ACS chemical biology

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Identification of SAP155 as the Target of GEX1A (Herboxidiene), an Antitumor Natural Product

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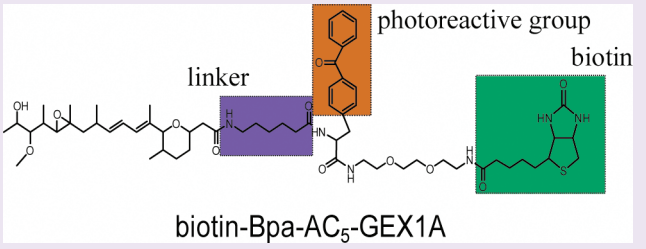
Supporting Information

ABSTRACT: GEX1A is a microbial product with antitumor activity. HeLa cells cultured with GEX1A accumulated $p27^{Kip}$ and its C-terminally truncated form p27*. GEX1A inhibited the pre-mRNA splicing of p27, producing p27* from the unspliced mRNA containing the first intron. p27* lacked the site required for E3 ligase-mediated proteolysis of p27, leading to its accumulation in GEX1A-treated cells. The accumulated p27* was able to bind to and inhibit the cyclin E-Cdk2 complex that causes E3 ligase-mediated degradation of p27, which probably triggers the accumulation of p27. By using a series of photoaffinity-labeling derivatives of GEX1A, we found that GEX1A targeted SAP155 protein, a subunit of SF3b responsible for pre-mRNA splicing. The linker length between the GEX1A pharmacophore and the photoreactive group was critical for detection of the GEX1A-binding protein. GEX1A serves as a novel splicing inhibitor that specifically impairs the SF3b function by binding to SAP155.



GEX1A (see Supplementary Figure S4) was isolated as a natural substance with *in vivo* antitumor activity from a culture broth of *Streptomyces* sp.[1] This compound causes cell cycle arrest in G1 and G2/M phases in the normal human fibroblast cell line WI-38.[2] These effects were confirmed in HeLa cells (Supplementary Figure S1), which were used throughout the experiments in this study.

The cell cycle in mammalian cells is driven by the periodic activation of cyclin-dependent kinases (Cdks). Activation of Cdks requires binding of the catalytic subunits Cdk4/6, Cdk2, and Cdk1 with their specific regulatory subunits known as cyclins D, E, A, and B. These kinase activities are further regulated in complex manners including cyclin-kinase inhibitors (CdkIs).[3] Since $p27^{Kip}$ (Cdk inhibitory protein, hereafter referred to as p27) is a key component of CdkIs of the G1 transition,[3] we examined the effects of GEX1A on the level of p27. Treatment of HeLa cells with GEX1A caused p27 accumulation in time- and dose-dependent manners (Figure 1, panels A, B). In addition to full-size p27 (27 kDa), a band with a smaller size (22 kDa, referred to as p27*) increased after the drug treatment. These two bands were both detected with an antibody against full-size p27 (amino acids 1−198). An antibody against the C-terminal peptide of p27 (amino acids 181−198) detected full-size p27 but not p27* (Figure 1, panel C), suggesting that p27* was a truncated form of p27 that lacked the C-terminal portion. It has been reported that cells treated with spliceostatin A, a newly identified splicing inhibitor, produce a C-terminally truncated form of p27.[4] Therefore, we examined whether GEX1A inhibited the pre-mRNA splicing of p27, producing p27*. Figure 2 (panel A) depicts the exon-intron disposition of the primary transcript of the *p27* gene. It also shows the mature mRNA (species 1) and unspliced RNA species (species 2−4) as well as their predicted RT-PCR products. Notably the first intron contained an in-frame translational termination codon. If RNA species containing intron 1 (species 3 and 4) are exported to the cytoplasm and somehow overcome the nonsense-mediated mRNA decay (NMD),[5] these RNAs could give rise to p27* consisting of the C-terminally truncated p27 (158 amino acids) and the peptide encoded by the intronic sequence (21 amino acids). The production of p27* in GEX1A-treated cells suggested that the drug perturbed the mRNA splicing. Therefore, we performed RT-PCR to examine whether treatment of cells with GEX1A led to the production of unspliced RNAs of p27. All of the predicted unspliced species were detected in cells cultured with GEX1A, while these species were almost undetectable in untreated cells and only the mature mRNA was found

