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Induction of sialic acid 9-O-acetylation by diverse gene products: implications for the expression cloning of sialic acid O-acetyltransferases

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Sialic acids can be modified by O-acetyl esters at the 7- and/or 9-position, altering recognition by antibodies, lectins and viruses. 9(7)-O-acetylation is mediated by a sialic acid–specific O-acetyltransferase, which has proven difficult to purify. Two groups have recently isolated cDNAs possibly encoding this enzyme, by expression cloning of human melanoma libraries in COS cells expressing the substrate ganglioside G_{D3}. Pursuing a similar approach, we have isolated additional clones that can induce 9-O-acetylation. One clone present in a melanoma library encodes a fusion protein between a bacterial tetracycline resistance gene repressor and a sequence reported to be part of the P3 plasmid. Expression of the open reading frame is necessary for inducing 9-O-acetylation, indicating that this is not a reaction to the introduction of bacterial DNA. Another clone from a rat liver cDNA library induced 9-O-acetylation on COS cells expressing α2–6-linked sialic acids, and encodes an open reading frame identical to the Vitamin D binding protein. However, truncation at the 5′ end eliminates the amino-terminal hydrophobic signal sequence, predicting cytosolic hyperexpression of a truncated protein. Thus, diverse types of cDNAs can indirectly induce sialic acid 9-O-acetylation in the COS cell system, raising the possibility that the real enzyme may be composed of multiple subunits which would not be amenable to expression cloning. Importantly, the cDNAs we isolated are highly specific in their ability to induce 9-O-acetylation either on α2–6-linked sialic acids of glycoproteins (truncated vitamin D binding protein) or on the α2–8-linked sialic acids of gangliosides (Tet^r fusion protein). These data confirm our prior suggestion that a family of O-acetyltransferases with distinctive substrate specificities exists in mammalian systems.

Key words: sialic acid/expression cloning/9-O-acetylation

Introduction

Sialic acids are found throughout the deuterostome lineage of the animal kingdom (Schauer, 1982). The most common sialic acid is N-acetyl-neuraminic acid (Neu5Ac), which is likely to be the metabolic precursor of most other members of the family (Schauer, 1991; Varki, 1992). One of the more common modifications of sialic acids is the presence of O-acetyl esters at the 7- and/or 9-position (Schauer, 1991; Varki, 1992). Increasing evidence suggests that these modifications affect embryonic development, and alter recognition of the parent sialic acid molecule by endogenous lectins, antibodies, and viruses (Cheresh *et al.*, 1984; Corfield *et al.*, 1986; Herrler and Klenk, 1991; Schauer, 1991; Varki, 1992, 1997; Varki *et al.*, 1991; Powell and Varki, 1995; Crocker and Feizi, 1996; Kelm *et al.*, 1996). The addition of these O-acetyl groups occurs after the sialic acid is transferred to a glycoconjugate, and is mediated by a sialic acid-specific Acetyl Coenzyme A (AcCoA)-dependent O-acetyltransferase activity that has been reported in a variety of systems (Schauer, 1978; Schauer, 1988; Diaz *et al.*, 1989; Higa *et al.*, 1989; Sjoberg *et al.*, 1992; Butor *et al.*, 1993; Hayes and Varki, 1993; Chammas *et al.*, 1996). O-Acetyl esters initially present at the 7-position can migrate to the 9-position under physiological conditions (Varki and Diaz, 1984; Kamerling *et al.*, 1987). This raised the possibility that the enzyme might transfer acetyl groups primarily to the 7-position, with subsequent migration to the 9-position. However, in the systems studied so far, transfer to both the 7- and the 9-position has been found (Schauer, 1988; Diaz *et al.*, 1989; Butor *et al.*, 1993). Unfortunately, despite years of effort by many groups, this O-acetyltransferase activity has so far proven intractable to solubilization and purification. One possible reason is that this enzyme may act by a novel mechanism, involving transmembrane transfer of acetate groups (Diaz *et al.*, 1989; Higa *et al.*, 1989). Thus, solubilization from the membrane seems to affect enzyme integrity, and activity assays involving exogenous acceptors have been very difficult to develop (Diaz *et al.*, 1989; Butor *et al.*, 1993).

