Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein

Fang Zhang, Raymond L. White, and Kristi L. Neufeld*

Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Building 555, Room 5263, Salt Lake City, UT 84112

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Mutation of the adenomatous polyposis coli (*APC*) gene is an early step in the development of colorectal carcinomas. APC protein is located in both the cytoplasm and the nucleus. The objective of this study was to define the nuclear localization signals (NLSs) in APC protein. APC contains two potential NLSs comprising amino acids 1767–1772 ($NLS1_{APC}$) and 2048–2053 ($NLS2_{APC}$). Both APC NLSs are well conserved among human, mouse, rat, and fly. $NLS1_{APC}$ and $NLS2_{APC}$ each were sufficient to target the cytoplasmic protein β-galactosidase to the nucleus. Mutational analysis of APC demonstrated that both NLSs were necessary for optimal nuclear import of full-length APC protein. Alignment of $NLS2_{APC}$ with the simian virus 40 large T antigen NLS ($NLS_{SV40\ T\text{-}ag}$) revealed sequence similarity extending to adjacent phosphorylation sites. Changing a serine residue (Ser^{2054}) to aspartic acid mutated the potential protein kinase A site adjacent to $NLS2_{APC}$, resulting in both inhibition of the $NLS2_{APC}$-mediated nuclear import of a chimeric β-galactosidase fusion protein and a reduction of full-length APC nuclear localization. Our data provide evidence that control of APC's nuclear import through phosphorylation is a potential mechanism for regulating APC's nuclear activity.

Adenomatous polyposis coli (APC) plays an important role in suppressing tumorigenesis in colorectal epithelial cells, and mutation of the *APC* gene is an early step in the initiation of colon cancer (1–5). One function of APC is to regulate cytoplasmic β-catenin, a key signal transducer of the Wnt signaling pathway. APC promotes the degradation of β-catenin by forming a β-catenin-destabilizing complex, together with axin and glycogen synthase kinase 3β (6–10). Colonic epithelial cells that express only a truncated form of APC, lacking the central β-catenin interaction and down-regulation domain as well as axin binding sites, fail to properly regulate intracellular β-catenin levels (6, 7, 11). The elevated level of cytoplasmic β-catenin allows nuclear translocation of β-catenin, where it, along with T cell factor/lymphoid enhancer factor, can activate transcription (12).

Previous studies have shown that wild-type, full-length APC protein is located in the cytoplasm of mammalian epithelial cells (13, 14). APC also was observed in the nucleus by immunofluorescence microscopy and biochemical fractionation in normal human epithelial cells, murine intestinal cells, and *Xenopus* A6 cells (15–18). In contrast, truncated APC protein resulting from a disease-causing mutation in *APC* did not localize to the nucleus (15). Recently, we have shown that APC contains two functional nuclear export signals that can facilitate movement from the nucleus to the cytoplasm (19). APC is thus a nucleocytoplasmic shuttling protein. This shuttling may play an important role in APC's tumor suppressor function, and understanding the mechanism and regulation of APC nuclear import may provide additional clues to the function and regulation of nuclear APC.

Nuclear translocation occurs through the nuclear pore complex (reviewed in ref. 20). Small proteins (<45 kDa) can diffuse into and out of the nucleus freely, whereas nuclear translocation of larger proteins usually requires the presence of a nuclear localization signal (NLS). Two major types of NLSs have been identified. The classical monopartite NLS is composed of a single cluster of basic amino acids, as exemplified by the simian virus 40 T-antigen (SV40 T-ag) NLS. The bipartite NLS is composed of two basic amino acids, a spacer region of 10–12 amino acids, and a basic cluster in which three of five amino acids are basic. Importin α recognizes both monopartite and bipartite NLSs (21), which then associate with importin β. The importins carry the cargo (NLS-containing protein) to the nuclear pore and translocate through the pore via an energy-dependent process. Recent reports suggest that NLS-mediated nuclear import may be regulated by phosphorylation. SV40 T-ag protein, for example, contains a CcN motif consisting of a casein kinase 2 (CK2) site, a cyclin-dependent kinase 2 (cdc2/cdk2) site, and a classical monopartite NLS, all involved in the phosphorylation-dependent regulation of its nuclear translocation (22–24). Phosphorylation at the CK2 site regulates the import rate of SV40 T-ag nuclear translocation, whereas phosphorylation at the cdc2/cdk2 site regulates maximal nuclear accumulation. The CcN motif is conserved in several nuclear proteins, including p53, c-Myc, cABL IV, and A-MYB (20).

In the present study, two potential classical monopartite NLSs ($NLS1_{APC}$ and $NLS2_{APC}$) were identified in APC. Either $NLS1_{APC}$ or $NLS2_{APC}$ was sufficient to target the cytoplasmic protein β-galactosidase (βgal) to the nucleus. Yet, both NLSs were necessary for optimal nuclear import of full-length APC. Alignment of $NLS2_{APC}$ with $NLS_{SV40\ T\text{-}ag}$ revealed extended similarity that included the NLS as well as adjacent phosphorylation sites. A mutagenesis study showed that phosphorylation of Ser^{2054} at the putative protein kinase A (PKA) site adjacent to $NLS2_{APC}$ negatively influenced the $NLS2_{APC}$-mediated nuclear import of a chimeric βGal fusion protein as well as the nuclear import of full-length APC.