Received: August 17, 2010
Accepted: December 2, 2010
Published: December 07, 2010

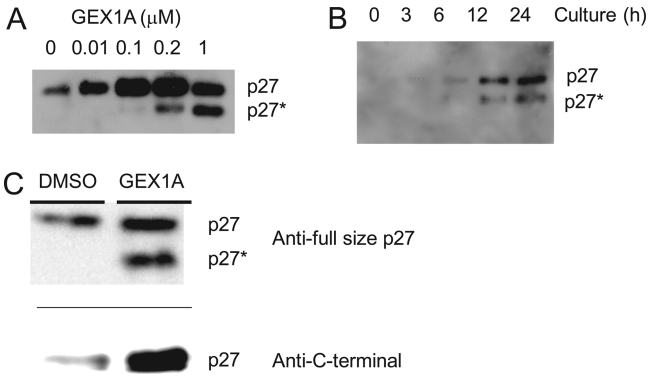


Figure 1. GEX1A causes accumulation of p27 and its C-terminally truncated form p27*. Both proteins were detected by Western blotting. (A) HeLa cells were incubated with the indicated concentrations of GEX1A for 24 h. p27 and p27* in the cell lysates were detected using an antibody against full-size p27. (B) HeLa cells were incubated with 0.2 μM GEX1A for the indicated periods. p27 and p27* in the lysates were detected using the antibody against full-size p27. (C) HeLa cells were incubated for 24 h with 1 μM GEX1A or with DMSO alone as a control. p27 and p27* in the lysates were detected using the antibody against full-size p27 (top) or an antibody against the C-terminal peptide of p27 (bottom).

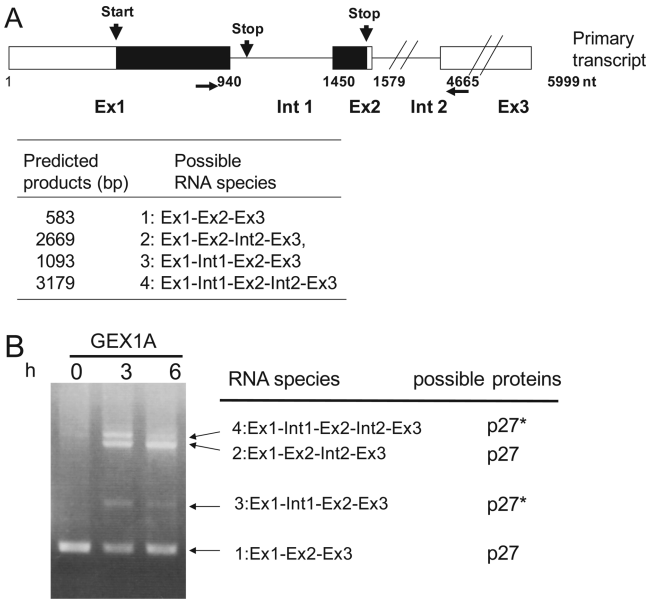


Figure 2. GEX1A inhibits the pre-mRNA splicing of p27. (A) Exon (Ex)-intron (Int) disposition of the p27 primary transcript and possible RNA species produced when splicing of the pre-mRNA is inhibited. The white rectangles indicate noncoding regions, and the black rectangles indicate coding regions. The sites for the start and stop of translation are shown. The horizontal arrows indicate the primer positions used for RT-PCR. The predicted RT-PCR products (bp) and the corresponding RNA species are also shown. (B) HeLa cells were incubated with 1 μM GEX1A for the indicated periods, and the p27-related RNA species were detected by RT-PCR. The middle column shows the RNA species. Possible proteins produced by these RNA species are shown in the right column.

(Figure 2, panel B). We confirmed that GEX1A is an inhibitor of universal pre-mRNA splicing by analyzing the transcripts of other three genes (*DNAJB1*, *BRD2*, and *RIOK3*). The unspliced RNA species of these genes were produced in dose-dependent manners (Supplementary Figure S2).

