β-catenin does not have nuclear export activity. In other words, R11-C can interact with NPC component required for its import, but it dose not interact with NPC component required for its export, which means that molecular interaction at NPC required for β-catenin import and export is not exactly the same. 2) R11-C is retained by some molecule(s) within the nucleus. Although this second possibility is difficult to assess clearly, since neither full-length ß-catenin, ß-catenin fragment R10-C, nor R11-C did not accumulate into the nucleus against concentration gradient when examined by confocal microscopy without fixing (data not shown), I considered that nuclear retention of R11-C is rather unlikely.

β-catenin is one of a simple example to examine the bi-directional translocation through NPC, since this molecule transit through NPC on its own and small GTPase Ran neither affect its import nor export. In addition, β-catenin is not significantly retained in the nucleus like transport receptor molecules in which number of cargo is usually present in nucleus and/or in cytoplasm. To examine the 1st. possibility, I investigated whether import and export of β-catenin can be competitively inhibited by β-catenin itself or different transport receptors under different conditions using digitonin-permeabilized cells, based on hypothesis that only sites which β-catenin shares with transport receptors upon its import and export would be NPC component. At each concentration of receptor molecule incubated, fixed number of molecules should occupy NPC binding sites. Therefore, if

molecular interaction at NPC required for import and export of β-catenin is exactly same, the inhibitory effect of competitor molecules against β-catenin import and export would expected to be identical at the same concentration of competitor molecules. However, my results show that import and export of β-catenin is competitively inhibited differently by different competitor molecules as described below.

First, a concentration of competitor molecules required for inhibition of β-catenin import and export was clearly different. β-catenin import was competitively inhibited by 3μM β-catenin whereas export of β-catenin was not inhibited up to 10μM concentration (Fig.6). All my evidences that export of β-catenin requires specific sequence, and is inhibited by WGA, importin β and CAS clearly show that the β-catenin export requires specific molecular interaction with NPC componets, and it is not a simple diffusion. In addition, β-catenin import can be competitively inhibited during 2nd incubation similarly as in the 1 st incubation, showing nature of NPC was not altered in 1 st and 2 nd incubation. Therefore, inability to inhibit the export of β-catenin by β-catenin itself up to 10μM concentration can be explained that binding sites necessary for β-catenin export did not reach the saturation in this assay at the concentration of β-catenin added. On the other hand, inhibition of β-catenin import shows that 3μM concentration of β-catenin could saturate NPC-binding site necessary for β-catenin import.

Importin β inhibited both import and export of β-catenin at much lower concentration around 100 to 300nM. Quantification of inhibitory effect examined under various concentration of importin β show that export inhibition requires lower concentration of importin β compared to those of import inhibition. CAS showed similar tendency to inhibit β-catenin import and export as importin β, although much higher concentration was required for the inhibition. Importin β can effectively inhibit import and export of CAS at 1μM concentration but CAS did not inhibit import and export of importin β up to 10μM (data not shown). This indicate that the affinity of importin β with NPC component(s)

could be very strong compared to those of CAS, and may explain why much higher concentration of CAS was required to inhibit β-catenin import and export. Importin β family proteins can inhibit β-catenin export at lower concentration than to inhibit the import also indicate that molecular interaction required for β-catenin export is weak compared to those required for import.

I found that the presence of ATP and Ran affects the NPC translocation of β-catenin; apparent repression in import, and stimulation in export (Fig.8). Apparent repression in import should due to the stimulation of export. The results were same when the experiments were performed in the absence of Ran if ATP was present (data not shown). Therefore, the effect observed in Fig.8 is Ran independent. I do not know at present where and how ATP affects the nuclear export of β-catenin, but since non hydrolizable ATP analogue AMPPCP did not stimulate the export of β-catenin (data not shown), some reaction involving ATP hydrolysis affects the export of β-catenin.

Nucleocytoplasm shuttling of β-catenin is Ran-independent (Yokoya et al., 1999, Wiechens and Fagotto, 2001, and also this study), whereas, in the case of transport receptor like importin β, presence of Ran-GTP affects its nucleocytoplasmic shuttling behavior (Kose et al., 1997, 1999a, b). There are lines of in vitro evidence that Ran-GTP causes the dissociation of importin β from FXFG motif containing NPC components (Görlich et al., 1996; Shah and Forbes, 1998; Bayliss et al., 2000). Interaction of importin β with NPC components may alter in the presence of both Ran and ATP, since RanGTP is generated during the incubation with semi permeabilized cells. When ATP and Ran were added during the in vitro transport assay, I found that importin β blocked export of β-catenin, but it did not inhibit the import of β-catenin (Fig.8). These results could be explained as follows: Because Ran-GTP causes the dissociation of importin β from FXFG repeats-containing NPC components, interaction of β-catenin with FXFG repeat-containing NPC components plays an essential role in the

import reaction of β-catenin, and thus releases the import inhibition of β-catenin by importin β in the presence of ATP and Ran. Conversely, in the export reaction of β-catenin, interaction of β-catenin with FXFG repeat-containing NPC components is not essential, but NPC components which bind to importin β under presence of Ran-GTP, would be important. These data argue that the interaction(s) between β-catenin and NPC component(s) differ in import and export reaction of β-catenin. To ascertain this hypothesis, I should identify NPC components that interact with β-catenin during its import and export reaction.

Materials and Methods

Cell culture

Hela cells were incubated in Dulbecoo's modified Eagle's minimum essential medium supplemented with 5% fetal bovine serum at 37℃. Cultured cells were plated on glass bottom microwell dishes (MatTek Corporation) for microinjection experiments or on eight-well multitest slides (ICN Biomedicals) for in vitro transport assay 36-48h before each experiment.

Cell fusion by the polyethylenglycol (PEG)

Hela cells were fused as follows: Appropriate cells were plated on glass bottom microwell dishes and incubated for 36-48h before polyethylenglycol (PEG) treatment. Cells were washed twice with pre-warmed serum-free medium, and then a few drops of 50% (w/v) PEG solution (PEG 1500, Roche) was added and the dishes were incubated for 2 minutes at room temperature. Cells were washed twice using pre-warmed serum-free medium, and medium with 10% fetal bovine serum was added. After incubation for 2-4h, homokaryons were used for nuclear microinjection experiments.

Expression purification of recombinant proteins

Expression and purification of recombinant mouse β-catenin (Yokoya et al., 1999), and importin β and the amino acid (aa) 1-449 mutant (Imp449) (Kose et al., 1997) were performed as described previously. To construct the expression vector of the N-terminus and armadillo repeats 1-9 (N-R9), armadillo repeats 10-12 (R10-12), armadillo repeats 10-12 and C-terminus (R10-C), armadillo repeats 11and12 to C-terminus (R11-C) and only C mutants (C), respectively, the region of aa 1-519, aa 520-664, aa520-781, aa 583-781 and aa 665-781 of mouse β-catenin was amplified by PCR using