Materials and Methods

Cell Culture. Cos7, 293, and L cells were grown in DMEM supplemented with 10% FBS.

Expression Vector Construction. Mutations were generated in the $NLS1_{APC}$ and/or $NLS2_{APC}$ of a Flag epitope-tagged APC expression construct (19) by using a PCR mutagenesis strategy (25). Specifically, APCmNLS1 contains substitutions of alanine for the four lysine residues ($Lys^{1768–1771}$) in $NLS1_{APC}$; APCmNLS2 contains substitutions of alanine for only the first two lysine residues ($Lys^{2049–2050}$) in $NLS2_{APC}$. PKA site mutants were generated by using a similar strategy. $APCNLS2mPKA_{S/A}$ con-

Abbreviations: APC, adenomatous polyposis coli; NES, nuclear export signal; NLS, nuclear localization signal; SV40 T-ag, simian virus 40 T antigen; βGal, β-galactosidase, PKA, protein kinase A; CK2, casein kinase II; cdc2/cdk2, cyclin-dependent kinase 2.

*To whom reprint requests should be addressed. E-mail: kristi.neufeld@hci.utah.edu.

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tains a substitution of alanine for serine (Ser^{2054}) at the putative PKA site adjacent to NLS2; and APCNLS2mPKA$_{S/D}$ contains a substitution of aspartic acid for the same serine (Ser^{2054}). Plasmid APC(mNES1,2) (19), which has mutant NES1$_{APC}$ and NES2$_{APC}$, was used as the backbone (APCmNES) to generate another series of NLS mutants and PKA site mutants. Protein products from the corresponding plasmids are referred to as APCmNESmNLS1, APCmNESmNLS2, APCmNESNLS1,2, APCmNESNLS2mPKA$_{S/A}$, and APCmNESmNLS2mPKA$_{S/D}$.

A Kozak sequence (GCC GCC ACC) and a start codon were inserted near the 5′ end of the multiple cloning site region in pCMV-βFUSa (26). Synthetic oligonucleotides coding for NLS1$_{APC}$ or NLS2$_{APC}$ were inserted into the pCMV-βFUSa plasmid immediately after the kozak sequence. All βGal expression constructs were generated by using the same strategy. The βGal-NLS1$_{APC}$, βGal-NLS2$_{APC}$, and βGal-NLS$_{SV40\ T\text{-}ag}$ expression constructs encode residues QLDGKKKKPTSPVKPIPQ (amino acids 1764–1781), SSLSIDSEDDLLQECISSAMPKKKKPSRLKGD (amino acids 2028–2058), and LFCSEEMPSSDDEATADSQHSTPPKKKRKVEDP (amino acids 103–135), respectively. βGal-(NLS2$_{APC}$)$_2$ has two copies of NLS2$_{APC}$ fused with βGal. βGal-NLS1,2$_{APC}$ includes amino acids 1064–2060 of APC, which includes both NLS1$_{APC}$ and NLS2$_{APC}$ fused to the N terminus of the *lacZ* gene.

Transfection. Cos7 and L cells plated on glass chamber slides were transfected by using Fugene 6 (Boehringer Mannheim) and Superfect (Qiagen, Chatsworth, CA), respectively, following the manufacturer's instructions.

Cell Fractionation and Immunoblotting. 293 cells, collected 36 h after transfection with Fugene 6, were rinsed three times with cold PBS and scraped into hypotonic buffer (10 mM Tris·HCl/0.2 mM $MgCl_2$). After a 5-min incubation on ice, cells were disrupted by passage through a 200-μl pipette tip until they were determined to be >99% lysed by trypan blue exclusion. Nuclei were pelleted by centrifugation at 1,000 × *g* for 10 min at 4°C. The supernatant fraction (cytosol) was collected, and the nuclear pellet was washed two times with cold PBS, resuspended in PBS with DNase I (200 units) and $MgCl_2$ (5 mM), and incubated on ice for 1 h. Nuclei then were incubated with lysis buffer (20 mM Tris·HCl/70 mM NaCl/1 mM EDTA/10% glycerol/1% TX-100/0.5% NP-40) on ice for 30 min. Proteinase inhibitors (Complete Mini, EDTA-free, Boehringer Mannheim) and phosphatase inhibitors (1 mM Na_3VO_4 and 50 mM NaF) were present throughout the fractionation. Proteins were separated on 4–12% gradient SDS/polyacrylamide gels (NOVEX, San Diego), transferred to Hybond nitrocellulose membranes (Amersham Pharmacia), and probed as described (15). βGal fusion proteins were detected with an anti-βGal antibody (1:1,000).

