First Synthetic Probe for the Detection and Quantification of a Protein with a Potential α,(2→8)Sialyltransferase Activity

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There is considerable interest in monitoring α,(2→8)sialyltransferase (ST8) levels; however, there are few specific and sensitive methods to directly detect and quantitate the protein. This paper reports the development of a synthetic probe composed of oxidized colominic acid coupled to biotinyl-L-lysine hydrazide to detect and quantify ST8 with putative "initiase" activity and its use in three solid-phase applications. The detection limit observed for ST8 purified from K562 cells was ∼2 pg by dot-blot analysis. In Western blots the probe bound and specifically recognized a protein band corresponding to ST8. In ELISA a linear dose response was obtained for pure protein in the range of 50−200 pg. Analysis of 3′-azido-3′deoxythymidine-treated cells by all three methods showed a reduction in ST8 compared to control cells; treated cells had 73% of control levels by ELISA. This probe will be useful for studies on the expression ST8 and its role in glycoconjugate biosynthesis.

INTRODUCTION

A large number of sialyltransferases (STs)[1] are required to terminally add sialic acid on glycoconjugates (*1*, *2*). In the case of humans, the genome encodes probably more than 20 different STs, but up until now about 15 different human ST cDNAs have been encoded and characterized (see the web site given in ref *3*). Among the various ST activities, two of them, namely, ST8Sia II (also known as STX or "initiase") and ST8Sia IV (also known as PST or "elongase"), mediate the so-called polysialylation process, a unique type of modification in which tens of sialic acid residues are joined through an α,(2→8) linkage in a linear array (*4*); usually, polysialic acid (PSA) chains formed by ST8Sia IV are longer than those synthesized by ST8Sia II (*5*−*7*). Due to these interesting and actual observations, remarkable efforts have been directed to develop simple, reliable, inexpensive, and specific assays for different STs in general, but at the moment none is available for the direct detection and quantification of ST8 viewed just as a protein. On the other hand, specific assays in which fluoresceinyl- or radiolabeled CMP−NeuAc is used as donor and asialoglycoproteins or gangliosides are used as acceptors, allow the determination of ST8 enzymatic activity (*8*−*15*). In addition, it has been also possible to set up a protocol for parallel screening of several samples using microtiter filtration plates (*16*). However, due to the heterogeneity of putative acceptor sites, assays that make use of labeled donors hardly discriminate the different ST activities. Nevertheless, the enzyme previously cloned by Livingstone and Paulson (*17*), as well as the subsequent ST8 purified from CHP-134 neuroblastoma cells (*18*), and the affinity-purified ST8 we isolated from K562 cells (*10*, *11*) all recognize solely fetuin as substrate (and not asialofetuin), suggesting that sialyl residues in either the α,(2→3) or α,(2→6) linkage are required for the enzymatic activity: for this reason the enzyme we purified is supposed to possess a potential ST8Sia II activity. Starting from these latter pieces of evidence and taking into strict account our recent studies (*10*, *11*), we report very sensitive and easy assays for the direct detection and quantification of a putative ST8Sia II in cell lysates such as the ones obtained from both control and AZT-treated K562 erythroleukemia human cells.

To this purpose a synthetic probe was prepared comprising oxidized colominic acid coupled to biotinyl-L-lysine hydrazide. In particular, the probe was successfully used in dot-blot, Western blot, and ELISA applications. Thus, many different samples supposed to contain a putative ST8 displaying "initiase"-like activity (ST8Sia II) could be inexpensively screened simultaneously with high sensitivity and specificity in a minimum of time.

EXPERIMENTAL PROCEDURES

Probe Synthesis. Colominic acid (poly[2,8-(*N*-acetylneuraminic acid sodium salt)]; $(C_{11}H_{16}NNaO_8)_n$, Fluka code 27698) was dissolved in 0.1 M sodium *m*-periodate (100 mM sodium acetate, pH 5.5; 2.5 mg/10 mL), and the solution was left at room temperature for 20 min in the dark. Glycerol 86−88% (250 μL) was added to stop the reaction (room temperature for 20 min in the dark). Four dialysis changes against PBS-cations (20 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 1 mM each $CaCl_2$ and $MgCl_2$) were then carried out. Subsequently, N_ϵ-(+)biotinyl-L-lysinehydrazide hydrochloride

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; BSA, bovine serum albumin; MET buffer: 50 mM MES buffer, pH 6.0, containing 1 mM dithiothreitol; NBT, nitro blue tetrazolium chloride; PBS-cations: 20 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 1 mM each $CaCl_2$ and $MgCl_2$; PBS-T, 20 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% (w/v) Tween-20; PSA, polysialic acid; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ST, sialyltransferase; ST8, α,(2→8)sialyltransferase; ST8Sia II or STX, α,(2→8)-sialyltransferase displaying "initiase" activity; ST8Sia IV or PST, α,(2→8)sialyltransferase displaying "elongase" activity; TTBS, 0.05 M Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% (w/v) Tween-20.