appropriate oligonucleotides (N-R9: 5'-GATCGGATCCATGGCTACTCAAGCTGACC-3' and 5'-CCTACCCCGGGAAGGG-CAAGGTTTCGAATCAATCC-3', R10-12: 5'-CCTACGGATCCTGC CCAGCAAATCATG-CG-3' and 5'-CCTACCCCGGGCTCAGACATTCGGAATAGGAC-3', R10-C: 5'-CCTACGGATCCTGCCCAGCAAATCATGCG-3' and 5'-CCTACCCCGGGCAGGT CAGTATCA-AACCAGGC-3', R11-C: 5'-CCTACGGATCCGACGTTCACAACCGGATTGTAATC-3' and 5'- CCTACCCCGGGCAGGTCAGTATCAAACCAGGC-3', C: 5'-CCTACGGATCCGACAA GCCACAGGATTACAAG-3' and 5'-CCTACCCCGGGCAGGTCAGTATCAAACCAGGC-3'). PCR product of N-R9 with His_6 at the C-terminus was inserted into BamHI and NotI site of the pGEX-6P-2 vector (Amersham Biosciences), and oligonucleotides encoding the FLAG epitope (DYKDDDDK) were ligated into the N-terminus of the N-R9 gene at the BamHI site. Other PCR products were inserted into BamHI and SmaI sites of the pGEX-2T vector (Amersham Biosciences), and the green fluorescent protein (GFP)-fragment amplified by PCR was then inserted into SmaI and EcoRI sites. Recombinant glutathion S-transferase (GST)-N-R9, GST-R10-12, GST-R10-C, GST-R11-C, GST-C and GFP-fused mutants, GST-C-GFP proteins were expressed by 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 12 h at 18 ℃ in *Escherichia coli* strain BL21(DE3). GST-R10-12-GFP, GST-R10-C-GFP, GST-R11-C-GFP and GST-FLAG-N-R9-His_6 proteins were expressed for 12 h at 18 ℃ in *Escherichia coli* strain DH5α. Recombinant proteins expressing bacteria were lysed in buffer A (50 mM Tris-HCl, pH 8.0, 50mM NaCl, 2 mM DTT, which contained 1μg/ml aprotinin, leupeptin and pepstatin) with freeze-thaw and sonication and clarified by centrifugation (45,000 rpm, 30 min). The resultant supernatant was incubated with glutathion-Sepharose at 4 ℃ for 2 h. After extensive washing in buffer B (20 mM phosphate, pH7.2, 20 mM NaCl, 2 mM DTT, which contained 1μg/ml aprotinin, leupeptin and pepstatin), other proteins

except for GST-FLAG-N-R9-His_6 were eluted by 20 mM glutathion from glutathion-Sepharose beads and purified on MonoQ column (Amersham Biosciences). These recombinant proteins were desalted with a PD10 column (Amersham Biosciences) equilibrated with buffer B, and concentrated by ultrafiltration using Microcon 50 (Amicon).

After the sepharose beads bound GST-FLAG-N-R9-His_6 protein were incubated with PreScission Protease (Amersham pharmacia Biotech), GST cleaved FLAG-N-R9-His_6 protein was purified with Ni-NTA-agarose. Gels were washed extensively with 50mM imidazole in buffer B, which contained 1mM 2-mercaptoetanol instead of DTT. Bound proteins were eluted with buffer B supplemented with 150mM imidazole. The recombinant FLAG-N-R9-His_6 protein was dialyzed against 20mM phosphate, pH7.2, 2 mM DTT, and 1μg/ml each of aprotinin, leupeptin and pepstatin.

Expression and purification of recombinant CAS (Hieda et al., 1999), importin α (Imamoto et al., 1995), GTPase Ran (Yokoya et al., 1999) were performed as described previously.

In vitro transport assay

Import assay: Digitonin-permeabilized HeLa cells were prepared as described previously (Kose et al., 1997). Unless described differently in figure legends, 10 μl of testing solution usually contained 10 pmol or 5 pmol of GFP-β-catenin, GFP-fused β-catenin mutants or FLAG-N-R9-His_6 in transport buffer (20 mM HEPES, pH7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, 1 μg/ml aprotinin, leupeptin and pepstatin) containing 2% BSA. Recombinant β-catenin, β-catenin mutants, importin β, CAS or importin α or Ran proteins, and energy source (1 mM ATP (Sigma, St. Louis, MO; A-6410), 5 mM phosphocreatine (Sigma, P-6502), 20 U of creatine kinase (Sigma, C-3755)) were included in the above 10 μl testing solution.

Digitonin-permeabilized cells were incubated with import mixtures containing β-catenin or other recombinant proteins. The import reaction was performed for 15 min at 30°C , and the cells were then washed twice with ice-cold transport buffer and fixed with 3.7% formaldehyde in transport buffer (minus DTT) for 10 min at room temperature.

Export assay: GFP-β-catenin and GFP-fused β-catenin mutants which enter the nucleus in the import assay were applied to export assay. After import reaction (first incubation), the cells were washed twice with ice-cold transport buffer, and were then reincubated in the transport buffer or 10 μl testing solution containing WGA (EY Laboratories, San Mateo, CA), recombinant β-catenin, GST-fused β-catenin mutants, importin β, CAS, importin α or Ran proteins (second incubation: export reaction). The export reaction was performed for 20 min at 20°C, and the cells were then washed twice with ice-cold transport buffer and fixed with 3.7% formaldehyde in transport buffer (minus DTT) for 10 min at room temperature.

Microinjection

Recombinant GFP-β-catenin or GFP-fused β-catenin mutants proteins were injected through a glass capillary into the cytoplasm or nucleus of cells plated on glass bottom microwell dishes. Where indicated, WGA or Cy3-labeled BSA (injection marker) was coinjected with GFP-β-catenin or GFP-fused β-catenin mutants proteins. After incubation for 30 min at 37°C, the cells were washed twice with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature.

Indirect immunofluorescence

To examine the localization of FLAG-N-R9-His_6, the fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, incubated with 3% skim milk in PBS for 20 min, and then incubated with 2 μg/ml mouse immunoglobulin G1 (IgG1) monoclonal anti-FLAG M2 antibody (SIGMA) or 1μg/ml β-CAT-7D11, a monoclonal antibody which interact with the N-terminus(exon 2) of β-catenin for 1 h at room temperature. The mouse antibody was detected with Cy3-labeled goat antibodies to mouse IgG (Jackson ImmunoResearch Lab., Inc.).

Conjugation of Cy3 with BSA

BSA was dissolved at 1 mg/ml in 0.1 M carbonate buffer, pH 9.5, and the protein solution (1ml) was mixed with the FluoroLink™ Cy3 monofunctional dye vial (amersham pharmacia biotech). After incubation for 30 min at room temperature, unconjugated dye was removed by gel filtration chromatography. Peak fractions containing Cy3-labeled BSA were collected and dialyzed against PBS.

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Figure 1. Constructions of deletion mutants of β-catenin The import and export activity of full-length and each mutant form of β-catenin were summarized on the right. ○, fragment has activity; ×, no activity; △, weak activity N.D.= not done.

Figure 2. R10-C is necessary and sufficient for import of β-catenin. Upper panels; 1mg/ml of recombinant full-length GFP-β-catenin (a), GST-R10-C-GFP (b) or FLAG-N-R9-His_6 (c) were injected into cytoplasm of Hela cells. After incubation for 30 min at 37℃, the cells were fixed with 3.7% formaldehyde in PBS. Lower panels; digitonin-permeabilized Hela cells were incubated with 1 μM of GFP-β-catenin (d), GST-R10-C-GFP (e) or FLAG-N-R9-His_6 (f). After incubation for 15 min at 30℃, the cells were fixed with 3.7% formaldehyde in transport buffer. GFP fusion proteins (a, b, d, e) were directly examined. FLAG fused protein (c, f) was visualized by indirect immunofluorescence with anti-FLAG mouse mAb (c) or anti-β-catenin β-cat-7D11 mouse mAb (f), and Cy3-labeled anti-mouse goat IgG.