Once mRNAs have been exported to the cytoplasm, they are subjected to quality control in the pioneering round of translation. NMD is one of the surveillance pathways that direct the degradation of translationally abnormal mRNAs.[5] NMD eliminates mRNAs containing a premature termination codon (PTC) for translation. The exon−exon junction complex (EJC) plays an important role in NMD. An EJC is deposited at −20 to −40 nucleotides upstream of an exon−exon junction.[6,7] In the normal mRNA, all of the EJCs are removed in the pioneering round of translation, which qualifies polyribosome formation for subsequent rounds of translation. When a PTC is present upstream of an EJC, a ribosome pauses at the PTC and does not reach the EJC to be removed. As a rule, the location of a PTC at >50−55 nucleotides upstream of an exon−exon junction has been thought to leave an EJC on mRNAs, which is a signal that elicits NMD.[8] In this context, the EJCs on the unspliced RNA species 2 of p27 (Ex1-Ex2-Int2-Ex3) as well as those on the mature mRNA (species 1) can be removed and species 2 may therefore contribute to p27 production in addition to the mature mRNA. Species 4 (Ex1-Int1-Ex2-Int2-Ex3) does not bind to an EJC and probably escapes NMD, producing p27*. However, species 3 (Ex1-Int1-Ex2-Ex3) may retain an EJC on exon 2 after the pioneering round of translation, leading to NMD-mediated degradation.

Next we discuss possible mechanisms of accumulation of p27* and p27. The level of p27 varies during the cell cycle. It is reduced in G1 and S phases so that the cell cycle can progress through these two phases.[9] The concentration of p27 is predominantly regulated by the ubiquitin-proteasome pathway.[10] Degradation of p27 through this pathway is triggered by the phosphorylation of Thr187 by cyclin E-Cdk2.[11] Since p27* lacks Thr187, it is capable of escaping from E3 ligase-mediated proteolysis, resulting in the accumulation of p27* in GEX1A-treated cells. How does GEX1A lead to the accumulation of p27? GEX1A did not increase p27 mRNA (see Figure 2, panel B). It is likely that the inhibition of cyclin E-Cdk2 by p27* (see below) triggers the accumulation of p27. The accumulation of p27 and p27* is probably exceptional, since the levels of most proteins would be decreased when splicing of their primary transcripts are inhibited.

p27 binds to the cyclin E or A-Cdk2 complex and inhibits its activity.[3] We therefore examined whether p27* was still able to bind to the Cdk2 complex. The Cdk2-associated p27 increased after treatment with GEX1A, and notably, p27* increased (Supplementary Figure S3, panel A). The Cdk2 activity in the immunoprecipitates obtained with either the anti-Cdk2 antibody or the anticyclin E antibody markedly decreased upon treatment with GEX1A (Supplementary Figure S3, panel B). Conceivably, p27* is functionally active and inactivates cyclin E-Cdk2, because the sites in p27 required for binding with the cyclin-Cdk complex are located in the N-terminal region,[12] which remains in p27*.

The excision of introns from pre-mRNAs is carried out by a multiprotein-RNA complex known as the spliceosome.[13,14] The core spliceosome consists of five small nuclear RNAs (snRNAs U1, U2, U4, U5, and U6) and associated proteins, forming snRNA-protein complexes (snRNPs). U2 snRNP consists of U2 snRNA, splicing factors SF3a and SF3b, and other proteins. The SF3b complex consists of seven spliceosome-associated proteins (SAPs), namely, SAP155, SAP145, SAP130, SAP49, SAP14a, SAP14b, and SAP10.[15] We designed three GEX1A derivatives as

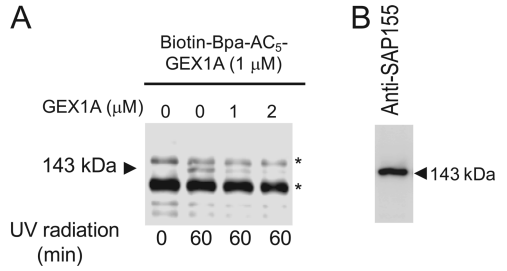


Figure 3. Photoaffinity labeling of the 143-kDa protein is inhibited by GEX1A. (A) Nuclear extracts from HeLa cells were incubated with 1 μM biotin-Bpa-AC$_5$-GEX1A in the absence or presence (1 or 2 μM) of GEX1A for 16 h. The samples were exposed to UV light (365 nm, 6 W) for 60 min. After SDS−PAGE, the proteins were electroblotted onto a membrane, and the photoaffinity-labeled proteins were detected by chemical luminescence using HRP-conjugated streptoavidin. The asterisks indicate nonspecific bands. (B) The same membrane was used for the detection of SAP155 with its antibody.

