Dynamic O-GlcNAcylation of the Small Heat Shock Protein αB-Crystallin†

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Received August 15, 1995; Revised Manuscript Received January 11, 1996⊗

ABSTRACT: αB-Crystallin, originally described as a structural lens protein, is now known to be a member of the small heat shock protein family and is expressed in a number of nonlens tissues. This highly conserved 20 kDa protein aggregates with homologous proteins, including αA-crystallin and the small heat shock protein HSP28, to form large heteromeric complexes. Recently, Roquemore et al. (1992) have established that both phosphorylated and unphosphorylated forms of lens αB-crystallin are modified with O-linked *N*-acetylglucosamine, a dynamic posttranslational modification abundant on nuclear and cytoplasmic proteins. In this paper, we have identified the major site of O-GlcNAcylation on lens αB as Thr 170. We have further shown that this modification is not restricted to lens αB-crystallin but occurs on αB isolated from rat heart tissue and human astroglioma cells. Two-dimensional electrophoresis of rat heart αB-crystallin revealed two O-GlcNAcylated forms with mobilities corresponding to the unphosphorylated form (αB2) and an unidentified, slightly more acidic form. Phosphorylated αB-crystallin (αB1) was not detected in the rat heart preparation. The major O-GlcNAcylation site on αB-crystallins from rat heart also appears to be at Thr 170. Metabolic pulse−chase labeling studies of U373-MG astroglioma cells indicated that turnover of the carbohydrate on αB-crystallin is not static but proceeds many-fold more rapidly than turnover of the protein backbone itself, consistent with a regulatory role for O-GlcNAc on this small heat shock protein.

Members of the small heat shock protein family are structurally homologous proteins, ranging in molecular mass from 15 to 30 kDa, whose expression is induced by elevated temperatures or other stress conditions. Small heat shock proteins respond similarly to heat shock, exhibiting rapid association with the nucleus followed by slow relocalization to the cytoplasm and self-assembly into high molecular weight aggregates called heat-shock granules. αB-Crystallin, a 20 kDa protein that migrates on polyacrylamide gels with an apparent M_r of 22K, is a newly recognized member of the small heat shock protein family (Klemenz et al., 1991b). Originally identified as a major structural component of the lens of the eye, αB-crystallin is now known to be expressed in a number of nonlenticular tissues, including heart, skeletal muscle, kidney, and brain (Bhat & Nagineni, 1989; Dubin et al., 1989; Iwaki et al., 1989, 1990b; Nagineni & Bhat, 1989; Longoni et al., 1990). In the lens, αB-crystallin associates with a related gene product, αA-crystallin, to form large heteromeric aggregates with reported molecular masses ranging from 300 to 1000 kDa. In nonlens tissues, αB-crystallin aggregates to form characteristic 15−20 nm heat shock granules (Klemenz et al., 1991b; Longoni et al., 1990) and appears to form large heteromeric complexes with the small heat shock protein HSP-28 in both human skeletal muscle (Kato et al., 1992) and the human astroglioma cell line U373-MG (Kato et al., 1993). αB-crystallins are overexpressed in the brain cells of scrapie-infected hamsters (Duguid et al., 1988) and in certain pathological disorders of the CNS such as Alexander's disease, in which αB is the major component of abnormal astrocytic inclusion bodies (Iwaki et al., 1989). αB-Crystallin also is expressed at elevated levels in NIH-3T3 fibroblasts transfected with Ha-*ras* and V-*mos* oncogenes (Klemenz et al., 1991a) and in retinoblastoma cells (Pineda et al., 1993). Furthermore, αB-crystallin forms insoluble complexes in ischemic heart tissue (Chiesi et al., 1990).