Figure 3. C-terminal portion of β-catenin plays an essential role in its nuclear migration. (A) 1mg/ml of recombinant full-length GFP-β-catenin, GST-R10-C-GFP, GST-R11-C-GFP, GST-C-GFP, GST-R10-12-GFP or GST-R9-12-GFP were injected into cytoplasm of Hela cells with (right panels) or without (left panels) 0.5mg/ml WGA. After incubation for 30 min at 37℃, the cells were fixed with 3.7% formaldehyde in PBS. (B) Digitonin-permeabilized HeLa cells were incubated with 1 μM of GFP-β-catenin, GST-R10-C-GFP, GST-R11-C-GFP or GST-C-GFP without (left panels: Buffer), or with (middle panels: WGA(+)) 0.5mg/ml WGA or 3 μM impβ449 (right panels: Impβ449(+)). After incubation for 15min at 30℃, cells were fixed with 3.7% formaldehyde in transport buffer.

Figure 4. Full-length β-catenin or deletion mutants, which accumulate into nucleus, mutually competitively inhibit with each others. (A) Digitonin-permeabilized HeLa cells were incubated with 1 μM of GST-R10-C-GFP, GST-R11-C-GFP or GST-C-GFP in the absence (upper panels) or presence (lower panels) of 3 μM of non-tagged full-length β-catenin. After incubation for 15min at 30℃, cells were fixed with 3.7% formaldehyde in transport buffer. (B) Digitonin-permeabilized HeLa cells were incubated with 1 μM of GFP-β-catenin in the absence (a), or presence of 10 μM of competitor. non-tagged full-length β-catenin (b), GST-R10-12 (c), GST-R10-C (d), GST-R11-C (e), GST-C (f) or GST (g) as competitor respectively. After incubation for 15min at 30℃, cells were fixed with 3.7% formaldehyde in transport buffer.

Figure 5. Sequence determination of region required for β-catenin export. (A) 1mg/ml of recombinant full-length GFP-β-catenin, GST-R10-C-GFP, GST-R10-12-GFP, GST-R11-C-GFP or GST-C-GFP (left panels) were coinjected with Cy3- labeled BSA (injection marker, middle panels) in the one of nucleus of homokaryons. After incubation for 30 min at 37℃, the cells were fixed with 3.7% formaldehyde in PBS. (B) After digitonin-permeabilized HeLa cells were incubated with 1 μM of GFP-β-catenin, GST-R10-C-GFP, GST-R11-C-GFP or GST-C-GFP (first incubation) and were then washed with transport buffer, the cells were reincubated with transport buffer with 0.5mg/mlWGA (upper panels) or without (lower panels) for export reaction (second incubation). After 2nd incubation (20 min at 20℃), the cells were fixed with 3.7% formaldehyde in transport buffer.

Figure 6. Export of GFP-β-catenin is not inhibited by concentration of full-length β-catenin

which inhibits import. Competition experiments for GFP-β-catenin import and export were performed using digitonin-permeabilized cells. In import assay, digitonin-permeabilized HeLa cells were incubated with 1 μM of GFP-β-catenin in the absence, or presence of full-length β-catenin (3 μM) as competitor. In export assay, after import reaction with 1 μM of GFP-β-catenin, the cells were reincubated with transport buffer in the absence, or presence of full-length β-catenin (10 μM) as competitor. The graphs show change of GFP intensity in nucleus by concentration of full-length β-catenin in vitro import and export assay. GFP intensity in nucleus in the absence of full-length β-catenin in import, and in the presence of 10 μM full-length β-catenin in export was set at 100%. Error bars show consequence of four independent experiments.

Figure 7. The difference of inhibitory effect against import and export of β-catenin by different transport receptors Competition experiments for β-catenin import and export were performed using digitonin-permeabilized cells. (A) In import assay (a-d), digitonin-permeabilized HeLa cells were incubated with 0.5 μM of GFP-β-catenin in the absence (a), or presence of importin β (1 μM, b) or CAS (10 μM, c), and with 1 μM of GFP-β-catenin in the presence of importin α (10 μM, d) as competitor. In export assay (e-g), after import reaction with 0.5 μM of GFP-β-catenin, the cells were reincubated with transport buffer in the absence (e), or presence of importin β (1 μM, f) or CAS (10 μM, g). In export assay (h), after import reaction with 1 μM of GFP-β-catenin, the cells were reincubated with transport buffer in the in the presence of importin α (10 μM, h). (B-D) show change of GFP intensity in nucleus by concentration of competitor in vitro import and export assay. Competitor was importin β (B), CAS (C) and importin α (D). Intensity in nucleus in the absence of competitor in import, and in the presence of 10 μM competitor in export was set at 100%. Error bars show consequence of

four independent experiments.

Figure 8. The inhibition of nuclear import and export of β-catenin by importin β in the presence of GTPase Ran and ATP The import assay; Digitonin-permeabilized cells was performed in the absence or presence of GTPase Ran and ATP. The cells were incubated with 1 μM of β-catenin with (lower panels) or without 3 μM of importin β (upper panels). The export assay; Digitonin-permeabilized cells were performed in the absence or presence of GTPase Ran and ATP-regenerating systems in second incubation. After import reaction with 1 μM of GFP-β-catenin, and the cells were then washed with transport buffer, the cells were reincubated with transport buffer in the absence (upper panels) or presence of 3 μM of importin β (lower panels).