10.1021/bc049970s CCC: $27.50
Published on Web 08/07/2004

($C_{16}H_{30}N_6O_3S$·HCl; Fluka code 14415; 2.5 mg/2 mL PBS-cations) was added and the solution incubated at room temperature for 16 h. After the solution had cooled at 0 °C and an equal volume of 30 mM sodium cyanoborohydride (Fluka code 71435) had been added, incubation at 0 °C for 45 min followed. Last, a dialysis step as above was carried out and the solution stored at 4 °C in the presence of 0.02% (w/v) sodium azide.

Cell Cultures. Human erythroleukemia cells (K562), kindly supplied by Dr. L. Di Renzo (Laboratory of Experimental and Pathologic Medicine, University of Rome 'La Sapienza'), were grown as already described (*10*).

Cell Homogenate Preparation and ST8 Purification. K562 cells (6.5×10^8) incubated alone or with 20 μM AZT for 3 h were washed twice with PBS (20 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl), resuspended in 1.45 mL of 50 mM MES buffer, pH 6.0, containing 1 mM dithiothreitol (MET buffer), and then homogenized. Sonication for 10 s × 10 and centrifugation at 1120g for 15 min followed. The supernatant solution was centrifuged at 23000*g* for 1 h and the pellet suspended in 500 μL of 0.1% (w/v) Triton CF-54 in MET buffer, pH 6.0, sonicated for 10 s × 10, and centrifuged at 23000*g* for 30 min. This second supernatant solution was used in all types of analyses.

ST8 was purified from K562 as already reported (*10*).

Dot-Blot. Different amounts of protein homogenates from control and AZT-treated samples, or pure ST8, were separately loaded into each well. The PVDF membrane was then washed in TTBS [0.05 M Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% (w/v) Tween-20] for 10 min and blocked with 1% (w/v) BSA. After three washes, the membrane was soaked in 25 mM HCl for 20 min, washed again, and then incubated with the probe solution (1.3 μg/mL, 1 h, 25 °C). Further washing steps were carried out, and incubation for 1 h with alkaline phosphatase−Extravidine conjugate (Sigma, Milan, Italy) diluted 1:300 000 followed. Blots were developed by using 10 mL of substrate solution composed of 9.9 mL of TBS, 45 μL of BCIP (13 mg/mL in dimethylformamide), and 55 μL of NBT [20 mg/mL in 70% (v/v) dimethylformamide].

SDS−PAGE and Western Blot. Aliquots of the second supernatant solution (8 μg) and pure ST8 (1 μg) were assayed by SDS−PAGE on a 10% gel according to the method of Laemmli (*19*); bands were visualized by Coomassie brilliant blue staining (Figure 2A). Cell lysates and pure ST8, prepared as above and resolved by SDS−PAGE, were blotted onto PVDF membranes as described elsewhere (*20*), with slight modifications. The membrane was washed, blocked, and treated as reported under Dot-Blot.

ELISA. Cell homogenates (100 μL), or the pure enzyme solutions, diluted in 0.08 M carbonate buffer, pH 9.6, were loaded to the wells of a plastic 96-well microtiter plate and left for 3 h at 37 °C. Wells were then washed four times with PBS-T [20 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% (w/v) Tween-20] followed by incubation with 25 mM HCl for 20 min. After a washing step, 100 μL of probe solution (1.3 μg/mL) was added to each well, and the reaction was allowed to go on for 1 h at 37 °C. The wells were then again washed four times with PBS-T before incubation (1 h at 37 °C) with alkaline phosphatase−Extravidine conjugate (Sigma, Milan, Italy) diluted 1:10 000. After the last washes, 100 μL of 1 mg/mL 4-nitrophenyl phosphate bis(cyclohexylamine) salt in 0.1 M glycine, pH 9.4, was poured into each well, and the absorbance was read at 415 nm after 1 h.

Protein Determination. Total protein concentration was determined according to the Bio-Rad protein assay (*21*).

RESULTS AND DISCUSSION

Polysialic acid (PSA) is a linear homopolymer ($n = \sim 8$ to > 100) of α,(2→8)-linked sialic acid joined, most of the time, to an N-linked carbohydrate core (*22*), although a novel member of α,(2→8)sialyltransferase (ST8Sia VI) that sialylates preferentially *O*-glycans has been recently identified (*23*).