An alternate approach practiced with great success in the glycosyltransferase field has been expression cloning, via detection of cell surface expression of the enzyme product. This approach involves transfection and hyperexpression of a cDNA library from a cell type known to express the enzyme or protein of interest (Aruffo and Seed, 1987; Seed and Aruffo, 1987). In the case of a cell surface protein product, enrichment of the episomally replicating plasmid is obtained by isolating cells expressing the protein, detected by monospecific antibodies (Aruffo and Seed, 1987; Seed and Aruffo, 1987). In the case of a glycosyltransferase, the product appearing on the cell-surface is a specific oligosaccharide, which is detected by an appropriate antibody or lectin (Natsuka and Lowe, 1994; Fukuda *et al.*, 1996). An absolute requirement for success in both cases is that the protein or enzyme to be cloned is the product of a single messenger RNA. This has fortunately proven to be the case for a large number of glycosyltransferase enzymes studied so far (Natsuka and Lowe, 1994; Fukuda *et al.*, 1996; Larsen *et al.*, 1989; Kukowska-Latallo *et al.*, 1990; Lowe *et al.*, 1990, 1991; Smith *et al.*, 1990; Nagata *et al.*, 1992; Nara *et al.*, 1994; Sasaki *et al.*, 1994; Smith and Lowe, 1994; Eckhardt *et al.*, 1995; Haslam and Baenziger, 1996; Mühlenhoff *et al.*, 1996; Nakayama and Fukuda, 1996; Nakayama *et al.*, 1996). An additional requirement for success in expression cloning of a glycosyltransferase cDNA is that the cell line used must express the precursor substrate structure that is a specific target for the enzyme to be

cloned. In the case of sialic acid O-acetylation, the target is sialic acid, which is present on the surface of the CHO and COS cells that are commonly used for expression cloning.

Neither of these two cell types constitutively expresses 9-O-acetylation (Shi *et al.*, 1996b). However, all of the sialic acids on these cell surfaces are α2–3-linked (Lee *et al.*, 1989). On the other hand, we had previously noted a strong preference for 9-O-acetylation to be found on α2–6 and α2–8-linked sialic acids in naturally occurring systems (Diaz *et al.*, 1989; Higa *et al.*, 1989; Sjoberg *et al.*, 1992). To increase the probability of success in expression cloning, we therefore stably transfected CHO cells or COS cells with cDNAs encoding the enzymes ST6Gal I (adds α2–6-linked sialic acids mainly to N-linked chains; Weinstein *et al.*, 1982, 1987; Grundmann *et al.*, 1990; Svensson *et al.*, 1990) or ST8Sia I (adds an α2–8-linked sialic acid to the α2–3-linked sialic acid of the ganglioside G_{M3}, converting it into G_{D3}; Nara *et al.*, 1994; Sasaki *et al.*, 1994). To our surprise, both these transfections induced the appearance of 9-O-acetylation on CHO cells (Shi *et al.*, 1996b), suggesting that these cells already had an endogenous O-acetyltransferase activity with preference for sialic acids in these linkages, thus making them unsuitable for expression cloning. Fortunately, similarly transfected COS cells did not show this induction, and hence could be used for this purpose.

In pursuing this strategy, we noticed that several apparently unrelated clones induced low levels of 9-O-acetylation. While we were puzzling over this result, two other groups published their reports using the identical approach. In each case, expression of human melanoma libraries in COS cells expressing the substrate ganglioside G_{D3} gave rise to clones capable of inducing 9-O-acetylation of the outer sialic acid residue on this substrate. In the first report, the deduced amino acid sequence of the 47.4 kDa cDNA product was found to be closely related to the milk fat globule membrane glycoprotein, a secreted mucin-type molecule (Ogura *et al.*, 1996). In the second instance, the 60.9 kDa cDNA product was predicted to be a very hydrophobic protein with multiple membrane-spanning regions, and studies with permeabilized cells indicated an enhanced incorporation of acetyl CoA into membrane compartments (Kanamori *et al.*, 1997). However, antibody localization of the latter protein indicated a wide-spread distribution in ER-like compartments. Furthermore, the proposed function of this protein (transport of acetyl CoA into the Golgi) does not fit with the mechanism previously proposed for the O-acetyltransferase enzyme (Higa *et al.*, 1989). Here, we report on our initial attempts at expression cloning of this elusive activity. Our results sound a cautionary note toward the use of this approach for solving this particular problem, and indicate that the real O-acetyltransferase has not been cloned by any group, to date. More importantly, they provide confirmatory evidence for the presence of more than one sialic acid O-acetyltransferase activity that can be induced in COS cells by different gene products.