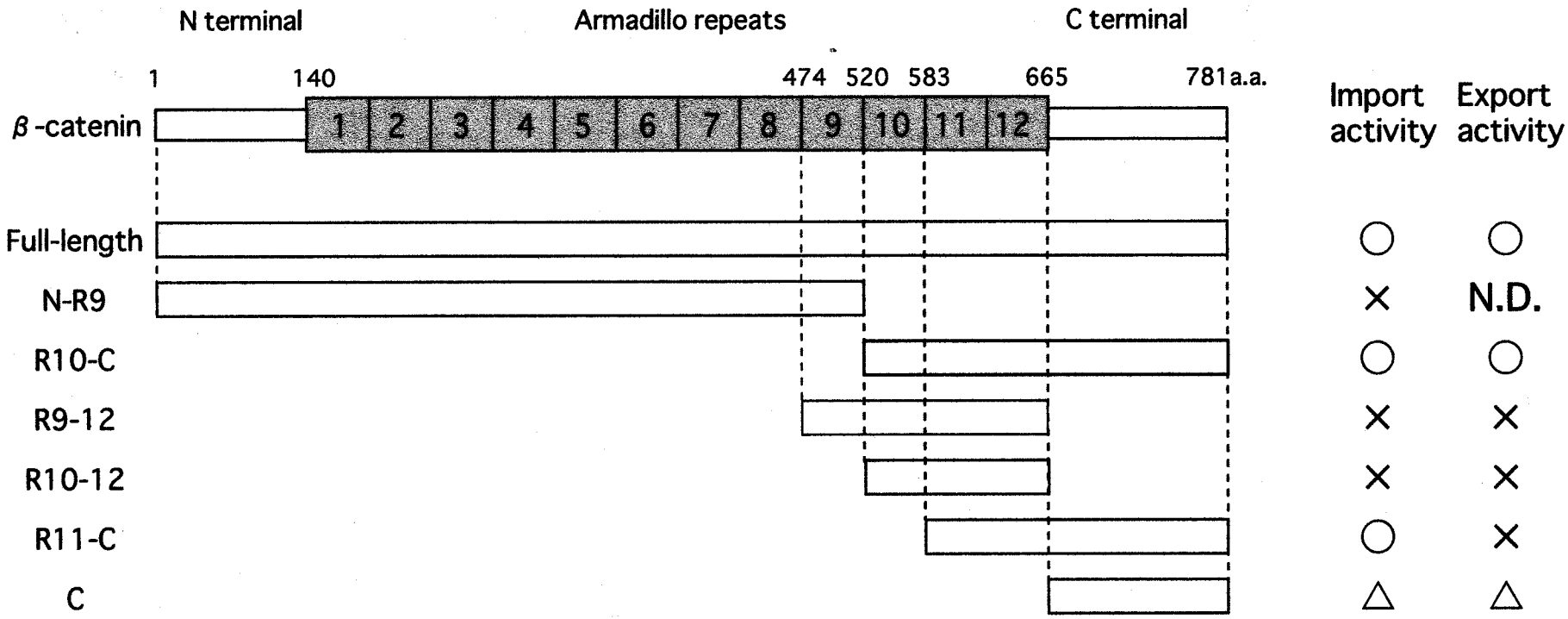

N terminal
Armadillo repeats
C terminal
1
140
474
520
583
665
781a.a.
β-catenin
1
2
3
4
5
6
7
8
9
10
11
12
Import activity
Export activity
Full-length
○
○
N-R9
×
N.D.
R10-C
○
○
R9-12
×
×
R10-12
×
×
R11-C
○
×
C
△
△