Immunofluorescence. Immunostaining was performed as described (15) by using anti-Flag (Eastman Kodak) 1:32,000 or anti-βGal (Promega) 1:1,000, and goat anti-mouse IgG-FITC (Southern Biotechnology Associates) 1:200. At least 100 transfected cells for each condition were scored for localization of flag-APC, the various flag-APC mutants, or the βGal fusion proteins. Results are presented as mean ± SD from at least three independent experiments.

Results

APC Possesses Two Potential Monopartite NLSs. To identify potential NLSs in APC, the complete amino acid sequence of APC was examined by using the PSORT computer program (27, 28). Two basic amino acid-rich, potential NLS regions were identified: amino acids 1767–1772 (NLS1$_{APC}$: GKKKKP) and amino acids 2048–2053 (NLS2$_{APC}$: PKKKKP) (Fig. 1*A*). NLS1$_{APC}$ and NLS2$_{APC}$ resemble classical monopartite NLSs, show sequence similarity to NLS$_{SV40\ T\text{-}ag}$ (Fig. 1*A*), and are highly conserved among human, mouse, rat, and fly APC (Fig. 1*B*).

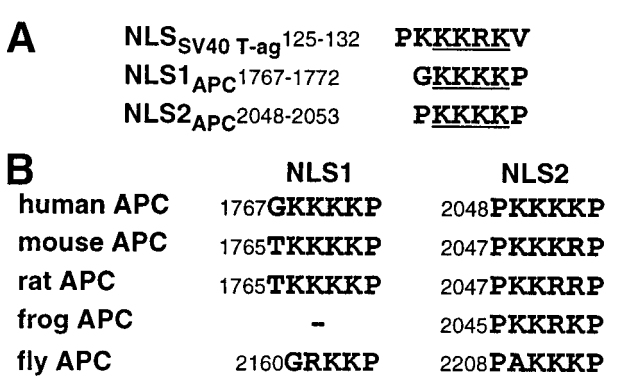

Fig. 1. APC possesses two potential monopartite NLSs. (*A*) Alignment of NLS1$_{APC}$ and NLS2$_{APC}$ with NLS$_{SV40\ T\text{-}ag}$ shows that both are classical monopartite NLSs. (*B*) Comparison of NLS$_{APC}$ in human, mouse, rat, frog, and fly. Both NLS1$_{APC}$ and NLS2$_{APC}$ are highly conserved among the different species.

Either NLS1$_{APC}$ or NLS2$_{APC}$ Is Sufficient to Translocate Cytoplasmic βGal Protein into the Nucleus. To determine whether NLS1$_{APC}$ or NLS2$_{APC}$ was sufficient to target a heterologous cytoplasmic protein for nuclear import, an expression construct for NLS1$_{APC}$ or NLS2$_{APC}$ fused with the N terminus of the cytoplasmic protein βGal (βGal-NLS1$_{APC}$ or βGal-NLS2$_{APC}$, respectively) was transiently transfected into mouse L cells. Cells transfected with the constructs had similar levels of fusion protein expression as determined by Western blot using a monoclonal anti-βGal antibody (data not shown). Subcellular localization of the chimeric protein was visualized with immunofluorescence microscopy. Wild-type βGal protein was seen predominantly in the cytoplasm (Fig. 2, βGal). Chimeric βGal fused with the NLS$_{SV40\ T\text{-}ag}$, on the other hand, was seen predominantly in the nucleus (Fig. 2, βGal-NLS$_{SV40\ T\text{-}ag}$). Both βGal-NLS1$_{APC}$ and βGal-NLS2$_{APC}$ showed a greatly increased nuclear localization compared with the control, distributing evenly between cytoplasm and nucleus (Fig. 2, βGal-NLS1$_{APC}$ and βGal-NLS2$_{APC}$). These results indicate that NLS1$_{APC}$ and NLS2$_{APC}$ are each sufficient to translocate the cytoplasmic protein βGal into the nucleus. To confirm specificity of both NLS$_{APC}$, mutations were introduced, changing the four lysines ($Lys^{1768–1771}$) of NLS1$_{APC}$ or the first two lysines ($Lys^{2049–2050}$) of NLS2$_{APC}$ to alanine. As anticipated, the mutants showed predominantly cytoplasmic staining (Fig. 2, βGal-mNLS1$_{APC}$ and βGal-mNLS2$_{APC}$), demonstrating that the Lys→$Ala^{1768–1771}$ and Lys→$Ala^{2049–2050}$ substitutions each abolished NLSs activity. This result indicated that either NLS1$_{APC}$ or NLS2$_{APC}$ was sufficient to target a heterologous protein for nuclear import. In addition, fusing both NLS1$_{APC}$ and NLS2$_{APC}$ to βGal increased the incidence of cells displaying nuclear βGal and the relative level of nuclear βGal (Fig. 2, βGal-NLS1,2$_{APC}$). This double NLS construct showed that NLS1$_{APC}$ and NLS2$_{APC}$ together had an additive effect on mediating nuclear import. Fusing a second copy of NLS2$_{APC}$ to βGal-NLS2$_{APC}$ showed a similar increase in nuclear translocation [Fig. 2, βGal-(NLS2$_{APC}$)$_2$], confirming that multiple NLS$_{APC}$ increased nuclear import.