However, at the moment it is not clear if the so-called "initiase" activity and the so-called "elongase" activity, both necessary to synthesize PSA, may be subsumed by the two above-mentioned enzymes or are the province of distinct sialyltransferases. It has been reported that cell lines from colon cancer express polysialyltransferases (mainly ST8 IV) without concomitant expression of PSA (*24*). Although different experimental approaches made possible the study on PSA biology (*22*, *25*−*27*), the cellular mechanism by which PSA affects cell interactions has not yet been defined. On the other hand, at present, specific and sensitive assays for ST8 displaying "initiase" activity are scarcely available except the one that makes use of fetuin as glycoprotein substrate acceptor (*10*, *11*, *18*). In the present study we report the first synthetic probe for the direct detection and quantification of a protein displaying a potential, putative ST8Sia II activity. To this purpose some typical methods such as dot-blot, Western blot, and ELISA were set up.

In particular, starting from the evidence that it is possible to purify ST8 basically by colominic acid/polyacrylohydrazido-agarose affinity chromatography (*10*, *11*, *18*), colominic acid was first oxidized and the reaction stopped by incubation with glycerol. Then, after dialysis, the colominic acid solution was recovered and reacted with a biotinylating compound such as biotinyl-L-lysine hydrazide that shows a better solubility in aqueous solution than either biotin hydrazide or biotin LC-hydrazide (*28*). Subsequently, the adduct solution was incubated with sodium cyanoborohydride to further stabilize the bond between the hydrazide and the aldehyde (produced after colominic acid oxidation) (*29*). An additional dialysis step was carried out to remove unbound material and the adduct solution stored a 4 °C. Because our recent studies have been focused on K562 cells expressing an ST8 consistent with an "initiase"-like activity sensitive to AZT (*10*, *11*), this cell system was used to check the ability of our synthetic adduct to recognize ST8Sia II, permitting both the detection and quantitation of the enzymatic protein. To this purpose, three different typical and basic methodological approaches were explored (i.e., dot-blot, Western blot, and ELISA). In all cases, protein extracts obtained after solubilization in Triton CF-54, from both control and AZT-treated K562 cells, were appropriately prepared and analyzed. In dot-blot format the same range of total proteins from both control and AZT-treated cells was exploited (from 0.5 to 20 μg). As shown in Figure 1, the sensitivity limit found for our ST8 purified from K562 cells was ∼2 pg (row ST8). Although by dot-blot analysis it was possible to determine the sensitivity limit of our probe, a different quantitative expression of ST8 between control cells and AZT-treated cells could be only slightly visually appreciated (Figure 1, rows C and T, respectively); furthermore, normal human serum did not give

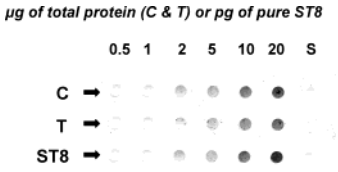

Figure 1. Dot-blot of control (C), AZT-treated (T) K562 cells, and α,(2→8)sialyltransferase (ST8) purified from K562 cells (*10*). S, normal human serum 1:250, 100 μL.

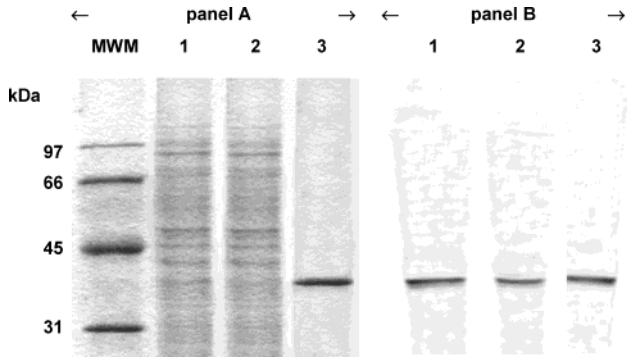

Figure 2. SDS−PAGE (A) and Western blot (B) analyses: (lane 1) control K562 cells; (lane 2) AZT-treated K562 cells; (lane 3) ST8 purified from K562 cells (*10*). MWM, molecular weight markers.

any reaction, indicating the lack of nonspecific binding of our probe at least toward human serum proteins (Figure 1, lane S).