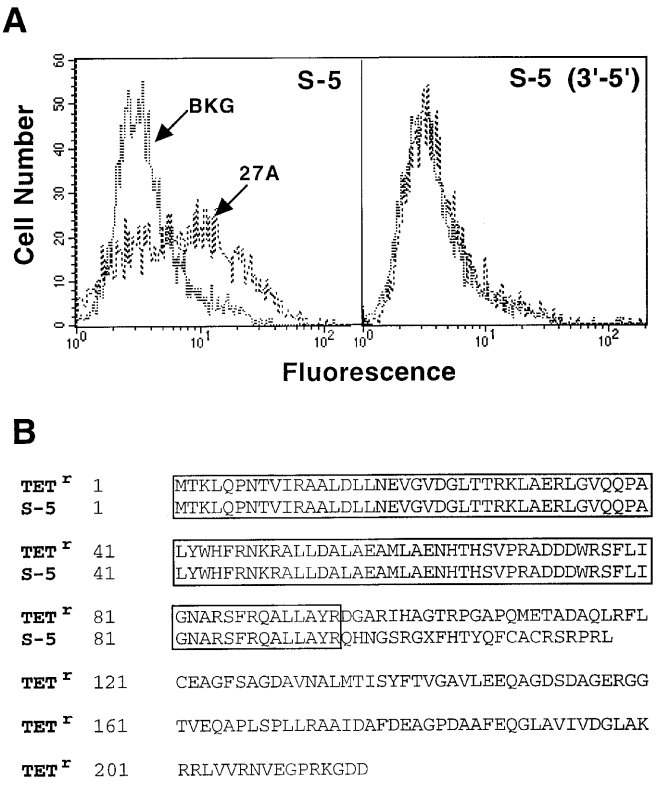


Fig. 1. A cDNA from a human melanoma library that induces 9-O-acetylation of ganglioside G_{D3} in COS.G_{D3} cells. (**A**) 1×10^6 COS cells that were stably expressing ST8Sia I enzyme (G_{D3} synthase) were transfected with 5 μg plasmid DNA (S-5) isolated from a human melanoma cDNA library as described under *Materials and methods*. Washed cells were stained with mAb27A followed by FITC-conjugated goat anti-mouse IgG (BKG = secondary antibody alone), and analyzed by flow cytometry. The right panel shows the results obtained when transfecting an equal amount of plasmid containing the same cDNA in a reversed (3′-5′) orientation. (**B**) Translation of the complete open reading frame of the cDNA insert (S-5) that induces 9-O-acetylation, showing the nearly identical match of the amino-terminal end (boxed region) to the reported sequence of the Pseudomonas Tet^r gene product (GenBank accession no. X75761). The complete predicted open reading frame of the isolated insert is identical to that found in Patent EP0330191 of GenBank accession no. I07249 (presumed to be derived from the P3 plasmid).

Results and discussion

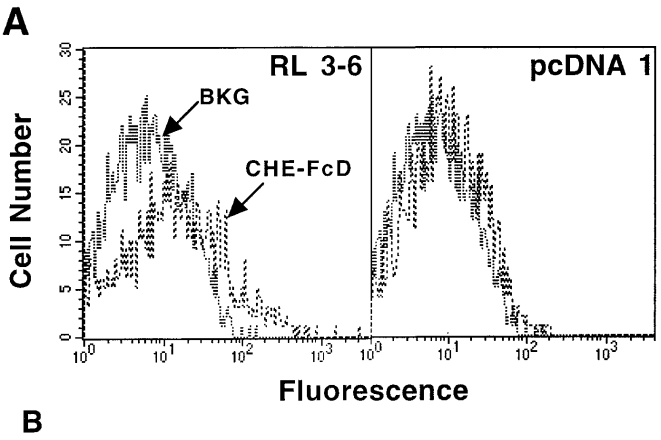
Isolation of cDNA clone from a human melanoma library capable of inducing 9-O-acetylation of α2–8 linked sialic acid on ganglioside G_{D3}