NLS1$_{APC}$ and NLS2$_{APC}$ Are Necessary for Optimal Nuclear Import of APC Protein. To determine whether NLS1$_{APC}$ and/or NLS2$_{APC}$ were necessary for the nuclear import of full-length APC, we constructed a series of APC expression plasmids containing mutations in the potential NLS elements of APC. These plasmids,

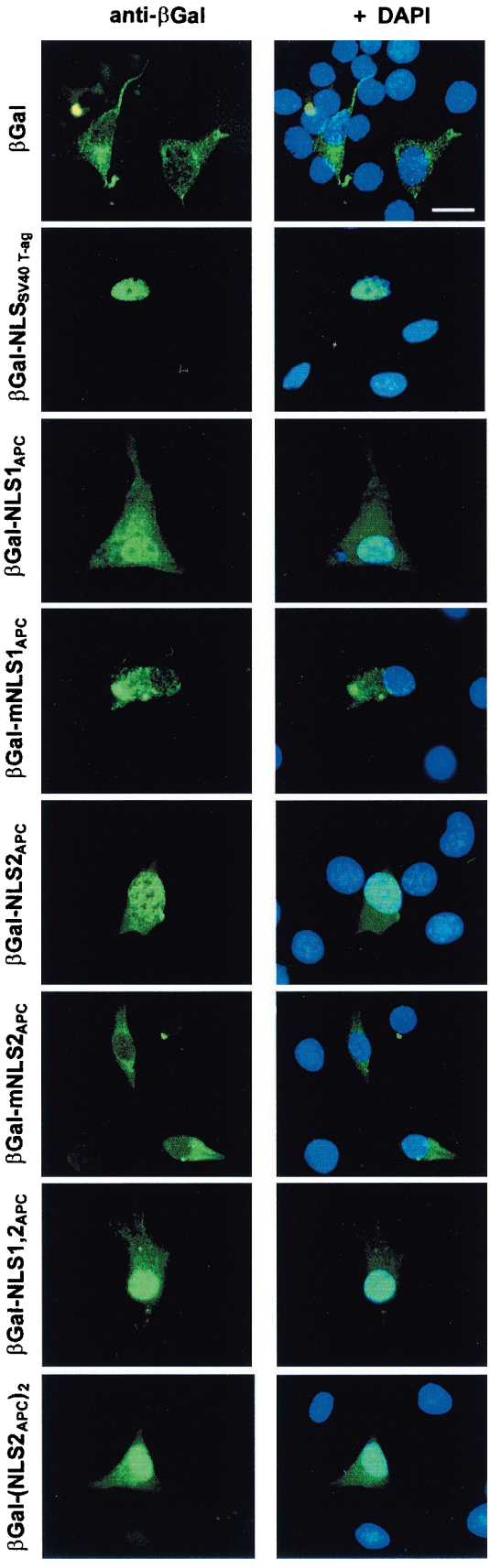

Fig. 2. Either NLS1$_{APC}$ or NLS2$_{APC}$ is sufficient to translocate the cytoplasmic protein βGal into the nucleus. βGal-fusion proteins were expressed in mouse L cells and detected by immunofluorescence microscopy. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Areas of overlap between the βGal fusion protein (green) and the nuclei (blue) appear an aqua color. (Bar = 10 μm.) For each construct, 100 cells were scored for βGal localization with conventional fluorescence microscopy. The results of four independent experiments are presented as the incidence of nuclear βGal staining ± SD as follows: βGal, 27(±6)%; βGal-NLS$_{SV40\ T\text{-}ag}$, 100(±0)%; βGal-NLS1$_{APC}$, 73(±11)%; βGal-mNLS1$_{APC}$, 30(±13)%; βGal-NLS2$_{APC}$, 69(±8)%; βGal-mNLS2$_{APC}$, 35(±4)%; βGal-NLS1,2$_{APC}$, 83(±6)%; and βGal-(NLS2$_{APC}$)$_2$, 91(±4)%.

