Altered Glycosylation of the env-sea Oncoprotein Inhibits Intracellular Transport and Transformation

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Abstract

The transforming gene product of the S13 avian erythroblastosis virus, env-sea, is a member of the growth factor receptor class of tyrosine kinases. The env-sea precursor protein gp155env-sea is proteolytically processed into the mature cleavage products gp85env-sea and gp70env-sea, which are subsequently terminally glycosylated and transported to the cell surface. Previous studies have shown that the abnormal glycosylation of gp155env-sea induced by the carbohydrate processing inhibitor castanospermine blocks the proteolytic cleavage of gp155env-sea and impairs its transforming ability. We have shown recently that an uncleaved but fully glycosylated sea-encoded protein retains the ability to transform chicken embryo fibroblasts, indicating that proteolytic processing is not essential for transformation by the env-sea tyrosine kinase. To address the question of how castanospermine blocks transformation by env-sea, differential sucrose gradient centrifugation was performed on env-sea-transformed cells treated with the inhibitor. This report shows that no surface forms of env-sea could be detected in inhibitor-treated cells, suggesting that castanospermine acts by blocking the transport of sea-encoded proteins to the cell surface.

Introduction

env-sea, the oncogene carried by the S13 avian erythroblastosis virus, is a member of the growth factor receptor family of protein tyrosine kinases (1). Within this family, the env-sea-encoded protein is most closely related, both in structure and by sequence homology, to the hepatocyte growth factor receptor (c-met) (2, 3). Like c-met, env-sea encodes a heterodimeric protein consisting of an extracellular α subunit, gp85env-sea, disulfide-linked to a transmembrane β subunit, gp70env-sea, containing an intracellular tyrosine kinase domain. However, in the case of the env-sea fusion protein, both the extracellular and the transmembrane domains are encoded by the env gene of S13, whereas the tyrosine kinase domain is encoded by cellular sea (c-sea)-derived sequences (1, 4). Thus, although the structure of this env-onc fusion protein superficially resembles that of a growth factor receptor tyrosine kinase, the extracytoplasmic domain has been replaced by env-encoded sequences. Therefore, the env-sea precursor, gp155env-sea, undergoes all of the posttranslational modifications characteristic of a retroviral env protein, including proteolytic processing and N-linked glycosylation.

Previous studies have shown that treatment of S13-transformed erythroblasts and fibroblasts with the glycosylation processing inhibitor castanospermine (5) causes the cells to revert to a normal phenotype (6). This inhibition of transformation is coincident with a dramatic reduction in the proteolytic processing of the precursor gp155env-sea to the mature products gp85env-sea and gp70env-sea. However, we have recently shown, through studies with a mutant lacking the cleavage site (Δcylv), that proteolytic processing is not essential for tyrosine kinase activity, N-linked glycosylation, or intracellular transport of the env-sea-encoded protein (7). In addition, this uncleaved, terminally glycosylated form of sea, which is specifically blocked in proteolytic processing, was found to transform fibroblasts as efficiently as the unmutated env-sea protein. Importantly, the transformed phenotype of Δcylv-transformed fibroblasts could be reverted by the addition of castanospermine. Therefore, the block to proteolytic processing induced by castanospermine is not responsible for the reversion of the transformed phenotype observed in the presence of this inhibitor.

Previously, both surface immunofluorescence and 125I surface labeling experiments were performed on inhibitor-treated cells (6). The immunofluorescence studies found env-sea surface expression to decrease in the presence of the drug, whereas the 125I surface labeling experiments showed a marked increase in env-sea-encoded proteins at the surface of inhibitor-treated cells. These results were taken to indicate that castanospermine did not significantly impair the intracellular transport of sea-encoded proteins. However, the 125I labeling experiments also showed that, even in the absence of the inhibitor, equivalent amounts of gp70env-sea and gp155env-sea could be detected at the cell surface. Our recent analysis of the processing and transport of env-sea, using differential sucrose gradient centrifugation (7), showed that only the mature, proteolytically processed env-sea protein, gp70env-sea, is efficiently transported to the cell surface. These latter results suggested that there may have been some labeling of intracellular membranes during the earlier surface iodination experiments. Therefore, we have now used subcellular fractionation to reexamine the effect of castanospermine on the intracellular transport of env-sea-encoded proteins.