After SDS−PAGE (Figure 2A), samples were blotted onto a PVDF membrane and revelation carried out as reported under Experimental Procedures (Figure 2B). Thus, when the same types of samples were analyzed by Western blot, a specific binding was observed for the protein band that showed a molecular mass of ∼40 kDa and corresponding to ST8, as previously reported (*10*). Interestingly, although the same amount of total proteins was loaded for both types of samples, control sample showed a higher intensity than AZT-treated sample, not only after SDS−PAGE (Figure 2A, lane 1) but also, and much more evidently, after Western blot (Figure 2B, lane 1). Moreover, apart from the net positive reaction observed for ST8, all other protein bands present in cell homogenates did not react with the probe (Figure 2B, lanes 1 and 2). Furthermore, control experiments carried out with different pure commercial sialyltransferases, that is, α,(2→3) (N), α,(2→3) (O), α,(2→6) (N) (Calbiochem), and loaded at the same amount of our pure ST (i.e., 1 μg) did not react versus our probe (data not shown). Eventually, when the pure protein (enzyme) was analyzed by ELISA, a linear dose-dependent response was obtained in the range of 50−200 pg (Figure 3, inset). A similar linear response was observed also for both control and AZT-treated samples, but these latter showed a decrease (73% of control) as calculated from the slope (Figure 3). Remarkably, the value of this decreased amount of protein (73% of control) strictly paralleled the decrease of ST8 activity we previously found in K562 cells treated with 20 μM AZT (75% of control) (*11*). Confirmatory evidence that the probe response in ELISA correlated with ST8 enzymatic activity was achieved through a "reverse" ELISA-like experiment. Briefly, streptavidin-coated microtiter plates (Molecular Probe) were first incubated with our probe and then with K562 cell homogenates from control or AZT-treated cells or with the pure ST8. After intermediate washing steps, elution-in-well by using colominic acid (25 mM) followed. The

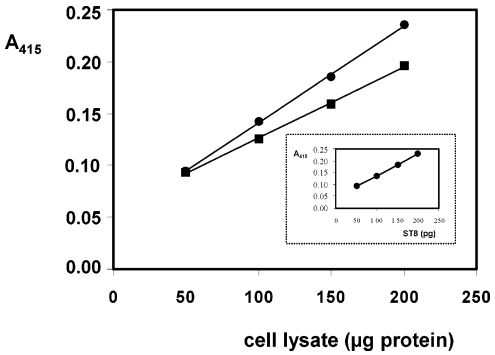

Figure 3. ELISA: absorbance values at 415 nm as a function of cell lysate protein amount in control (●) and AZT-treated (■) K562 cells. (Inset) Standard curve obtained with the pure enzyme ST8 purified from K562 cells (*10*). Data are means of three separate determinations with SD < 3%.

recovered solution was subsequently dialyzed, concentrated, assayed for its enzymatic activity (*10*, *11*), and subjected to dot-blot, Western blot, and ELISA. In all cases, compared to the ST8 enzyme affinity purified, superimposed results were obtained (data not shown). Of course, for an absolute quantitation in other cell systems, the pure protein is required.

It is worth noting that all data here reported, representing the best results achieved, were obtained when incubation with the probe was carried out after incubation with 25 mM HCl (20 min, room temperature). In our opinion this "acidic step" improved all assays probably because the enzymatic protein was freed of some bound anionic compound. This view is partly supported by the evidence that ST8 displaying "initiase"-like activity (ST8Sia II) strongly recognizes and binds to a highly negatively charged molecule such as colominic acid (coupled to polyacrylohydrazido-agarose) and it is eluted from the affinity column by using high colominic acid concentration (*10*, *11*, *18*). However, it should be underscored that although colominic acid binds to ST8Sia II, it does not serve as substrate (*10*, *18*). Last, independently from the analyses we carried out (dot-blot, Western blot, ELISA), the decreased amount of the putative ST8 we found in AZT-treated cells, compared to control cells, was in line with our recent study (*11*).

At the moment further studies are in progress to better characterize the synthetic probe we obtained, mainly from the spectroscopic point of view.

In conclusion, because considerable efforts have been directed to developing simple, reliable, specific, and inexpensive assays for different STs in general, but at present none is available for the direct detection and quantitation of ST8, especially in cell homogenates, we have developed a promising synthetic probe for the direct, rapid, inexpensive, and sensitive detection and quantitation of ST8. Our probe combines the advantage and detection technology of solid-phase reaction, being versatile and capable of simultaneous processing of multiple samples. The probe we synthesized could represent an attractive alternative to the currently used approaches to study ST8, and it is predicted to be a powerful tool for further investigation of ST8.

ACKNOWLEDGMENT

We thank Prof. A. Bozzi for helpful advice during manuscript preparation. This work was supported by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Rome, Italy).

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BC049970S