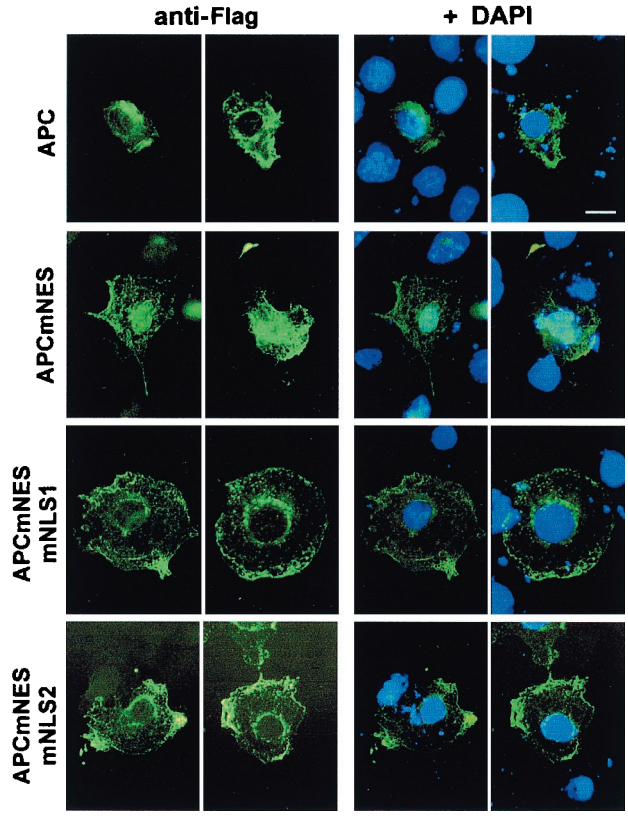

Fig. 3. Both NLS1$_{APC}$ and NLS2$_{APC}$ are necessary for optimal nuclear import of full-length APC. The various ectopic flag-tagged APC proteins were localized in Cos7 cells by immunofluorescence microscopy. (Bar = 20 μm.) Cells were scored as above with the results of three independent experiments presented as the incidence of nuclear APC staining ± SD. APC, 18(±1)%; APCmNES, 42(±7)%; APCmNESmNLS1, 17(±10)%; and APCmNESmNLS2, 14(±15)%.

which encode full-length APC with an N-terminal Flag epitope tag, were transfected into Cos7 cells and expressed protein localized by immmunofluorescence microscopy. As expected, tagged, wild-type APC protein was primarily cytoplasmic in the great majority of the transfected cells (Fig. 3, APC). Although a minority also showed some nuclear staining, it appeared that because of the low level of nuclear staining, it would be difficult to determine any reduction caused by mutation of the NLSs. However, as previously shown (19), mutation of APC's two nuclear export signals (NESs) greatly increases the incidence of nuclear staining (Fig. 3, APCmNES), providing a better opportunity to study nuclear import activity. Mutational analysis of NLS function was, therefore, carried out against a background of NES mutations.

Cells expressing APCmNES protein bearing additional mutations in NLS1 (APCmNESmNLS1) or NLS2 (APCmNES-

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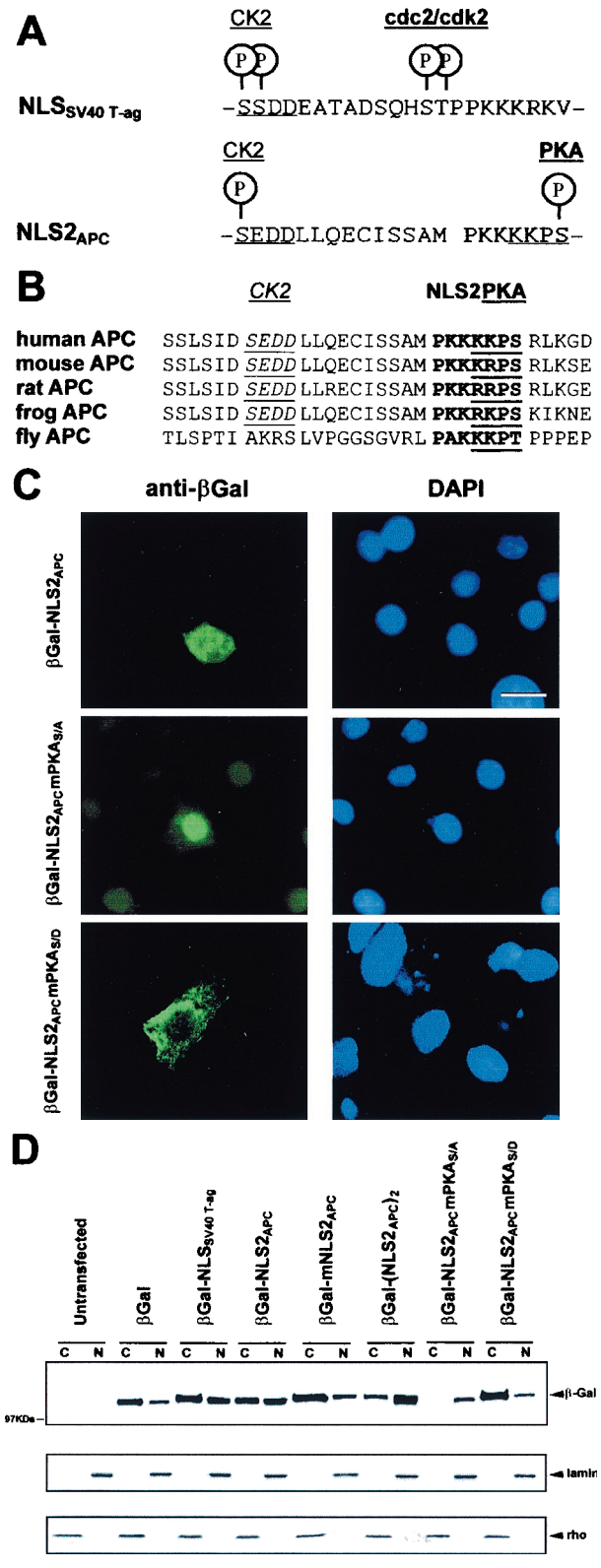