In this report, we show that castanospermine treatment of both env-sea- and Δcylv-transformed cells resulted in a dramatic reduction of all cell-surface forms of env-sea as measured by differential sucrose gradient centrifugation. These results indicate that complex carbohydrate modification of env-sea may be necessary for the protein to obtain a conformation compatible with transport. In addition, the data suggest that the observed inhibitory effect of castanospermine on env-sea transfor-
Inhibition of transformation by castanospermine. A, morphology of G418-resistant Δclvg CEF plated at approximately 3 × 10^5 cells/90-mm diameter dish and incubated at 37°C × 100. B, morphology of G418-resistant Δclvg CEF plated at approximately 3 × 10^5 cells/90-mm diameter dish and incubated at 37°C in the presence of 300 μM castanospermine for 24 h prior to photographing × 100. C, morphology of Δclvg CEF plated at approximately 4 × 10^5 cells/90-mm diameter dish and incubated at 37°C in the presence of 10 μM swainsonine for 24 h prior to photographing × 100. D, morphology of uninfected CEF plated at approximately 2 × 10^5/90-mm diameter dish and incubated at 37°C × 100.

Transformation is due to a block in intracellular transport and that cell surface localization is necessary for transformation by the env-sea oncogene product.

Results

Castanospermine Inhibits Transformation by env-sea and Δclvg. Previous studies have shown that the altered glycosylation of the env-sea-encoded protein that occurs in the presence of the α-glucosidase I inhibitor, castanospermine, blocks transformation by env-sea (6). We have recently shown that the transforming ability of an uncleaved form of sea in CEF is also inhibited by castanospermine (7). In these studies, the block of env-sea transformation induced by castanospermine was quantified by colony formation, which showed that Δclvg-infected CEF, similar to env-sea CEF, were inhibited in their ability to form colonies when plated in the presence of castanospermine. In addition, we show here that the transformed, fusiform morphology of Δclvg CEF (Fig. 1A) reverted to a flattened morphology, characteristic of uninfected primary CEF, when grown in the presence of 300 μM castanospermine (Fig. 1B). These same cells were unaffected by the addition of 10 μM swainsonine (Fig. 1C), which blocks trimming at a later stage of carbohydrate processing than castanospermine and leads to an increase in hybrid oligosaccharide chains. These data confirm that castanospermine causes a reversion of the transformed phenotype in both env-sea- and Δclvg-infected CEF.

Protein Expression in the Presence of Castanospermine. To determine the effect of castanospermine on protein expression in both env-sea- and Δclvg-infected CEF, cells were grown in the presence or absence of 300 μM castanospermine and then tested for sea protein expression by immunoblot. As shown in Fig. 2A, env-sea CEF expressed all three env-sea-encoded proteins: the precursor protein, gp155mm*, the mature cleavage product, gp70mm**, and a minor amount of the uncleaved, terminally glycosylated form of env-sea, termed gp171mm (Fig. 2A, Lane 1). However, as described previously (7), Δclvg CEF expressed only gp155mm* and an increased amount of gp171mm (Fig. 2A, Lane 6). No gp70mm** could be detected in these cells, as expected since this mutant is specifically blocked in proteolytic processing. In the presence of castanospermine, both env-sea CEF and Δclvg CEF expressed only the modified form of the gp155mm* precursor, namely gp157mm* (Fig. 2A, Lanes 4 and 8). These data indicate that the inhibitor blocked the formation of the mature env-sea proteins in both env-sea- and Δclvg-
infected CEF. A similar pattern was observed in HD3 erythroblasts (B) infected with env-sea and Δclvg (data not shown).