Little is known about the posttranslational control of the small heat shock proteins. However, in human HSP 28 and *Drosophila melanogaster* HSP 26/27, stress-induced phosphorylation occurs concomitantly with transient translocation to the nucleus (Arrigo et al., 1988; Arrigo & Welch, 1987; Lee et al., 1990; Welch, 1985; Vincent & Tanguay, 1982; Crete & Landry, 1990; Rollet & Best-Belpomme, 1986; Landry et al., 1991, 1992). Like other small heat-shock proteins, αB-crystallin exists in both phosphorylated and unphosphorylated forms, designated αB1 and αB2, respectively. Recently, Roquemore et al. (1992) have established that, in the lens, α-crystallins are further posttranslationally modified with O-linked *N*-acetylglucosamine (O-GlcNAc), a form of dynamic glycosylation unique to nuclear and cytoplasmic proteins. The major site of O-GlcNAcylation on bovine lens αA-crystallin was identified as Ser 162, an amino acid near the C-terminus of the molecule. The O-GlcNAcylation site on αB-crystallin was not established, nor was it determined whether αB was glycosylated *in vivo*

† This work was supported in part by Public Health Service Grant NIH R01 CA-42486 to G.W.H. from the National Cancer Institute and by funding from the BCMB program NIH Training Grant 5T3 GM07445 at Johns Hopkins University School of Medicine.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

0006-2960/96/0435-3578$12.00/0

outside of the lens. Although current models of O-GlcNAc function are speculative, compelling cumulative evidence suggests that O-GlcNAc is a dynamic regulatory modification analogous to phosphorylation. Independently of protein synthesis, cellular O-GlcNAc levels fluctuate during the cell cycle (Chou & Omary, 1993; W. J. Kelly, E. P. Roquemore, and G. W. Hart, unpublished observations) and vary in response to mitogenic stimulation (Kearse & Hart, 1991). Relatively few of the hundreds of nuclear and cytoplasmic proteins modified with O-GlcNAc have been identified, but all of those identified undergo reversible phosphorylation/dephosphorylation, all of them form heteromeric complexes with other proteins, and most of them translocate between the cytoplasm and the nucleus [for a review, see Haltiwanger et al. (1992)]. It has been suggested that O-GlcNAc might play a role in regulating the interprotein interactions and subcellular localizations of these proteins. A definitive reciprocal relationship between O-GlcNAcylation and phosphorylation has been demonstrated for several proteins, including the C-terminal domain of RNA polymerase II (Kelly et al.., 1992) and the transactivation domain of the c-myc oncogene protein (Chou et al., 1995a,b). O-GlcNAcylation of c-myc occurs at Thr 58, which is also a known glycogen synthase kinase 3 phosphorylation site and a mutational "hot spot" in human lymphomas. In this paper, we identify the major site of αB-crystallin O-GlcNAcylation. We further demonstrate that O-GlcNAcylation of αB-crystallin is not a phenomenon restricted to the lens but also occurs in heart tissue and in a human astroglioma cell line, locations where αB-crystallin functions as a heat shock protein. Finally, we document the rapid turnover of O-GlcNAc on αB-crystallin in the human astroglioma cell line U373-MG.

MATERIALS AND METHODS

Materials. Polyclonal antiserum to bovine α-crystallin was generously provided by J. S. Zigler, Jr. (National Eye Institute, Bethesda, MD) and recognizes both αA and αB crystallins.

Radioisotopes. UDP-[4,5-^{3}H]galactose (40 Ci/mmol, NEN DuPont), L-[4,5-^{3}H]leucine (154 Ci/mmol, Amersham), and D-[6-^{3}H]glucosamine (40 Ci/mmol, ICN Biochemicals) were used. Unless specified, all chemicals were of reagent grade or better.

Preparation of Lens αB-Crystallin. Lenses were dissected from Sprague-Dawley rats, decapsulated, and frozen at −80 °C until needed. Whole lenses from rhesus monkeys were provided by J. S. Zigler, Jr. (Laboratory of Mechanisms of Ocular Disease, National Eye Institute, Bethesda, MD), decapsulated, and stored at −80 °C. Water-soluble α-crystallins were purified from crude lens homogenates by chromatography on a Sephacryl S-200 sizing column as previously described (Roquemore et al., 1992). αB-Crystallins were separated from αA-crystallins by reverse-phase HPLC using a Dynamax column in 0.1% TFA according to the method described by Swamy and Abraham (1991).