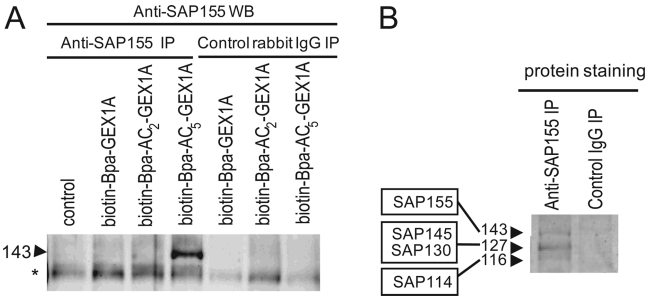


Figure 4. The photoaffinity-labeled protein of 143 kDa is SAP155. (A) Nuclear extracts from HeLa cells were incubated with 1 μM GEX1A derivatives for 16 h. The samples were exposed to UV light for 60 min and immunoprecipitated with an anti-SAP155 antibody or a control IgG. The proteins in the immunoprecipitates were separated by SDS−PAGE and electroblotted onto a membrane, and the photoaffinity-labeled proteins were detected as in Figure 3. The asterisk indicates a nonspecific band. (B) Nuclear extracts from HeLa cells were immunoprecipitated with the anti-SAP155 antibody or control IgG. Proteins in the immunoprecipitates were separated by SDS−PAGE and visualized by silver staining. Three protein bands of 143, 127, and 116 kDa were analyzed by in-gel digestion and subsequent LC−MS/MS. These bands were identified as SAP155, SAP145 + SAP130, and SAP114, respectively (see Supplementary Table S11).

probes to identify the spliceosome proteins interacting with GEX1A (Supplementary Figure S4). A photoreactive amino acid (*p*-benzoylphenylalanine, Bpa) was introduced into GEX1A at the carboxylic acid with linkers of various lengths between this amino acid and GEX1A. The photoreactive amino acid was converted to a triplet state by UV light and thereby covalently bound to C−H bonds in the binding protein. A biotin moiety was also included for detection of the labeled protein with HRP-conjugated streptavidin. The GEX1A derivatives were confirmed to be the expected compounds by mass spectrometry (MS) (Supplementary Figure S5). All of the derivatives showed inhibition of splicing but with different potencies (Supplementary Figure S6). These different potencies may be derived from differences in the membrane permeability, intracellular stability and affinity for the target protein.

To identify the spliceosome proteins that bound to the GEX1A derivatives, nuclear extracts of HeLa cells treated with the derivatives were subjected to the photocross-linking reaction. After the separated nuclear proteins were electroblotted onto a membrane, the labeled proteins were detected. Among the three photoreactive GEX1A derivatives, only biotin-Bpa-AC$_5$-GEX1A yielded a labeled protein band of 143 kDa (Supplementary Figure S7). The formation of this 143-kDa protein band was almost completely inhibited by the presence of a 2-fold excess of GEX1A (Figure 3, panel A). When the same membrane was subjected to immunoblotting, the 143-kDa band corresponded to SAP155, a subunit in the SF3b complex (Figure 3, panel B).

To confirm that the photoaffinity-labeled 143-kDa band was SAP155, the nuclear extracts after treatment with the GEX1A derivatives were immunoprecipitated with an anti-SAP155 antibody. After the precipitated proteins were separated and electroblotted onto a membrane, the labeled proteins were detected. A clear band of 143 kDa was observed only when biotin-Bpa-AC$_5$-GEX1A was used for the labeling (Figure 4, panel A), consistent with the notion that this ligand is able to bind to the 143-kDa protein. The proteins immunoprecipitated from the nuclear extracts using the anti-SAP155 antibody were separated and visualized by protein staining. Three bands of 116, 127, and 143 kDa were observed (Figure 4, panel B). These protein bands were identified by in-gel trypsin digestion and subsequent LC−MS/MS analysis. The MASCOT program matched the 143-kDa MS/MS ion sequences to SAP155 (Supplementary Table S1). These results indicate that GEX1A inhibits splicing of the pre-mRNA through binding with SAP155.