**Phosphorylation in the Presence of Castanospermine.**

Env-sea displays constitutive tyrosine kinase activity in vitro and in vivo (4), and analysis of a temperature-sensitive isolate of env-sea has indicated that this kinase activity is essential for transformation (9). Previous studies showed that treatment of env-sea-transformed cells with castanospermine did not inhibit the tyrosine kinase activity of env-sea as measured by an immune complex kinase assay (6). To confirm this result for the proteolytic processing mutant, we performed in vitro kinase assays on lysates from both env-sea- and Δclvg-infected HD3 cells. As shown in Fig. 1, both gp70^env^ and gp155^env^ as well as the uncleaved, terminally glycosylated form of env-sea expressed in erythroblasts, termed gp178^env^, displayed tyrosine kinase activity (Fig. 1, Lane 2). When these cells were treated with castanospermine prior to lysis, both gp157^env^ and a minimal amount of gp68^env^, presumably the result of residual cleavage of the abnormally glycosylated gp157^env^ (6), were capable of auto-phosphorylation (Fig. 1, Lane 4).
A similar pattern was observed with Δclvg HD3 cells; both gp155<sup>−180</sup> and gp178<sup>−</sup> displayed tyrosine kinase activity in the absence of castanospermine (Fig. 3, Lane 6), and, when cells were grown in the presence of the inhibitor, gp157<sup>−</sup> was still capable of autophosphorylation (Fig. 3, Lane 8). However, as expected due to the deletion of the cleavage site, no gp70<sup>−180</sup> tyrosine kinase activity could be detected in the immune precipitate from Δclvg HD3 cells. These data confirm our previous studies (6) and indicate that the in vitro kinase activity of both env-sea and Δclvg is unaffected by altered glycosylation.

To determine the effect of castanospermine on in vivo tyrosine phosphorylation, the sea immunoblot in Fig. 2A was reprobed with a monoclonal antibody to phosphotyrosine. As shown in Fig. 2B, all three env-sea-encoded proteins were phosphorylated on tyrosine in vivo. However, as described previously (7), the mature complex sugar-containing forms of env-sea, namely gp70<sup>−180</sup> and gp171<sup>−</sup>, contain a much higher stoichiometry of phosphotyrosine than the precursor protein gp155<sup>−180</sup> (Fig. 2B, Lane 2). Likewise, in lysates from Δclvg-transformed CEF, although both env-sea-encoded proteins contained phosphotyrosine, the major phosphoprotein detected was gp171<sup>−</sup> (Fig. 2B, Lane 6). For both Δclvg and env-sea CEF, treatment with castanospermine resulted in a dramatic reduction in total env-sea phosphotyrosine content due to the loss in expression of the mature, highly phosphorylated forms of env-sea (Fig. 2B, Lanes 4 and 8). However, the effect of the inhibitor on both the expression and tyrosine phosphorylation of the immature precursor protein was negligible (Fig. 2, A and B, Lanes 4 and 8). A similar in vitro and in vivo phosphorylation pattern was observed in lysates from both infected erythroblasts and rat fibroblasts, indicating that the effect of castanospermine on phosphorylation was not species or cell type specific (data not shown). These data suggested that castanospermine was blocking the formation of the mature, highly phosphorylated forms of env-sea which are expressed on the surface of env-sea-transformed cells (7).

Analysis with endoglycosidase H, which digests only immature carbohydrate chains (10), confirmed that whereas both gp70<sup>−180</sup> and gp178<sup>−</sup> were resistant to endoglycosidase H, both the normal env-sea precursor, gp155<sup>−180</sup>, and gp171<sup>−</sup>, the sole form of env-sea expressed in castanospermine-treated cells, were sensitive to this treatment (Ref. 7; Fig. 4). These data indicate that, as predicted, treatment of both fibroblasts and erythroblasts with castanospermine (5) blocks the processing of the env-sea precursor gp155<sup>−180</sup> into its mature, complex carbohydrate forms.