Carbohydrate Detection and Analysis. The methods for O-GlcNAc detection and analysis used in this paper, and briefly summarized below, are described in further detail by Roquemore et al. (1994). Terminal GlcNAc residues were detected by the enzymatic addition of [^{3}H]galactose according to the method of Holt and Hart (1986). Protein samples were reacted in the presence of 2−3 μCi of UDP-[^{3}H]-galactose and 50−75 milliunits of autogalactosylated bovine milk galactosyltransferase. [^{3}H]Galactosylated proteins were separated from unincorporated UDP-[^{3}H]galactose by Sephadex G-50 chromatography in 50 mM ammonium formate and 0.1% SDS. Linkage of carbohydrate to protein was assessed by resistance to peptide:N-glycosidase F (PNGase F) concomitant with sensitivity to base-catalyzed β-elimination. β-Elimination products were further analyzed by chromatography on a 2-m TSK sizing column equilibrated in 0.2 M ammonium acetate and 10% ethanol. Products eluting as disaccharides from the TSK column were further analyzed by high pH anion exchange chromatography with pulsed amperometric detection on a CarboPak MA-1 column using a 25-min isocratic gradient of 200 mM sodium hydroxide at a flow rate of 0.4 mL/min.

Enrichment of αB-Crystallin from Rat Heart. The following method for enrichment of αB-crystallin from rat heart tissue was based on the procedure described by Kato et al. (1992). Hearts were dissected from Sprague-Dawley rats, rinsed with phosphate buffered saline (PBS), and stored at −80 °C until needed. All purification steps were performed at 0−4 °C. For each preparation, approximately 100 g of rat heart were thawed and homogenized in a Waring blender in 5 volumes of homogenization buffer containing 0.5 M GlcNAc, 0.05 M sodium phosphate, pH 7.0, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was then centrifuged at 20000*g* for 40 min, and the supernatant was filtered through cheese cloth. Proteins in the filtrate were precipitated by the gradual addition of ammonium sulfate to 40% saturation and pelleted at 25000*g* for 45 min. The ammonium sulfate pellet was brought to a final volume of 100 mL in DEAE column buffer (0.01 M potassium phosphate, pH 8.0, 0.05 M GlcNAc, 0.5 mM PMSF) and dialyzed for 18 h in 12 000−14 000 MW cutoff Spectrapor membrane (Spectrum Medical Industries, Houston, TX) against two changes of DEAE column buffer (4 L each). Particulate matter was removed by a brief centrifugation, and the supernatant was passed through cheese cloth to remove floating material. The filtrate was applied to a 2.7 × 23-cm DEAE-Sepharose column equilibrated with DEAE column buffer. Proteins were eluted with a linear gradient of 0.01−0.4 M potassium phosphate, pH 8.0. Fractions containing a M_r = 22 000 Coomassie Blue-staining band were pooled, and proteins were precipitated by addition of ammonium sulfate to 50% saturation. The resulting pellet was resuspended in 5 mL of Sephacryl S-200 column buffer (0.1 M Tris, 0.2 M sodium chloride, pH 7.3) and applied to a 2.2 × 46-cm S-200 column flowing at a rate of 10 mL/h. α-Crystallins collected from the void volume of the S-200 column were lyophilized and stored at −20 °C.

Two-Dimensional Electrophoresis. Radiolabeled α-crystallin preparations were resolved in the first dimension by nonequilibrium pH-gradient electrophoresis (O'Farrell et al., 1977) on a Bio-Rad mini-Protean 2-D system at 400 V for 4 h. Tube gels were then run in the second dimension on 15% polyacrylamide gels. Gels were then either electroblotted for Western analysis or stained with Coomassie Blue, impregnated with En^{3}Hance (NEN DuPont, Boston, MA), dried, and exposed to preflashed X-ray film.

Western Blot Analysis. Proteins were electroblotted to prewetted 0.45 m polyvinylidine difluoride (PVDF) membranes (Millipore) for 2 h at 12 V in buffer containing 25

mM Tris, 192 mM glycine, and 20% methanol. Blots were blocked with 10% nonfat dried milk (Carnation brand) in TBST (0.05% Tween-20, 137 mM sodium chloride, 20 mM Tris, pH 7.6) and probed with anti-α-crystallin antiserum (1:5000) as previously described (Roquemore et al., 1992). Immunoreactive proteins were detected by enhanced chemiluminescence (Whitehead et al., 1979).