Although all derivatives showed inhibitory effects on pre-mRNA splicing (Supplementary Figure S6), only biotin-Bpa-AC$_5$-GEX1A was able to label SAP155, a subunit of the SF3b complex. A close proximity of the photoreactive group to the surface of the SF3b subunit may be necessary for efficient cross-linking. The length of the linker between the Bpa residue and GEX1A appeared to be critical for the cross-linking, and biotin-Bpa-AC$_5$-GEX1A had the longest linker of 7 Å.

The primary transcripts of most eukaryotic genes are retained in the nucleus until their introns are removed by the spliceosome. GEX1A inhibited pre-mRNA splicing, producing unspliced RNA species of the *p27* gene. At least one of the unspliced RNAs containing the first intron is exported to the cytoplasm where it is translated to produce p27*. Furthermore, GEX1A exerts its inhibitory effect on the splicing through binding with SAP155, a subunit of SF3b. Presumably GEX1A-derived inactivation of SF3b makes it possible for unspliced pre-mRNAs to emerge into the cytoplasm. These results support a previous proposal [4] that SF3b has a dual function in pre-mRNA processing, namely the removal of introns and the nuclear retention of unspliced RNAs.

Recently, two natural products were reported to inhibit pre-mRNA splicing. Spliceostatin A may interact with SAP130 or SAP155,[4] and pladienolide B binds to SAP130.[16] Regarding their structures, spliceostatin A is considerably different from GEX1A, while pladienolide B is similar to GEX1A except that GEX1A lacks a macrolide ring (Supplementary Figure S8). A photoreactive derivative of pladienolide B was cross-linked to SAP130 but not SAP155. These distinct results between GEX1A and pladienolide B imply that an interface between SAP155 and SAP130 in the SF3b complex is located near the binding sites for these inhibitors. Detailed mapping of the GEX1A-binding site is underway. Crucial roles of SAP155 in the splicing machinery have been amply documented.[17,18] Moreover, the functions of SAP155 are regulated by its phosphorylation-dephosphorylation balance.[19] Thus elucidation of the GEX1A-binding site in

SAP155 is important for better understanding of not only the inhibitory mechanism but also the regulation of SAP155. Chemical-derived inactivation of SF3b appears to disrupt the cellular function required for nuclear retention of unspliced pre-mRNAs (ref 4 and this paper). Such chemicals would be very useful for elucidating the molecular mechanism that links the inhibition of splicing and the leakage of unspliced pre-mRNAs. All of the three compounds have antitumor activity, indicating that the splicing machinery is a novel target for antitumor drugs and that further modification of these chemicals may lead to the development of drugs with high efficacy.

METHODS

GEX1A, Cells, and Western Blotting. GEX1A was supplied by Kyowa Hakko Kirin Co. Ltd. and was dissolved in DMSO. The final concentration of DMSO was adjusted to 0.1%. Controls contained 0.1% DMSO alone. HeLa cells were cultured in RPMI medium containing 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin. The cells were incubated in NP40 lysis buffer consisting of 50 mM HEPES pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM β-glycerophosphate, 2 mM Na_3VO_4, 0.2 mM EDTA, 10 mM NaF, 1 mM DTT, 1% Halt Protease Inhibitor Cocktail (Pierce), and 1 mM PMSF. The lysates were centrifuged and the protein concentrations of the supernatants were determined by the Quick Start Protein Assay (Bio-Rad). The proteins were analyzed by Western blotting with the following primary antibodies against human proteins purchased from Santa Cruz Biotechnology: antifull-size p27 (amino acids 1−198) (1:200), anti-C-terminal peptide of p27 (amino acids 181−198) (1:100), and anti-β-actin (1:1000). As secondary antibodies, HRP-conjugated antimouse IgG (1:5000; Calbiochem) and HRP-conjugated anti-rabbit IgG (1:5000; Invitrogen) were used. When the antibody against the C-terminal peptide of p27 was used, a solution for signal enhancement (Can Get Signal Immunoprecipitation Solution; Toyobo) was used according to the manufacturer's protocol.