Fig. 4. NLS2$_{APC}$-mediated nuclear translocation of βGal chimera is regulated by phosphorylation at a PKA site. (*A*) Alignment of NLS2$_{APC}$ with NLS$_{SV40\ T-ag}$ reveals similarity of NLS sequence as well as the adjacent putative phosphorylation sites. (*B*) The NLS2$_{APC}$ region is highly conserved among different species. NLS2 sequences are bold; the CK2 and PKA sites are underlined. (*C*) βGal-NLS2$_{APC}$ localizes to both the cytoplasm and nucleus of L cells. βGal-NLS2$_{APC}$mPKA$_{S/A}$ is predominantly nuclear and βGal-NLS2$_{APC}$mPKA$_{S/D}$ predominantly cytoplasmic. (Bar = 10 μm.) Cells were scored as above, and the incidence of nuclear staining for each βGal fusion is as follows: βGal-NLS2$_{APC}$, 69(±8)%; βGal-NLS2$_{APC}$mPKA$_{S/A}$, 100(±0)%; and βGal-NLS2$_{APC}$mPKA$_{S/D}$, 29(±6)%. (*D*) Fractionation and Western immunoblot analysis of 293 cells transiently transfected with various constructs confirmed the subcellular localization of the chimeric proteins. Fractions are labeled as follows: C, cytosol; N, nuclear. The various cell fractions were characterized by striping and reprobing the blot for the marker proteins: lamin as a nuclear marker and rho as a cytoplasmic marker.

mNLS2) showed significantly reduced nuclear staining compared with cells expressing APCmNES (Fig. 3). The localization pattern of the NLS mutant APC proteins thus was altered from the characteristic nuclear and cytoplasmic distribution of the APCmNES protein to a predominantly cytoplasmic pattern. Similar results were observed with Cos7 cells transfected with the several APC NLS mutants introduced into a wild-type APC expression construct, although the differences were less dramatic (data not shown). Because mutation of either NLS$_{APC}$ resulted in a predominantly cytoplasmic localization of APCmNES, it appears that neither NLS1$_{APC}$ nor NLS2$_{APC}$ alone can effectively target APC to the nucleus. Rather, both NLS1$_{APC}$ and NLS2$_{APC}$ are necessary for the optimal nuclear import of full-length APC.

Potential CK2 and PKA Phosphorylation Sites Flanking NLS2$_{APC}$ Are Similar to the CcN Motif of SV40 T-ag and Are Highly Conserved in APC from Different Species. The similarity between NLS2$_{APC}$ and NLS$_{SV40\ T-ag}$ extends beyond the monopartite NLS, with alignment of NLS2$_{APC}$ with NLS$_{SV40\ T-ag}$ revealing similarity in their adjacent CK2 phosphorylation sites. Furthermore, both show potential phosphorylation sites immediately adjacent to their NLS sequences (NLS2$_{APC}$: PKA site and NLS$_{SV40\ T-ag}$: cdc2/cdk2 site, Fig. 4*A*). Like the NLS$_{APC}$ sequence, the CK2$_{APC}$ and PKA$_{APC}$ sites are both evolutionarily conserved among human, mouse, rat, and frog APC proteins (Fig. 4*B*), suggesting their possible functional significance. Thus, the extended similarity between the NLS2$_{APC}$ region and SV40 T-ag CcN motif suggests a possible conservation not only of NLS function, but also of phosphorylation-mediated regulation of nuclear import.

Substitution of a Negatively Charged Amino Acid for Ser^{2054} in the Potential PKA Site Is Sufficient to Block NLS2$_{APC}$-Mediated Nuclear Import. In SV40 T-ag, phosphorylation within the CcN motif by CK2 increases the nuclear import rate, whereas phosphorylation by cdc2/cdk2 decreases the maximal extent of nuclear accumulation. If NLS2$_{APC}$-mediated nuclear import is similarly inhibited by phosphorylation at the PKA$_{APC}$ site, mutation of this site would be expected to increase nuclear import activity. To test this idea, we created a βGal-NLS2$_{APC}$ fusion protein carrying a substitution of an alanine for the first serine (Ser^{2054}) immediately after NLS2$_{APC}$ (βGal-NLSZ$_{APC}$mPKA$_{S/A}$). In contrast to the nuclear and cytoplasmic distribution of the βGal-NLS2$_{APC}$ chimera, βGal-NLS2$_{APC}$mPKA$_{S/A}$ showed a predominantly nuclear staining pattern (Fig. 4*C*). Thus, the Ser^{2054} to Ala substitution significantly increased the nuclear targeting ability of NLS2$_{APC}$, indicating that Ser^{2054} may be functionally important for regulating NLS2$_{APC}$-mediated nuclear import. More specifically, it suggests that phosphorylation of Ser^{2054} may negatively regulate the NLS2$_{APC}$-mediated nuclear import, analogous to the phosphorylation of SV40 T-ag by cdc2/cdk2. To further test this hypothesis, we created a βGal-NLS2$_{APC}$ fusion protein carrying a substitution of an aspartic acid for the Ser^{2054} (βGal-NLSZ$_{APC}$mPKA$_{S/D}$), which might mimic the negative charge of phosphorylation at the PKA site. As predicted and in contrast to the result with the previous construct, the βGal-NLS2$_{APC}$mPKA$_{S/D}$ protein showed a predominantly cytoplasmic staining pattern in transfected cells (Fig. 4*C*). This altered subcellular localization indicates that a negative charge after the NLS2$_{APC}$ may be sufficient to block NLS2$_{APC}$-mediated nuclear