Figure.1

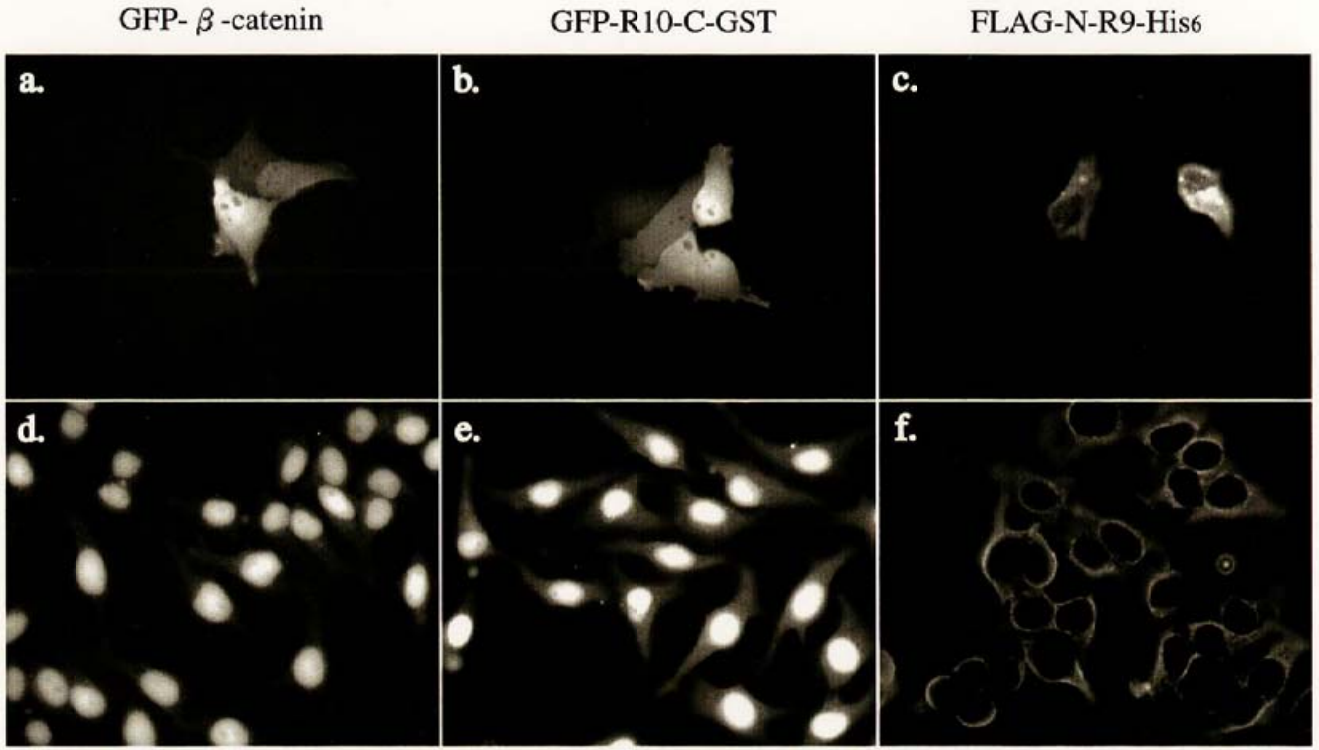


Figure 2

A

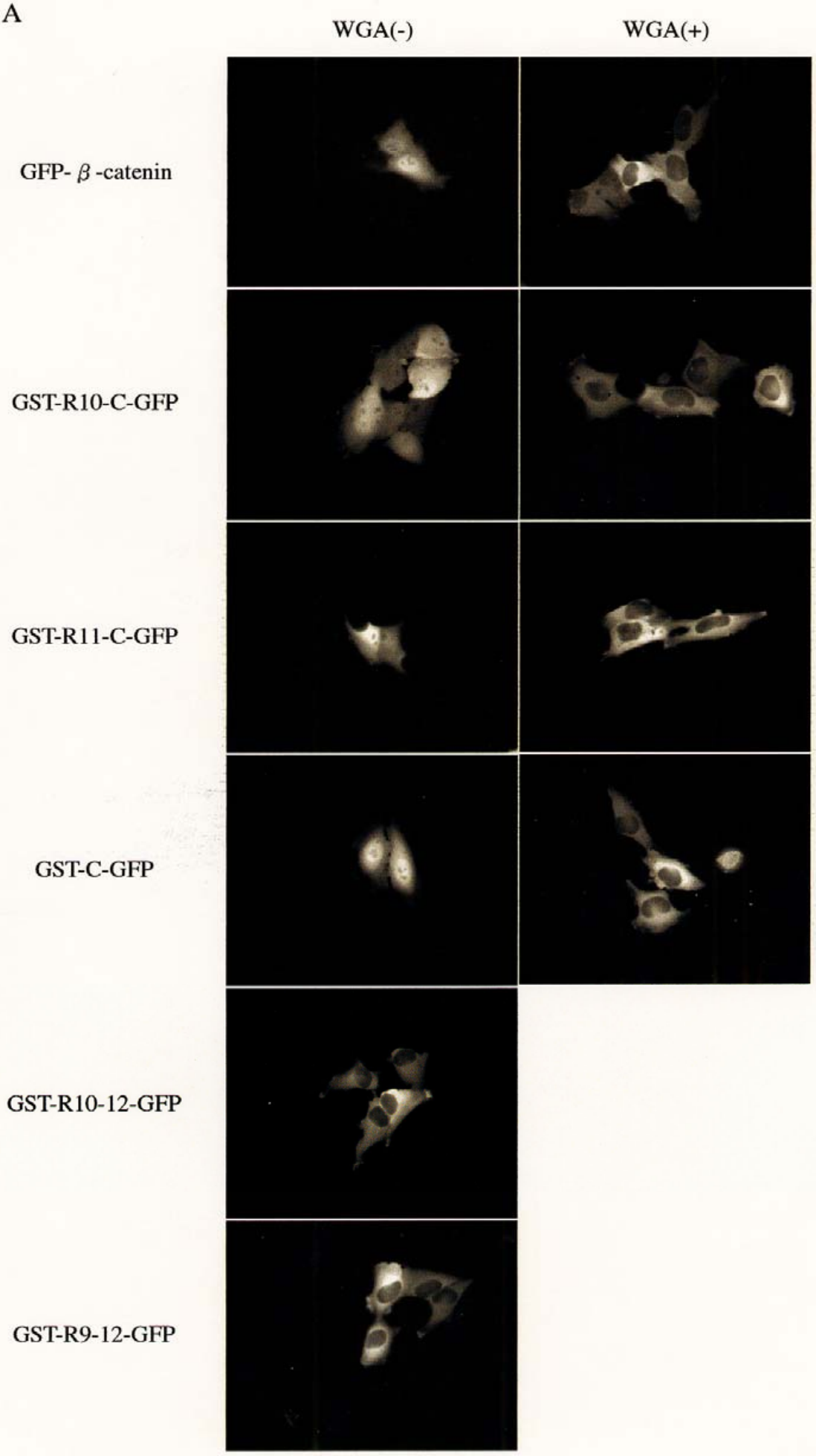
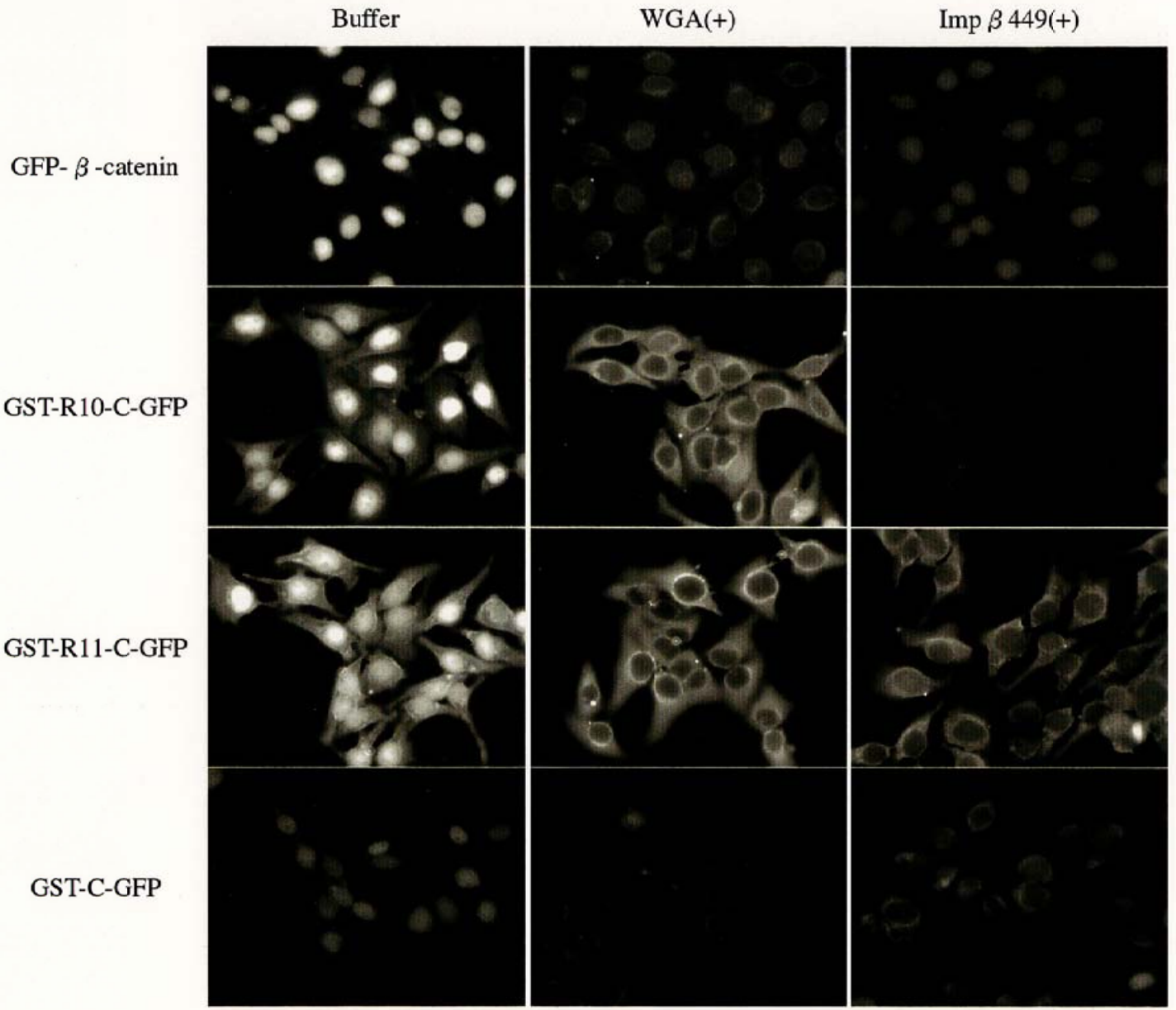

WGA(-)
WGA(+)
GFP- β -catenin
GST-R10-C-GFP
GST-R11-C-GFP
GST-C-GFP
GST-R10-12-GFP
GST-R9-12-GFP

Figure.3

B


Buffer
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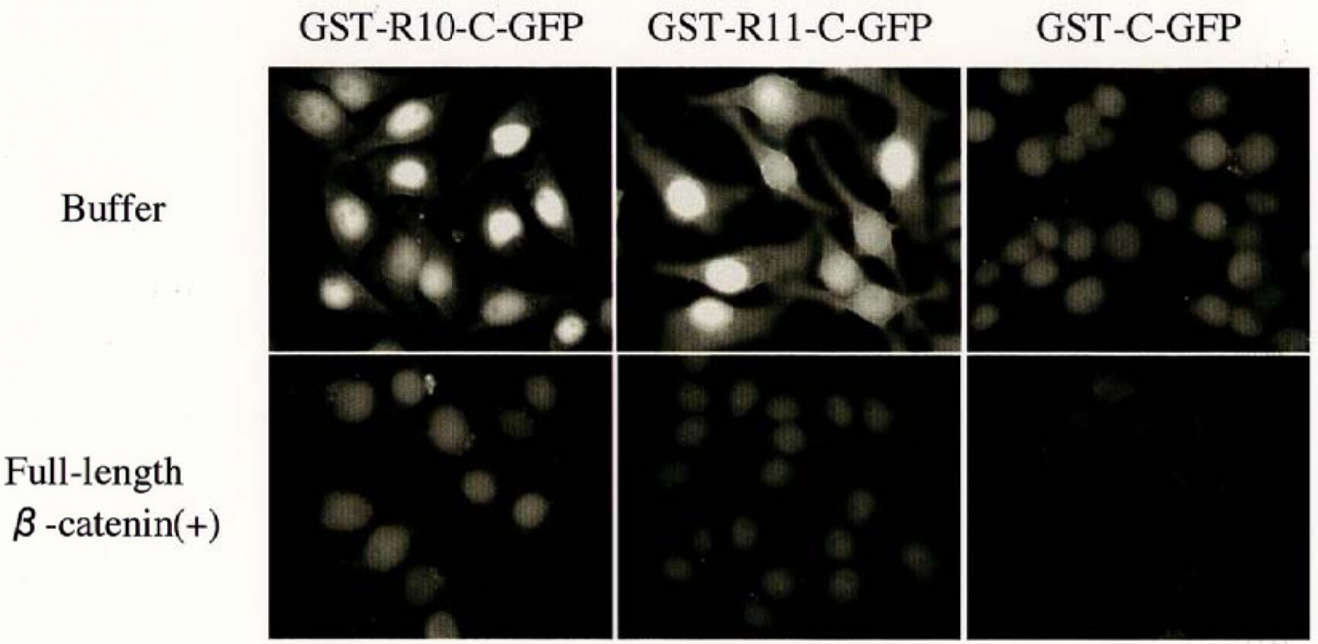
Imp β 449(+)
GFP- β -catenin
GST-R10-C-GFP
GST-R11-C-GFP
GST-C-GFP