Human melanoma cells are known to 9-O-acetylate the terminal α2–8 linked sialic acid on ganglioside G_{D3} (Cheresh *et al.*, 1984). Since COS cells express the precursor G_{M3}, we first stably transfected these cells with G_{D3} synthase (ST8Sia I), and then attempted expression cloning with a human melanoma cDNA library prepared in the pCDM8 vector. To isolate cells expressing 9-O-acetylation, we used FACS after staining with 27A, a MAb specific for 9-O-acetyl G_{D3} (Dekan *et al.*, 1990; Reivinen *et al.*, 1992). FACS isolation, plasmid extraction, and retransfection were carried out as described in *Materials and methods*. After two rounds of enrichment by FACS, final isolation of clones was carried out by sibling selection of 20 pools using 27A and checking by flow cytometry. We finally obtained one clone that induced high levels of cell surface 9-O-acetyl-G_{D3} (Figure 1A, left panel). Upon complete sequencing, this clone was found to encode a fusion protein between Tet^r (a repressor of the bacterial tetracycline resistance gene Tet^a; Hillen and Berens, 1994) and an unknown sequence previously reported as part of the bacterial P3 plasmid (Figure 1B, GenBank accession numbers X75761 and I07249). This cDNA must have been serendipitously incorporated into the melanoma cDNA library during its preparation using the parent pCDM8 plasmid, which was originally grown in MC1061 *E.coli* that is known to harbor the P3 plasmid. To rule

out the possibility that this induction of O-acetylation was a nonspecific reactive response to introduction of bacterial DNA, we also transfected a clone containing the reversed (3′-5′) sequence. As shown in Figure 1A (right panel), this clone does not cause induction of O-acetylation, indicating that expression of the open reading frame is necessary for this response. The presence of several hydrophobic stretches suggests that the gene product might be associated with membranes. However, given the mechanism of action of the Tet^r repressor gene product (a transcription repressor), the induction is more likely to be the consequence of an alteration in O-acetyltransferase gene expression by this protein.

Isolation of a rat liver cDNA clone capable of inducing 9-O-acetylation of α2–6 linked sialic acids

Another batch of COS cells were stably transfected with ST6Gal I to give expression of terminal α2–6 linked sialic acids. These cells were then transfected with a cDNA library from rat liver, where such residues are known to be 9-O-acetylated (Diaz *et al.*, 1989; Hayes and Varki, 1993). To isolate cells expressing 9-O-acetylation, we used CHE-FcD, a recombinant soluble chimera of the Influenza C hemagglutinin-esterase fused to the hinge and Fc regions of human IgG_1 and treated with DFP (Klein *et al.*, 1994). The process of FACS isolation, plasmid recovery, and retransfection was very similar to that described above. After two rounds of enrichment by FACS, final isolation of clones was carried out by sibling selection of 20 pools by flow cytometry detection using CHE-FcD. Several cDNA clones induced expression of very low levels of 9-O-acetylation, but were not studied further. One clone that gave a higher level of 9-O-acetylation (see Figure 2A) was sequenced from the 5′ and 3′ ends. Both ends showed a nearly complete identity with the previously reported cDNA (Figure 2B) encoding the rat vitamin D-binding protein (GenBank accession number M12450), which is synthesized by the liver (Cooke, 1986; Haddad, 1995). Of note, the isolated clone was truncated by 6 bases, eliminating the first two amino acids of the open reading frame of the native protein (see Figure 2B). This predicts a start site from the second methionine at normal position (M123), which interestingly coincides with the junction between the first two domains of the native protein (Cooke, 1986). Sequencing from the 3′ end showed a complete identity with the 3′ end of the native cDNA encoding the same protein (including the polyA tail; see Figure 2B). Since the N-terminal signal sequence was eliminated by the truncation and there are no other extended hydrophobic sequences, this gene product cannot enter the ER-Golgi pathway and must be expressed in the cytosol. Since this cDNA could not be encoding the O-acetyltransferase itself and was essentially identical to the rat vitamin D binding protein at both 5′ and 3′ ends, we chose not to complete the sequencing of the central portion. Interestingly, partial sequencing of one other clone that gave very low levels of 9-O-acetylation showed identity to the previously reported cDNA for rat preproalbumin (data not shown, identical to GenBank accession numbers V01222,J00698). As with the first clone, this one was incomplete at the 5′ end, and complete at the 3′ end (data not shown). Again, the amino-terminal end of native protein was eliminated by the truncation, predicting a start site from the methionine at normal position 148. It is of particular note that the closest reported homologue of the vitamin D binding is albumin (Cooke, 1986). Thus, two homologous proteins with known lipid binding properties (vitamin D binding protein and albumin) are capable of inducing 9-O-acetylation on COS cells expressing



B

5′end

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RL3-6:       AGGGTTCTGGTTCTCCTGCTGGCCTTAGCCTTTGGGCATGCCCTAGAGAGAGGCCGAGAT 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP: ATGAAGAGGGTTCTGGTTCTCCTGCTGGCCTTAGCCTTTGGGCATGCCCTAGAGAGAGGCCGAGAT 66
         M  K  R  V  L  V  L  L  L  A  L  A  F  G  H  A  L  E  R  G  R  D