RT-PCR. Total RNA was extracted using an RNeasy Mini Kit (Qiagen). RT-PCR was conducted with ReverTra Ace-α (Toyobo) using 0.3 μg of RNA. The primers used for p27 were 5′-GCAAGTACGAGTGGCAAGAGG-3′ for exon 1 and 5′-TCCAACGCTTTTAGAGGCAGA-3 for exon 3.

Chemical Synthesis. Photoaffinity-labeling GEX1A derivatives were synthesized using linker moieties (β-alanine or 6-aminohexanoic acid), *p*-benzoylpehenylalanine (Bpa), and biotin-PEO-amine (EZ-Link Biotin Reagents; Thermo Fisher Scientific). In each step, the carboxyl group in a GEX1A derivative was esterified with 3 equiv of *N*-hydroxysuccinimide (HOSu) by treatment with 3 equiv of water-soluble carbodiimide in dimethylformamide (DMF) containing 3 equiv of triethylamine (TEA). A GEX1A-HOSu derivative was mixed with an amine component (3−4 equiv of a linker moiety) in a DMF/water mixed solvent containing 3 equiv of TEA. After reaction for 4−22 h at 30 °C, each product was purified using a Cosmosil AR-II 5C18 column (Nacalai Tesque) and an HPLC (Gilson). The HPLC was carried out at a flow rate of 1 mL/min with a linear gradient of 10−50% CH_3CN in 10 mM sodium acetate (pH 5.7). The eluates from the HPLC column were monitored at 220 nm, and their contents were analyzed by ESI IT-TOF mass spectrometry (LCMS-IT-TOF System; Shimadzu).

Nuclear Extract Preparation. Cold PBS-washed HeLa cells (1×10^9 cells) were suspended in two volumes of buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) and homogenized by 30 strokes of a glass Dounce homogenizer. After centrifugation of the homogenate, the precipitate was squeezed by 10 strokes of the glass homogenizer in 2.5 mL of buffer A containing 420 mM NaCl. After 30 min of stirring, the insoluble matter was removed by centrifugation. The supernatant was dialyzed against buffer B (20 mM Tris-HCl pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol). Aliquots were stored at −80 °C until analysis.

Immunoprecipitation. An anti-SAP155 antibody (2 μg; Bethyl Laboratories) was added to a nuclear extract and mixed gently on a rotator. Protein G-agarose was added and mixed. The agarose beads were precipitated and washed with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween-20). Coprecipitated proteins were eluted into the SDS−PAGE sample buffer by boiling the beads for 5 min.

Protein Identification by MS. Protein bands in SDS−PAGE gels visualized by staining with a Silver Stain MS Kit (Wako Pure Chemical Industries) were excised and rinsed with 50 mM $NaHCO_3$/50% acetonitrile. The gel particles were dried in a vacuum concentrator and rehydrated in 50 μL of 50 mM $NaHCO_3$ buffer containing 10 ng/μL sequencing grade trypsin (Sigma-Aldrich). After removing the excess trypsin solution, the gel particles were overlaid with 30 μL of 50 mM $NaHCO_3$ and incubated for 16 h at 37 °C to extract trypsin-digested peptides. The supernatants containing the peptides were subsequently used for MS analysis. Each sample was loaded onto a nano-LC equipped with a PicoFrit column (New Objective). A Prominence Nano binary pump system (Shimadzu) was programmed to elute the peptides with a gradual ramp of a linear gradient of 5−90% acetonitrile with 0.1% formic acid over 60 min. The flow rate in the column was 200 nL/min, and the column end was directly connected to the nanospray tip of an LCMS-IT-TOF System (Shimadzu). The mass spectrometer was set to a cycle of one full mass scan, followed by three tandem mass scans of the three most intense ions. All the tandem mass spectra obtained were searched for in the Swiss-Prot database using the MASCOT program (Matrix Science).

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We thank S. Ikeda, Y. Mukai, and A. Yamamoto for helpful discussions. This research was supported in part by Grants-in-Aid to M.H. from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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