Figure.3

A


GST-R10-C-GFP
GST-R11-C-GFP
GST-C-GFP
Buffer
Full-length
β -catenin(+)

B

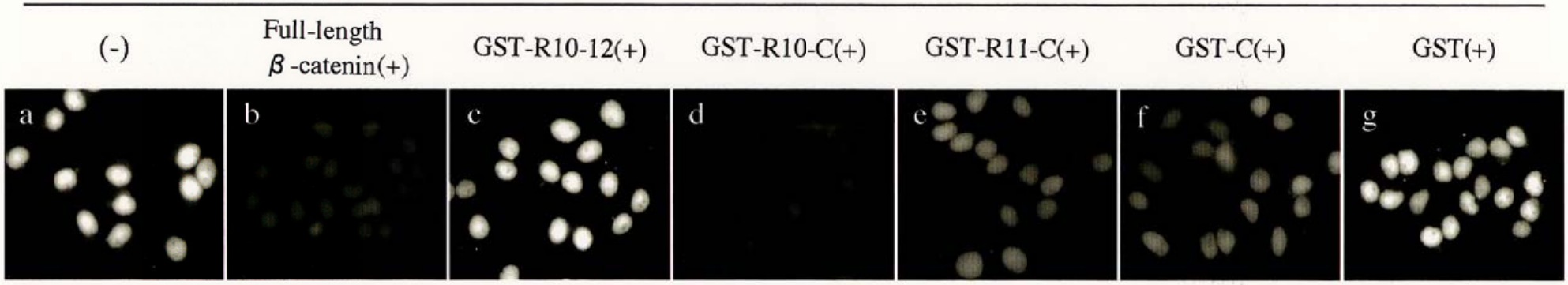

Competitor
(-)
Full-length
β -catenin(+)
GST-R10-12(+)
GST-R10-C(+)
GST-R11-C(+)
GST-C(+)
GST(+)
a
b
c
d
e
f
g

Figure 4

A

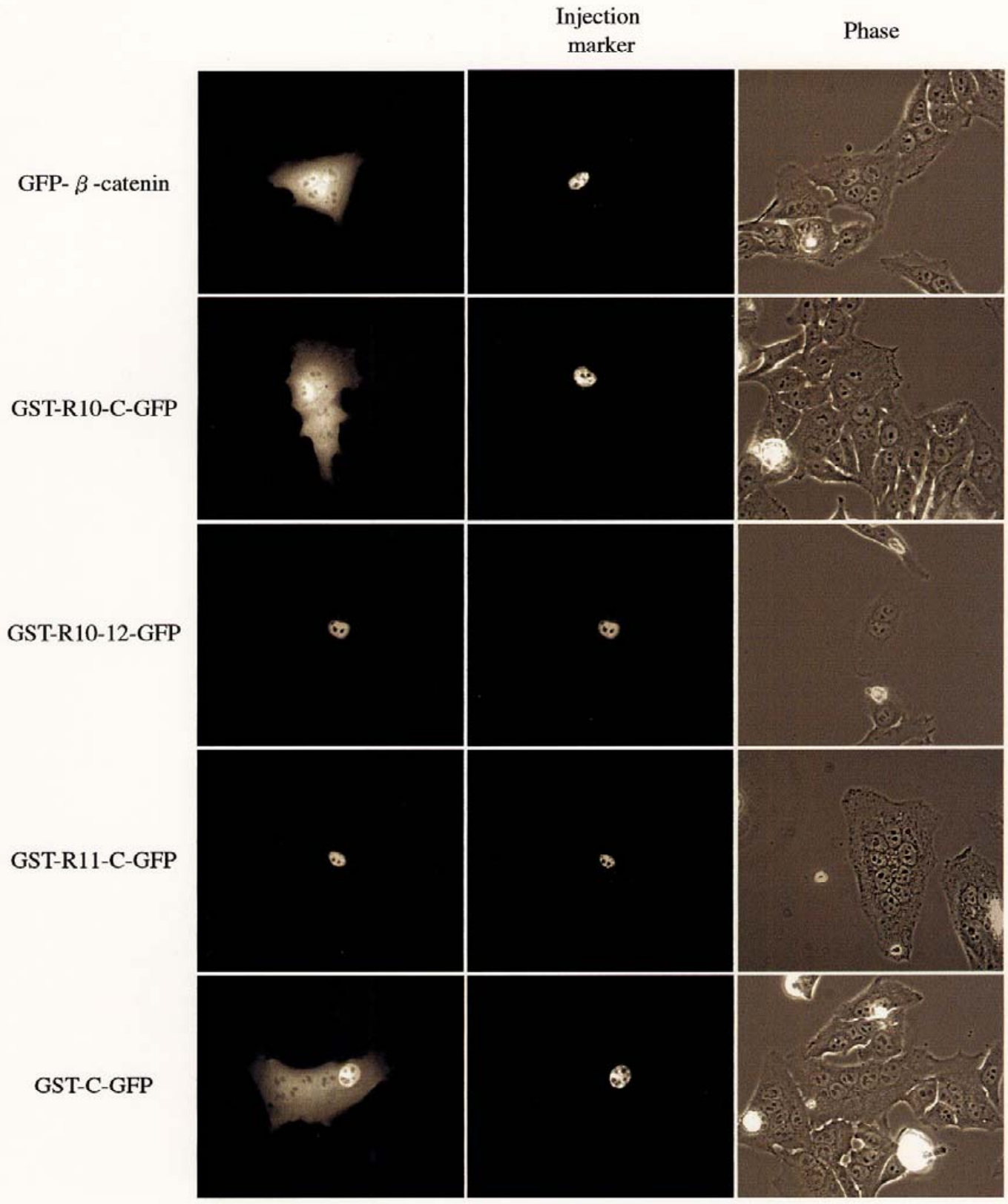

Injection marker
Phase
GFP- β -catenin
GST-R10-C-GFP
GST-R10-12-GFP
GST-R11-C-GFP
GST-C-GFP