RL3-6:       TATGAGAAGGATAAAGTTTGCCAGGAACTCTCCACGCTGGGGAAAGATGACTTCAGATCT 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       TATGAGAAGGATAAAGTTTGCCAGGAACTCTCCACGCTGGGGAAAGATGACTTCAGATCT 126
              Y  E  K  D  K  V  C  Q  E  L  S  T  L  G  K  D  D  F  R  S

RL3-6:       CTATCACTAATCCTATACAGCAGGAAGTTTCCCAGTAGCACCTTTGAACAGGTCAGTCAG 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       CTATCACTAATCCTATACAGCAGGAAGTTTCCCAGTAGCACCTTTGAACAGGTCAGTCAG 186
              L  S  L  I  L  Y  S  R  K  F  P  S  S  T  F  E  Q  V  S  Q

RL3-6:       CTTGTGAAGGAAGTTGTCTCCTTAACTGAGGAATGCTGTGCTGAGGGAGCAGATCCCAAC 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       CTTGTGAAGGAAGTTGTCTCCTTAACTGAGGAATGCTGTGCTGAGGGAGCAGATCCCAAC 246
              L  V  K  E  V  V  S  L  T  E  E  C  C  A  E  G  A  D  P  N

RL3-6:       TGCTATGACACCAGGACTTCGGAGCCGTCTATTAAGTCCTGTGAAAGTGACGCCCCCTTT 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       TGCTATGACACCAGGACTTCGGAGCTGTCTATTAAGTCCTGTGAAAGTGACGCCCCCTTT 306
              C  Y  D  T  R  T  S  E  L  S  I  K  S  C  E  S  D  A  P  F

RL3-6        CCCGTTCACCCCGGGACTTCCGAGTGTTGCACGAAGGAGGGGCTAGAGCGGAAACTCTGC 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       CCCGTTCACCCCGGGACTTCCGAGTGTTGCACGAAGGAGGGGCTAGAGCGGAAACTCTGC 366
              P  V  H  P  G  T  S  E  C  C  T  K  E  G  L  E  R  K  L  C

              M  A  A  L  S  H  Q  P  Q  E  F
RL3-6:       ATGGCTGCCCTCAGTCACCAGCCACAGGAATTT......
             |||||||||||||||||||||||||||||||
RVDBP:       ATGGCTGCCCTCAGTCACCAGCCACAGGAATTT......
              M  A  A  L  S  H  Q  P  Q  E  F
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3′end

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                C  S  I  N  S  P  P  R  Y  C  S  S  Q  I  D  A  E  M  R  D
RL3-6:       TGCTCCATAAACTCTCCTCCACGCTACTGTAGCTCACAGATTGATGCAGAAATGAGAGAC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       TGCTCCATAAACTCTCCTCCACGCTACTGTAGCTCACAGATTGATGCAGAAATGAGAGAC 1421
                C  S  I  N  S  P  P  R  Y  C  S  S  Q  I  D  A  E  M  R  D

                I  L  Q  S  *
RL3-6:       ATCCTGCAGTCCTGATGCAGGGTATATGCAATGCCTTGGACCTAGAACTGGACCACTCTG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       ATCCTGCAGTCCTGATGCAGGGTATATGCAATGCCTTGGACCTAGAACTGGACCACTCTG 1481
                I  L  Q  S  *

RL3-6:       GAAAAATTGCCAGATAACAAAATCAAGCAAATACCACAGAGATGGCCTTCCAGGAGGACC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       GAAAAATTGCCAGATAACAAAATCAAGCAAATACCACAGAGATGGCCTTCCAGGAGGACC 1541

RL3-6:       ACCAGGATAATTCCATTTTTCCCATCTACAATGTTTTTTGATGAATTATAAAAATAAATT 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       ACCAGGATAATTCCATTTTTCCCATCTACAATGTTTTTTGATGAATTATAAAAATAAATT 1601

RL3-6:       GAAATATGGTGCAAACTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RVDBP:       GAAATATGGTGCAAACTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. A cDNA from a rat liver library that induces 9-O-acetylation of sialic acid on ST6Gal I expressing COS cells. (**A**) COS cells that were stably expressing ST6Gal I enzyme were transfected with a cDNA (RL 3–6) isolated from a rat liver library as described under *Materials and methods*. The washed cells were stained with CHE-FcD followed by FITC-conjugated goat anti-Human IgG (BKG = secondary antibody alone), and analyzed by flow cytometry. The right panel shows the results obtained when transfecting an equal amount of the vector (pcDNA3) without an insert. (**B**) 5′ end of the cDNA insert that induces 9-O-acetylation, showing the nearly identical match to the reported sequence of the rat vitamin D binding protein (GenBank accession number M12450). Note that the predicted open reading frame of the isolated insert does not include the initiator methionine of the native protein, and therefore must begin at the second methionine encoded by bases 367–369. Sequencing of the 3′ end of the insert showed identity to the 3′ end of the rat vitamin D binding protein, including the translational stop site and the poly-A tail.