Castanospermine Affects Transport of sea. In keeping with the results described above, many researchers have found that castanospermine blocks the transport and processing of several cell surface-expressed and secreted glycoproteins (11–14). In order to address the possibility of an inhibitor-induced transport block more directly, we have used differential sucrose gradient centrifugation to examine the subcellular localization of env-sea in castanospermine-treated cells, env-sea-transformed erythroblasts were used for this analysis since high levels of env-sea protein expression and processing could be obtained in these cells. In all cases tested, fractionation of env-sea-infected HD3 cells gave similar results.

As shown in Fig. 5, fractionation of homogenates from env-sea-transformed erythroblasts grown in the absence of inhibitors resulted in the previously described pattern.
Thus, the terminally glycosylated cleavage product, gp70\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde}, was the primary form of env-sea detected in the plasma membrane- and smooth membrane-enriched fractions (Fig. 5, Lanes 3 and 4), whereas the precursor protein gp155\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde} fractionated primarily with intracellular membranes (Fig. 5, Lane 5). No env-sea proteins could be detected in the cytoplasmic fraction (Fig. 5, Lane 6). Fractionation of homogenates from the same cells, grown in the presence of 300 \(\mu\)M castanospermine, indicated that gp157\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde} was localized exclusively in intracellular membranes (Fig. 5, Lane 11); however, the residually expressed cleavage product, gp68\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde}, appeared to be transported normally (Fig. 5, Lane 9). These results suggest that, although drastically inhibiting the processing of env-sea, castanospermine did not cause an absolute block in the transport of the residual cleavage product to the cell surface.

To confirm this apparent inhibition of env-sea transport, we analyzed the subcellular localization of the proteolytic processing mutant, \(\Delta\text{clg}\), in the presence of castanospermine. As mentioned above, cleavage of env-sea is not required for transport to the cell surface or for transformation (7). As shown in Fig. 6A, the only form of env-sea expressed in inhibitor-treated \(\Delta\text{clg}\) HD3 cells, namely gp157\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde}, was confined to intracellular membranes (Fig. 6B, Lanes 5 and 6), indicating that castanospermine induced an absolute block of env-sea protein transport in these cells. As a control to show that castanospermine did not cause a general block of protein transport in the HD3 erythroblasts, fractionation samples from the \(\Delta\text{clg}\) HD3 cells were analyzed in parallel for expression of the well-characterized v-erbB glycoproteins constitutively expressed in the HD3 cell line used for this study. As shown in Fig. 6A, gp72\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde} [the aberrantly glycosylated form of gp74\textsuperscript{\textasciitilde\textasciitilde\textasciitilde\textasciitilde} observed in the presence of castanospermine (15)], could still be detected in the plasma membrane-enriched fraction. In addition, preliminary studies with swainsonine suggest that this mannosidase II inhibitor (16) has no inhibitory effect on either the proteolytic processing or the intracellular transport of env-sea-encoded proteins. This latter finding is consistent with our previous results (6) showing that swainsonine does not block the transforming activity of env-sea. The fractionation data indicate, therefore, that castanospermine impairs the intracellular transport of env-sea-encoded proteins, suggesting that addition of complex carbohydrates may be necessary for the proteolytic processing and cell-surface localization of env-sea.

**Discussion**

Glucosidase-1 inhibitors, such as castanospermine, have been shown to affect the intracellular processing and/or transport of a number of cell surface glycoproteins, including v-fms (13, 17), the insulin receptor (11), the low-density lipoprotein receptor (12), the Friend mink cell murine leukemia virus envelope protein (18), and the E2 glycoproteins of Sindbis virus and mouse hepatitis virus (19, 20), although having no effect on the transport of v-erbB (15), influenza hemagglutinin (21), vesicular stomatitis virus G-protein (21), or the Rous sarcoma virus env protein (22). This suggests that the importance of N-linked glycosylation for correct intracellular transport is protein specific.