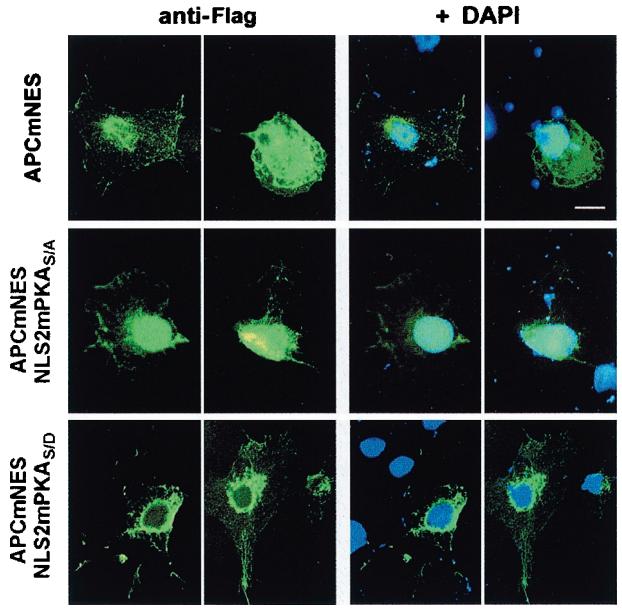

Fig. 5. Phosphorylation of Ser^{2054} within the PKA site adjacent to NLS2$_{APC}$ negatively regulates the nuclear import of full-length APC. Cos7 cells expressing APCmNES, APCmNESNLS2mPKA$_{S/A}$, or APCmNESNLS2mPKA$_{S/D}$ were analyzed by immunofluorescence microscopy. (Bar = 20 μm.) Cells were scored, and the incidence of nuclear staining for each construct is shown as follows: APCmNES, 42(±7)%; APCmNESNLS2mPKA$_{S/A}$, 42(±11)%; and APCmNESNLS2mPKA$_{S/D}$, 19(±14)%. DAPI, 4′,6-diamidino-2-phenylindole.

targeting of the βGal fusion protein and that phosphorylation at the PKA site adjacent to NLS2$_{APC}$ (Ser^{2054}) may negatively regulate NLS2$_{APC}$-mediated nuclear import.

Cell fractionation and Western immunoblot (Fig. 4*D*) confirmed the subcellular localizations indicated by immunofluorescence microscopy. Transfected 293 cells were separated into cytosolic and nuclear fractions. Because each transfection resulted in a slightly different level of βGal protein expression, we compared the relative cytosolic to nuclear ratios for each βGal chimera. βGal protein was observed predominantly in the cytosolic fraction. βGal-NLS$_{SV40\ T\text{-}ag}$ protein was observed more in the nuclear fraction. Addition of wild-type, but not mutant, NLS2$_{APC}$ to the N terminus of βGal resulted in an increased nuclear localization, with two copies of NLS2$_{APC}$ giving a more pronounced nuclear shift. A Ser^{2054} to Ala substitution at the potential PKA site significantly increased the nuclear localization, whereas Ser^{2054} to Asp substitution decreased the nuclear localization.

Ser^{2054} Regulates Nuclear Import of Full-Length APC. To determine whether Ser^{2054} is critical for modulating nuclear import of full-length APC, Ser^{2054} was changed to an alanine or aspartic acid residue in the APCmNES expression construct. Each construct was transfected into Cos7 cells, and the subcellular localization of the expressed protein was visualized by immunofluorescence microscopy. Although we observed some cells with predominantly nuclear APCmNES protein (Fig. 5 *Left*), the majority of cells showing nuclear staining displayed a more even distribution between the cytoplasm and the nucleus (Fig. 5 *Right*). However, APCmNESNLS2mPKA$_{S/A}$ protein was predominantly nuclear. In contrast, APCmNESNLS2mPKA$_{S/D}$ localized primarily to the cytoplasm (Fig. 5). These trends were reflected in the overall incidence of nuclear staining as well (see Fig. 5 legend). This result shows that Ser^{2054} can functionally regulate APC's nuclear translocation; a negative charge after NLS2$_{APC}$ inhibits nuclear import of full-length APC. To confirm this observation, the two PKA mutants were introduced into wild-type APC protein. Similarly, compared with the parental Flag-APC, APCNLS2mPKA$_{S/D}$ showed a decreased incidence of nuclear staining from 18% to 5%, whereas APCNLS2mPKA$_{S/A}$ did not show a significant change (22%) (data not shown). On an individual cell basis, both APCmNESNLS2mPKA$_{S/A}$ and APCNLS2mPKA$_{S/A}$ showed more nuclear staining than cytoplasmic, whereas APCmNESNLS2mPKA$_{S/D}$ and APCNLS2mPKA$_{S/D}$ showed predominantly cytoplasmic staining, with a strong perinuclear component (Fig. 5). Comparing the relative intracellular distribution of the three proteins together with quantitative population-based scoring of the incidence of nuclear staining demonstrated that Ser^{2054} can regulate the nuclear import of full-length APC; a negative charge at this site immediately adjacent to NLS2$_{APC}$ inhibits the nuclear translocation of APC protein.