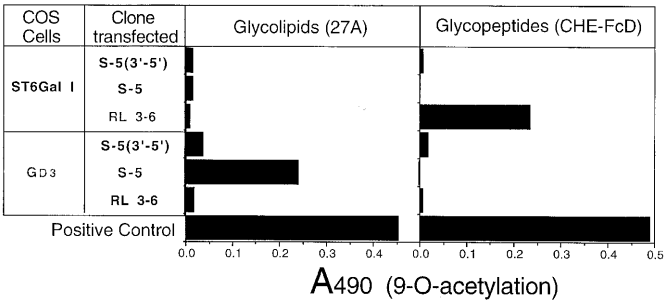

Fig. 3. Differential effects of two cDNA clones in inducing sialic acid 9-O-acetylation on different classes of glycoconjugates. The different cDNAs were transfected into modified COS cells as shown: S-5, Tet^r fusion protein, RL 3–6, truncated rat vitamin D binding protein, S-5 (3′-5′), S-5 cDNA in a reversed orientation. Total ganglioside extracts or glycopeptides from the transfected cells were studied by ELISA for the presence of 9-O-acetylation with MAb 27A or probe CHE-FcD as described under *Materials and methods*. Positive controls are: gangliosides, human melanoma glycolipids, glycopeptides, rat liver glycopeptides.

α2–6 linked sialic acids when expressed in a truncated form in the cytosol. It is also important to note that the same clones did not induce 9-O-acetylation in parental COS cells that have primarily α2–3 linked sialic acids (data not shown).

The isolated clones selectively induce 9-O-acetylation on specific classes of glycoconjugates

It is obvious that none of these isolated clones encode the sialic acid O-acetyltransferase itself. Rather, they must induce the expression and/or activity of an endogenous activity by indirect mechanisms. If these mechanisms were very non-specific (e.g., altering the availability of the donor AcCoA) each isolated clone should induce 9-O-acetylation on all the different classes of glycoconjugates. To explore this possibility, we transfected the clones encoding the truncated vitamin D–binding protein or the Tet^r repressor gene into the host COS cells that expressed either α2–6 linked or α2–8 linked sialic acids. As a negative control, we used the 3′-5′ reversed sequence of the truncated Tet^r repressor gene. As shown in Figure 3, the two clones selectively induced O-acetylation either on glycoproteins (the vitamin D binding protein in cells with α2–6 linked sialic acids) or on gangliosides (the Tet^r repressor gene in cells with α2–8 linked sialic acids). Transfection of the 3′-5′ oriented cDNA of the Tet^r-related protein did not induce 9-O-acetylation (see Figure 3). These data indicate that each gene product must be selectively inducing the activity of a distinct and specific 9-O-acetyltransferase.