We have shown here that treatment with castanospermine efficiently blocked both the proteolytic processing and the cell surface expression of sea-encoded proteins in env-sea-infected cells. In addition, the intracellular transport of a proteolytic processing-defective mutant, \(\Delta\text{clg}\), was also found to be inhibited by castanospermine. These results are in contrast to those found with another avian retroviral protein, the RSV env glycoprotein (22). RSV env, although highly homologous in structure to the env portion of the env-sea protein, is still proteolytically processed and transported in the presence of the glucosidase-1 inhibitor, N-methyl-1-deoxy-
nojirimycin. This difference may reflect the variability in both the extent and the positioning of N-linked glycosylation sites which exists among glycoproteins from different avian retroviral subgroups. The env-sea-encoded protein belongs to subgroup A, whereas the RSV env protein used in the inhibitor studies is encoded by the Schmidt-Ruppin B virus. It remains unclear, then, whether castanospermine treatment would block transport of the RSV env protein encoded by a subgroup A virus. Thus, it is possible that the different positioning and number of N-linked glycosylation sites in env-sea may have a greater effect on protein folding, and hence transport, than it does in RSV env.

The mechanism by which castanospermine retards the migration of certain glycoproteins from the ER to the Golgi has not yet been determined; however, stable multimerization and/or correct folding of a number of glycoproteins, including the influenza virus HA protein (23), vesicular stomatitis virus G-protein (24), and the Rous sarcoma and human immunodeficiency virus glycoproteins (25-29), appears to be a necessary step for transport of these proteins out of the ER. Thus, castanospermine may block the transport of env-sea-encoded proteins out of the ER by disrupting the correct folding and/or multimerization of the protein.

Intracellular localization plays a critical role in both the normal biological function of cellular protooncogenes and the transforming activity of their oncogenic counterparts. Cell-surface localization of the oncogenic receptor tyrosine kinases, v-erbB (30) and v-fms (13, 17, 31), is essential for transformation by the transfembrane forms of these proteins. Recently, experiments with the v-src tyrosine kinase have suggested that, although partially transforming mutants have been described for v-src which are not associated with adhesion plaques (32), a full conversion to the transformed phenotype may require association with the cytoskeleton (33). In addition, numerous studies have been performed with other membrane-associated oncoproteins, including v-ras (34, 35) and v-fps (36), which provide evidence that processing and intracellular localization are necessary for the transforming ability of many oncoproteins.

One role of the env domain of env-sea may therefore be to target the cell tyrosine kinase to its site of action in the cell. This hypothesis is supported by the finding that the transformation block induced by castanospermine coincides with an inhibition in the transport of env-sea-encoded proteins to the cell surface. Further evidence for the importance of membrane association comes from our recent analysis of a myristylated form of sea, termed mvr-sea, which was targeted to the cytoplasmic face of intracellular membranes by a myristylation signal sequence, and, although containing no env sequences, could efficiently transform CEF (37). In addition, the transformed phenotype of mvr-sea-infected CEF was found to be unaffected by treatment with castanospermine (data not shown). Thus, the phenotypic reversion induced by castanospermine in env-sea- and Δyclg-transformed cells appears to be due to the altered intracellular localization of env-sea, and not to a secondary effect on a downstream target of the sea tyrosine kinase. Finally, we reported previously that castanospermine induces a more complete inhibition of transformation in Δyclg CEF than in env-sea CEF (94% inhibition in Δyclg CEF compared to 76% in env-sea CEF, as measured by colony formation in soft agar) (7). If cell surface expression of env-sea is required for transformation, the residual transforming activity of env-sea CEF could be explained by the finding, described here, that residual gp68 could still be detected at the cell surface in castanospermine-treated env-sea erythroblasts, whereas no surface forms of sea could be detected in Δyclg HD3 cells grown in the presence of the inhibitor.

In conclusion, the findings presented here suggest a functional role for complex sugars in the intracellular transport and proteolytic processing of the env-sea protein. In addition, the data support a model in which cell surface localization of a tyrosine kinase active form of env-sea is necessary for transformation.