Figure.5

B

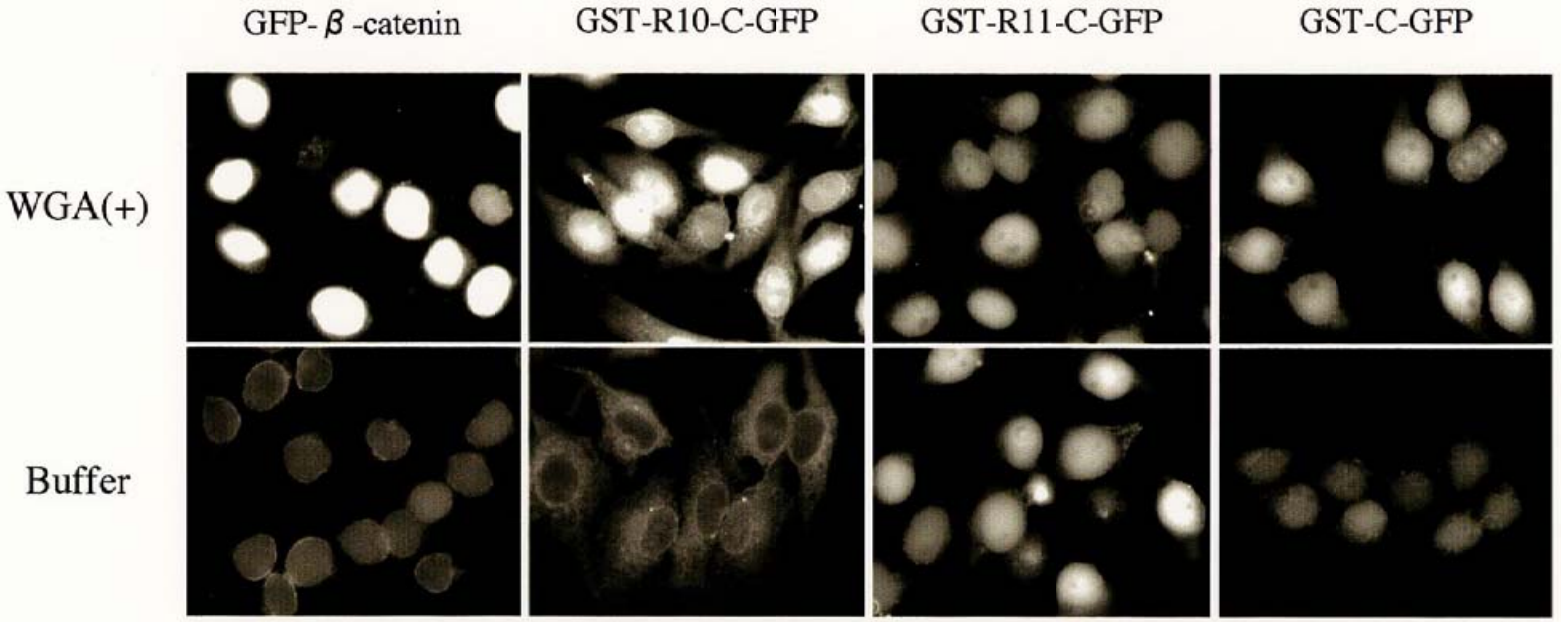


Figure 5

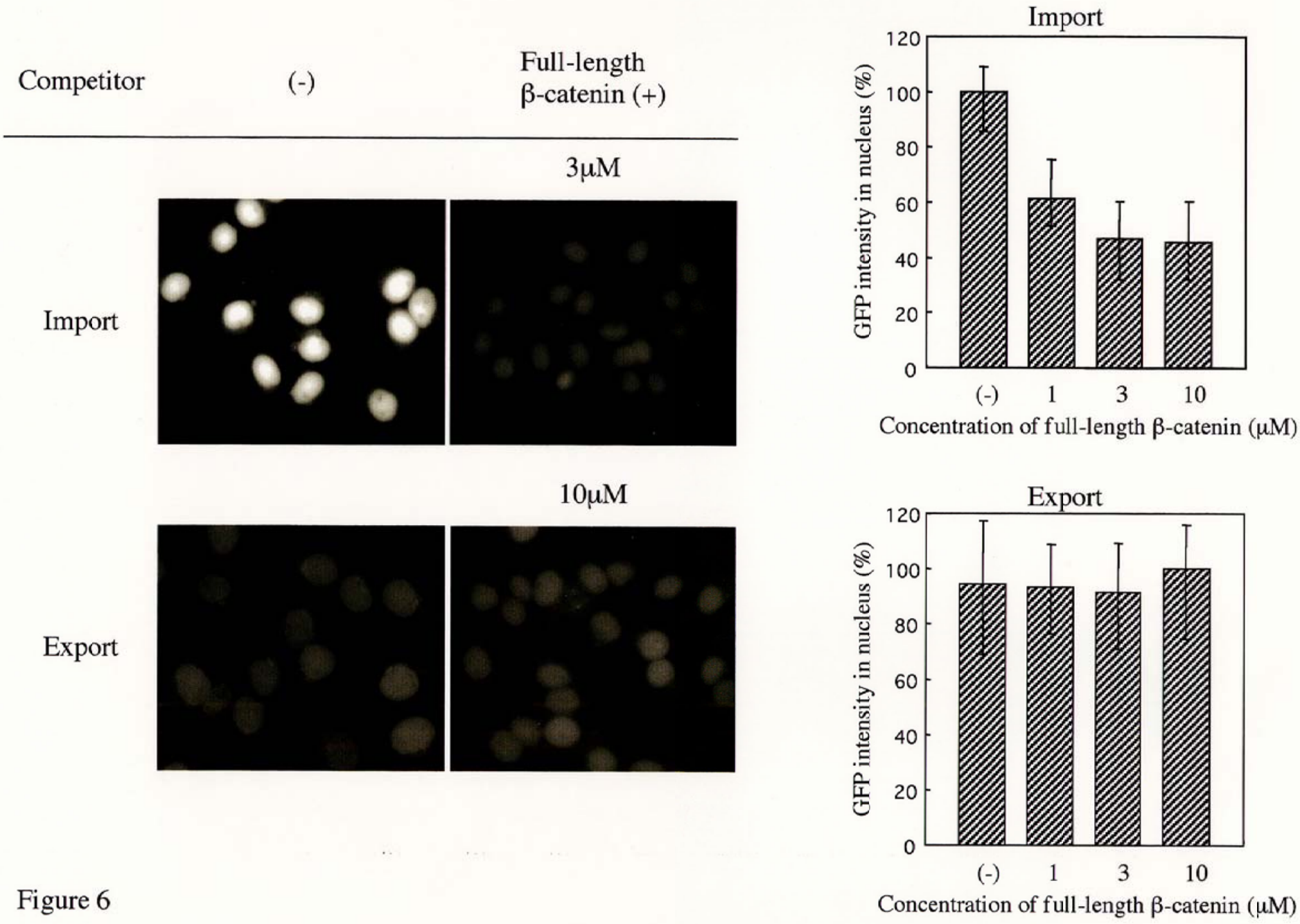
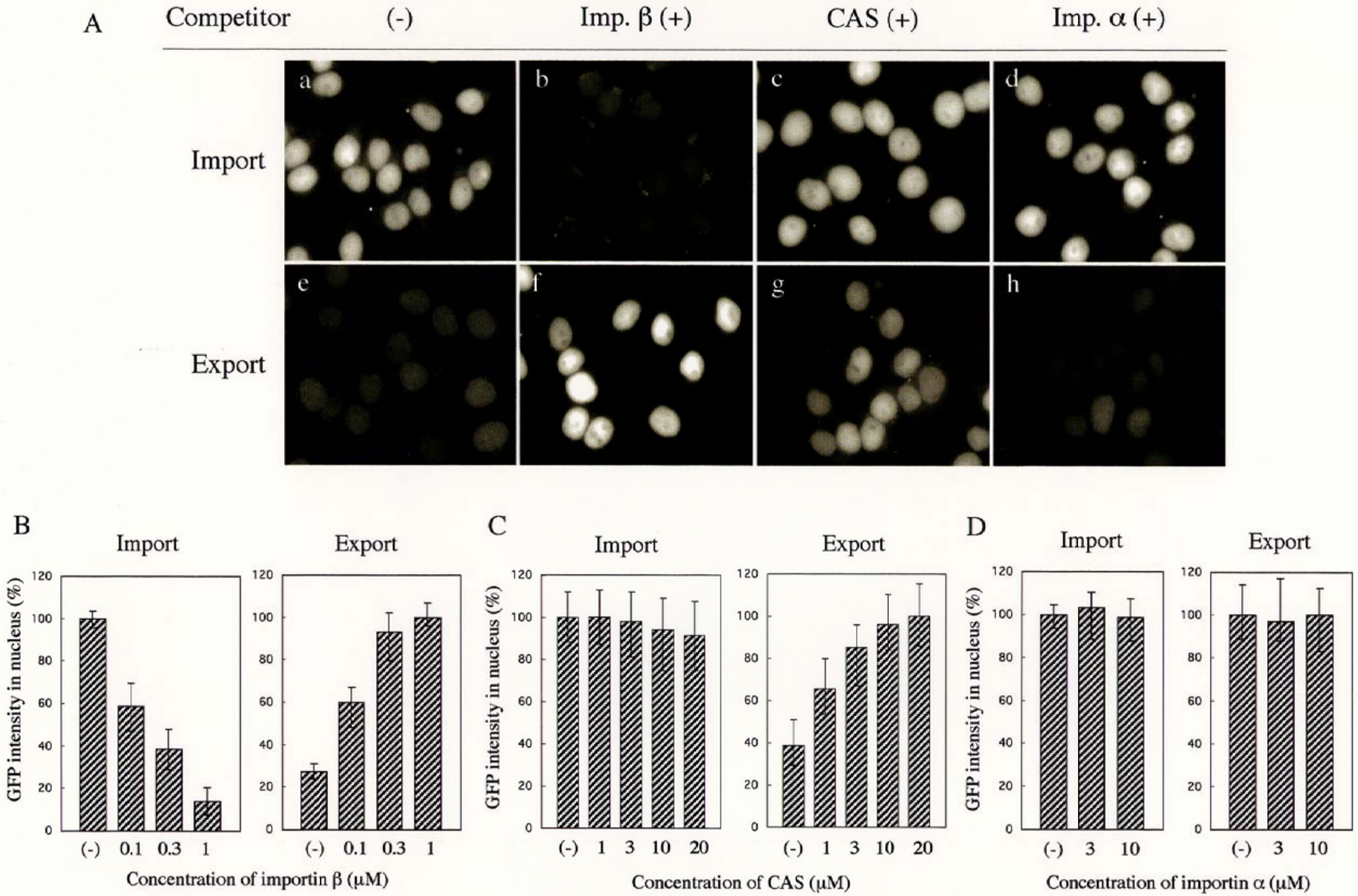