Conclusions and perspectives

While these results can be considered disappointing with regard to cloning the O-acetyltransferase, several very useful conclusions emerge. First, two other groups have isolated different clones capable of inducing 9-O-acetylation in COS cells with α2–8 sialic acid linkages (Ogura *et al.*, 1996; Kanamori *et al.*, 1997). Only one of these clones does have hydrophobic sequences of the type that would be expected for the true sialic acid O-acetyltransferase (Kanamori *et al.*, 1997). However, this protein (called AT-1) does not have the expected Golgi localization (Sjoberg *et al.*, 1992; Hayes and Varki, 1993; Chammas *et al.*, 1996), and its proposed activity (AcCoA transport) does not fit with the previously proposed mechanism for the enzyme (transmembrane transfer of the acetyl group; Diaz *et al.*, 1989; Higa *et al.*, 1989). Our data indicate a likely explanation: that several different cDNAs can indirectly induce 9-O-acetylation in COS cells. The failure to isolate the true 9-O-acetyltransferase cDNA might be explained by (1) low frequency of representation in the libraries being screened; (2) a very large size, outside the limits of the insert sizes in the libraries used; or (3) the existence of more than one polypeptide component required to reconstitute the enzyme. The last possibility is supported by the extreme instability of the native enzymatic activity to purification and the novel transmembrane mechanism proposed for its action (Higa *et al.*, 1989). Regardless, these data sound a note of caution to the many groups now attempting to clone this activity using the expression cloning method. Fortunately, some very useful information has emerged from this work. The fact that the cDNAs isolated induce 9-O-acetylation on specific classes of glycoconjugates strongly supports our prior predictions based on more indirect evidence (Sjoberg *et al.*, 1992; Butor *et al.*, 1993; Shi *et al.*, 1996b) that there must be more than one distinct form of mammalian sialic acid-specific 9-O-acetyltransferase. The present data indicate the existence of at least two forms of the activity—one working on α2–6 linked sialic acids of N-linked sugars chains (Butor *et al.*, 1993; Hayes and Varki, 1993; Shi *et al.*, 1996b), and another working on α2–8 linked sialic acids of gangliosides (Sjoberg *et al.*, 1992; Shi *et al.*, 1996b). Based on other independent data, we can also now suggest a third enzyme working on as yet unidentified Sia linkages of mucin-type O-linked chains (Shi *et al.*, 1996a; Krishna and Varki, 1997). The cloning of the cDNAs encoding these activities is needed to clarify if the size of this enzyme family is even larger. This will also provide the critical tools to explore the selective expression and functions of the different types of 9-O-acetylation during development and differentiation and in the interactions with microbes.

Materials and methods

Materials

Most of the materials used were from the Sigma Chemical Co. The following materials were obtained from other sources indicated: fluorescein isothiocynate-conjugated goat anti-mouse IgG Ab, Pierce; Proteinase K, Life Technologies, Inc.; peroxidase-conjugated goat anti-mouse IgG Ab, Bio-Rad; fluorescein isothiocynate-conjugated goat anti-human IgG, CalTag Laboratories; diisopropyl fluorophosphate (DFP), Aldrich; fetal calf serum, Hyclone; Xenobind microwell plates, XENOPORE Corp. Protein assays were determined with the bicinchonic acid protein assay reagent kit (Pierce) using BSA as a standard. All other chemicals were of reagent grade or better, and were obtained from commercial sources.

Cell Lines, monoclonal antibodies, and probes

Human melanoma cells (Melur) were from David Cheresh, Scripps Research Institute, and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The mouse IgG_3 anti-G_{D3} monoclonal antibody R-24 (American Type Culture Collection; Pukel *et al.*, 1982), and the IgG_3 anti-9-O-acetyl-G_{D3} monoclonal antibody 27A (M. Farquhar, UCSD) (Dekan *et al.*, 1990; Reivinen *et al.*, 1992) were used as hybridoma supernatants. The soluble chimeric protein CHE-

Fc, consisting of the extracellular domain of Influenza C hemagglutinin-esterase fused to Fc portion of human IgG_1, was generated as described previously (Klein *et al.*, 1994). The modified form CHE-FcD was made by treating CHE-Fc with 1mM diisopropyl fluorophosphate to irreversibly inactivate the esterase (Klein *et al.*, 1994). The CHE-Fc molecule specifically releases 9-O-acetyl esters from Sias, whereas CHE-FcD specifically recognizes and binds to 9-O-acetylated Sias (Klein *et al.*, 1994).

Extraction of gangliosides and glycopeptides from cultured cells

Washed cell pellets were sequentially extracted by $CHCl_3$/MeOH(2:1, 1:1, and 1:2,v/v), using brief homogenization. Extracts were pooled, dried down, and resuspended in 3 volumes of ice-cold deionized water, homogenized at 4°C, and the homogenate added dropwise to 8 volumes of methanol at room temperature (RT) with constant stirring. Chloroform (4 volumes) was then added and mixed. After centrifugation, the supernatant was collected and adjusted to a final chloroform/methanol/water ratio of 4:8:5.6 (v/v/v). After phase separation, gangliosides were enriched in the hydrophilic upper phase, which was dried down, resuspended in methanol, and kept at -20°C until use. The denatured proteins left behind after the lipid extraction were digested completely with Proteinase K. The supernatant was then concentrated using Centricon-10 (Amicon, Beverly, MA), in order to remove free amino acids.