Materials and Methods
Cell Culture and Antibodies. All cells were grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum and 2% chicken serum, unless otherwise indicated. Primary CEF were obtained from 7-8-day-old chicken embryos (Spafas), as described previously (38). Bone marrow cells were obtained from the femurs of 7-10-day-old chicks as described previously (39). The sea-specific antibody (Rb123) which was generated against the PstI to Cfi fragment of v-sea has been characterized previously (40). The monoclonal antibody SE2 directed against phosphotyrosine was generously provided by Genentech, San Francisco, CA. The rabbit v-erbB antibody kn14 and the rat monoclonal v-erbB antibody 20.3.6 were generated against a 588-base pair BamHI fragment encoding the v-erbB kinase domain, as described previously for kn14 (41). QT64dh is a quail packaging cell line which was kindly provided by Dr. A. Stoker (42). The erythroblast cell line HD3, which is transformed by the avian ts 34 AEV-ES4 erythroleukosis virus, was described previously (8).

Plasmid Construction and Transfections. env-sea and Δyclg plasmid DNA were cloned into the pNeo-MAV retroviral expression vector (43) as described previously (7). Ten μg of plasmid DNA were transfected into either QT64dh or secondary CEF by the calcium phosphate method, as described by Chen and Okayama (44). Transfectants were selected in Dulbecco's modified Eagle's medium containing 400 μg/ml G418. G418-resistant pools of HD3 cells expressing either pMAVsea or pMAVΔyclg (7) were generated by cocultivation of 3 × 10⁵ HD3 cells with stably transfected QT64dh cell lines. Infected HD3 cells were selected in Dulbecco's modified Eagle's medium containing 1 mg/ml G418. CEF expressing pMAVsea or pMAVΔyclg were generated by infection of secondary CEF with 6 ml of virus supernatant from G418-resistant pools of transfected QT64dh. Infected CEF were selected in Dulbecco's modified Eagle's medium containing 400 μg/ml G418. env-sea-transformed erythroblasts were generated by cocultivation of chicken bone marrow cells with G418-resistant pools of pMAVsea stably transfected QT64dh cells.

Subcellular Fractionation. The subcellular fractions designated P100 and S100 were isolated by crude cell fractionation as described previously (45). The P100 membrane fraction was then subfractionated by equilibrium centrifugation on a discontinuous sucrose density gradient exactly as described previously (7), except that
all solutions were buffered in 10 mM Tris (pH 7.4) containing 1 mM benzamidine-1 mM ethylene glycol-bis-(β-
-mercaptoethanol)-N,N',N''-tetraacetic acid-0.5 mM phenylmethylsulfonyl fluoride.

Immunoprecipitations and Immunoblots. Cells were lysed in 0.5% Nonidet P-40-0.5% deoxycholate-25 mM Tris (pH 8)-50 mM NaCl. Lysates were immunoprecipitated and separated by SDS-PAGE as described previously (9) and then immunoblotted as described previously (7).

In Vitro Kinase Assay. In vitro immune complex kinase assays were performed exactly as described previously (7).

Endoglycosidase Treatment. Cells were lysed and immunoprecipitated as described above. Immunoprecipitates were resuspended in 15 µl of 0.5% SDS-0.1% β-
-mercaptoethanol and boiled for 5 min. The boiled lysates were then incubated in 40 mM NaPO₄ (pH 5.5) in the presence of 2.5 mM endoglycosidase H (Genzyme, Cambridge, MA) for approximately 18 h. As a control for nonspecific proteolytic degradation, parallel samples were treated exactly as above except no enzyme was added.

Glycosylation Inhibitors. Castanospermine (Boehringer Mannheim, cat. no. 1296 728) was resuspended in 1X sterile phosphate-buffered saline at a final concentration of 100 mM (19 mg/ml) and stored at −20°C in 100-
-µl aliquots. Cells were incubated at 37°C for 24 h, unless otherwise indicated, with 300 µM castanospermine. Swainsonine (Boehringer Mannheim, cat. no. 810 169) was resuspended in 1X sterile phosphate-buffered saline at a final concentration of 10 mM (1.7 mg/ml) and stored at −20°C in 50-µl aliquots. Cells were incubated at 37°C for 24 h with 10 µM swainsonine.

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References