Competitor
(-)
Full-length β-catenin (+)
3μM
Import
10μM
Export
Import
GFP intensity in nucleus (%)
120
100
80
60
40
20
0
(-)
1
3
10
Concentration of full-length β-catenin (μM)
Export
GFP intensity in nucleus (%)
120
100
80
60
40
20
0
(-)
1
3
10
Concentration of full-length β-catenin (μM)

Figure 6


A
Competitor
(-)
Imp. β (+)
CAS (+)
Imp. α (+)
Import
Export
a
b
c
d
e
f
g
h
B
Import
Export
GFP intensity in nucleus (%)
0
20
40
60
80
100
120
(-) 0.1 0.3 1
Concentration of importin β (μM)
C
Import
Export
GFP intensity in nucleus (%)
(-) 1 3 10 20
Concentration of CAS (μM)
D
Import
Export
GFP intensity in nucleus (%)
(-) 3 10
Concentration of importin α (μM)

Fugure 7

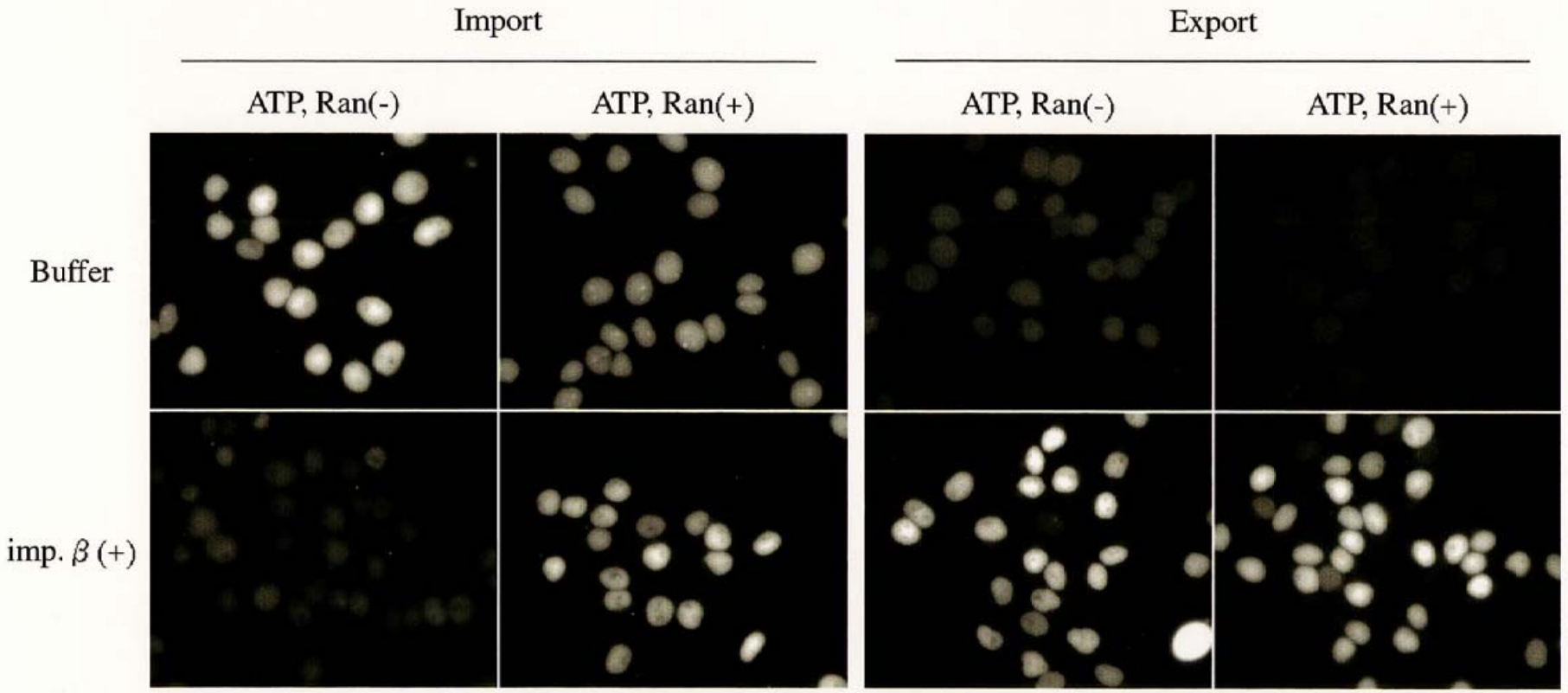


Figure 8