Discussion

We have identified two functional NLSs in the central domain of APC protein. Both NLSs are necessary for the optimal nuclear import of full-length APC protein. These data are consistent with the previous discovery that APC protein can be located in the nucleus (15). We have previously shown that the truncated APC protein (amino acids 1–1638), which lacks both NLS1$_{APC}$ and NLS2$_{APC}$, can localize to the nucleus of mouse embryo fibroblasts (29). In a recent report, the truncated APC protein (amino acids 1–1309), although typically observed in the cytoplasm, was localized to the nucleus of cells treated with the nuclear export inhibitor leptomycin B (30). Thus, it is likely that the amino terminal region of APC protein possesses yet a third domain able to facilitate the nuclear import of some truncated APC proteins. This is one possible explanation for why APC protein bearing inactivating mutations in both NLS1$_{APC}$ and NLS2$_{APC}$ was observed in the nucleus of a few cells when both NES$_{APC}$ also were inactivated [APCmNESmNLS1,2, 13(±5)%; data not shown]. As the current study demonstrates, multiple NLSs can increase nuclear import. We and others previously have shown that APC also possesses at least two functional NESs (19, 30, 31). It is likely that multiple NLSs and NESs are responsible for nucleocytoplasmic shuttling of APC, ultimately determining its final subcellular localization. Furthermore, we have demonstrated that the first serine (Ser^{2054}) after NLS2$_{APC}$ can regulate the nuclear import of APC; a negative charge at Ser^{2054} inhibits NLS2$_{APC}$-mediated nuclear translocation. This result suggests that phosphorylation of Ser^{2054} by PKA may negatively regulate the nuclear import of APC, thus providing a potential mechanism to regulate shuttling, subcellular localization, and nuclear activity of APC protein.

Of particular relevance to this study, APC can be phosphorylated *in vitro* by PKA (32). Although APC has been reported to be highly phosphorylated *in vivo* (33, 34) and also can be phosphorylated *in vitro* by recombinant p34cdc2 (35), the specific consequences of hyper- or hypophosphorylation have not yet been characterized. In addition to SV40 T-ag, an increasing number of nuclear proteins have been found to modulate their nuclear translocation through phosphorylation (20). Among these examples, most proteins, such as SV40 T-ag, are regulated by phosphorylation immediately adjacent to the N terminus of the NLS (22–24, 36). APC provides an example of a protein whose nuclear import is inhibited by a negative charge immediately adjacent to the C terminus of an NLS.

Phosphorylation mediates several steps in the Wnt signaling pathway. Phosphorylation of β-catenin, axin, glycogen synthase kinase 3β, and APC leads to ubiquitination and rapid turnover of β-catenin protein (7). Expression of the protein phosphatase 2A (PP2A) regulatory subunit, B56, in HEK293 cells results in a decrease in β-catenin protein abundance and activity (37). PP2A can dephosphorylate serine residues phosphorylated by

PKA (38, 39). If, as shown in this paper, nuclear import of APC is negatively regulated by phosphorylation of Ser^{2054}, then it is possible that dephosphorylation by a protein phosphatase, such as PP2A, might lead to increased nuclear transport of APC, potentially impacting nuclear β-catenin activity.

Axin/conductin has been shown to be an integral cofactor in the APC-mediated down-regulation of β-catenin. Mapping the three axin binding sites in APC protein led to speculation that many of the observed phenotypes associated with specific truncations in APC could be explained by the loss of axin binding sites (40). Here, we have demonstrated the functionality of two NLSs in the middle portion of the APC protein. Further analysis of the APC protein sequence reveals that the last two axin-binding sites are close, if not immediately adjacent, to these two NLSs. Thus truncations of the APC protein, which eliminate the last two axin binding sites, also eliminate the two NLSs. It remains possible, therefore, that APC protein may need to not only bind axin, but also efficiently enter the nucleus to effectively down-regulate β-catenin. Here we present evidence that phosphorylation of APC regulates the nuclear import of this tumor suppressor protein and suggest that it may affect signaling through the Wnt pathway.

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