ELISA plate assays for 9-O-acetylated gangliosides and glycopeptides

Total ganglioside extracts or glycopeptides were studied by ELISA. Lipids were applied to the plate in 45% methanol, and dried at RT. Glycopeptides were adsorbed onto 96-well "Xenobind" plates. The effects of base on reactivity were assessed by treating with 0.1 M NaOH at 4°C for 30 min (control experiments show that base treatment does not cause loss of antigen from the wells, data not shown). After treatment, the plates were extensively washed with PBS, blocked with 2% BSA in PBS for 2 h, and then incubated with either a MAb 27A or with CHE-FcD at 4°C for 2 h. After washing three times with 1% BSA, horseradish peroxidase–conjugated goat anti-mouse IgG (MAb probe) or goat anti-human IgG antibodies (CHE-FcD probe) was added (each at 1:1000 dilutions) for 1 h. After washing, the reaction was developed with orthophenylenediamine dihydrochloride (OPD). Background levels measured by adding the secondary antibody alone were subtracted in all cases.

Construction of stable transfectants for targeting cells

Plasmids carrying the ST6Gal I and ST8Sia I were constructed as reported previously (Shi *et al.*, 1996b). Stable expression of the same cDNAs were obtained by transfecting pcDNA3-based constructs (containing the *neo* gene) into parental COS cells using the Lipofectamine reagent (BRL). After 72 h, cells were passaged 1:10 into selective medium containing 1mg/ml of geneticin (G418; GIBCO Labs, Grand Island, NY). G418-resistant cells were subcloned by limiting dilution, and levels and linkages of sialylation expressed by individual clones of cells were analyzed. Two clones named COS·G_{D3} and COS·ST6Gal I were isolated and used as target cells for expression cloning.

Flow cytometric analysis of O-acetylation on transfected cells

Transfected cells (10^6) were released from the culture plate with trypsin or 0.5 mM EDTA in PBS, incubated with mAb 27A (recognizes 9-O-acetyl-G_{D3}) or CHE-FcD (recognizes O-acetylated Sias), followed by FITC-conjugated goat anti-mouse IgG or goat anti-human secondary antibody. After final washing, the cells were analyzed by flow cytometric analysis on a Becton Dickinson FACScan machine.

Isolation of cDNA clones inducing 9-O-acetylation on ganglioside G_{D3}

A mammalian expression vector-based (pcDM8) human melanoma cDNA library was a gift from Drs. John Lowe and Robert Larsen. COS·G_{D3} cells(2.4×10^7) were transfected with 160 μg of this library using Lipofectamin. After 68 h, the 9-O-acetylated G_{D3} positive cells were isolated by fluorescence-activated cell sorting (FACS) using mAb 27A and a FACStar unit (Becton Dickinson). Plasmid DNA was isolated (Aruffo and Seed, 1987; Seed and Aruffo, 1987) from the sorted cells, and then expanded in the host bacteria MC1061/P3 cells in the presence of ampicillin and tetracycline. The plasmids obtained were transfected into COS·G_{D3} cells again and another cycle of isolation of 9-Oacetylated G_{D3} positive cells was done. Recovered plasmids were divided into pools of plasmids, and each pool tested for its capability to induce expression of O-acetylated G_{D3} by re-transfection into COS·G_{D3} cells. Once a particular pool was identified to induce the expression of O-acetylation, plasmids in that pool were further divided into subpools containing a smaller number. Further narrowing of the pools eventually gave the isolation of a single cDNA that induces O-acetylation or is the O-acetyltransferase itself. The nucleotide sequencing of the insert was determined in both directions by the dideoxy nucleotide chain termination method, using the services of UCSD CFAR Molecular Biology Core Facility.

Isolation of a cDNA clone inducing 9-O-acetylation on α2,6-sialylated N-linked glycoproteins

A mammalian expression vector-based (pcDNA3) rat liver cDNA library was purchased from Invitrogen (San Diego, CA). COS·ST6Sia I cells (2.4×10^7) were transfected with 160 μg of this library using Lipofectamin. After 68 h, 9-O-acetylated Sia positive cells were isolated by FACS (FACStar, Becton Dickinson) after staining with CHE-FcD, which recognizes 9-O-acetylated sialic acids. Plasmid DNA was isolated from the sorted cells and then expanded in the host bacteria DH5α cells in the presence of ampicillin. Pools and subpools of plasmids were obtained using the same approach described above. Further narrowing down of the pools eventually gave the isolation of cDNA clones that induced varying amounts of 9-O-acetylation. The nucleotide sequencing of these inserts was determined in both directions as described above.

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Abbreviations

CHE-Fc, chimeric protein made of InfCHE (Influenza C hemagglutinin-esterase with the fusion peptide eliminated by mutation) and the Fc portion of human IgG_1; CHE-FcD, DFP-treated CHE-Fc (esterase activity irreversibly inactivated).

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