Preparation and Purification of Tryptic Peptides. Lens α-crystallins were reduced and alkylated as previously described (Swiedler et al., 1983), then digested for 24 h at room temperature with constant stirring in 0.1 M ammonium bicarbonate with the addition of an estimated 2% (w/w) TPCK-treated trypsin (Worthington) at the start of the incubation and again after 8 h of incubation. The reaction was terminated by lyophilization. Rat heart α-crystallins were electroeluted from 15% SDS−PAGE gel slices using an Amicon Centrilutor at 400 V for 2 h. Approximately 150 mg of total protein were reduced, alkylated, and digested with a total of 286 mg of TPCK-treated trypsin as described above. Peptides were resolved by reverse-phase HPLC on a C-18 column. Radiolabeled peptides were detected by scintillation counting.

Laser Desorption Mass Spectrometry. Mass spectrometry was performed on a Lecroy model 9400A matrix-assisted laser desorption time-of-flight mass spectrometer with a 600 ps, 1.2 mJ nitrogen laser as previously described (Dong et al., 1993; Chevrier & Cotter, 1991).

Cell Culture. U373-MG astroglioma cells, derived from a human grade III glioblastoma/astrocytoma (Ponten & Westermark, 1978), were obtained from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, MEM nonessential amino acids (Gibco), and penicillin−streptomycin. The medium was changed every 2−3 days.

Metabolic Incorporation of [^{3}H]Glucosamine and [^{3}H]-Leucine into Cytosolic αB-Crystallins. U373-MG cells were seeded to 100 or 150 mm dishes and grown to confluence. Dishes were preincubated in glucose-free or leucine-free DMEM supplemented with 5% dialyzed FBS and 1 mM pyruvate for 30 min. Cells were then labeled in parallel with either [^{3}H]glucosamine (75−100 μCi/mL) or [^{3}H]leucine (50−100 μCi/mL) for 2−12 h. Unlabeled glucosamine was added to the [^{3}H]leucine-labeling cells in an amount equal to the amount of [^{3}H]glucosamine used in parallel dishes. Similarly, unlabeled leucine was added to the [^{3}H]glucosamine-labeling cells. One [^{3}H]glucosamine-labeled dish and one [^{3}H]leucine-labeled dish were harvested for each time point. After incubation, cells were washed twice with PBS and frozen immediately at −80 °C. To harvest cells, dishes were warmed to room temperature, and cells were lysed by the addition of RIPA [10 mM Tris, pH 8.15, 50 mM sodium chloride, 0.05% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.02 M PMSF, protease inhibitor cocktails 1 and 2 (Holt & Hart, 1986)]. Lysed cells were then scraped from the dish with a rubber policeman and immediately transferred to an ice-cold eppendorf tube. Lysates were sonicated and centrifuged at 12000*g* for 20 min at 4 °C. α-Crystallins were then immunoprecipitated from the supernatants as described below. The immunoprecipitated products were resolved by SDS−PAGE on 15% polyacrylamide gels. Gels were stained with Coomassie Blue, impregnated with En^{3}Hance, and exposed to X-ray film. Alternatively, to quantitate radiolabel incorporation directly, Coomassie Blue-stained α-crystallins migrating with the expected apparent M_r = 22K were excised from the polyacrylamide gel and pressed through stainless steel wire mesh into 200 mL of 0.1 M sodium hydroxide/0.5 M sodium borohydride. This solution was allowed to incubate with constant mixing for 18 h at 37 °C. The supernatants from this base-catalyzed β-elimination reaction were neutralized with 40 μL of 8.7 M acetic acid, dried, and extracted twice with 20% methanol under nitrogen gas. The remaining gel pieces were further extracted with 200 mL of 1% SDS/1% β-mercaptoethanol for 18 h at 37 °C with constant mixing. The β-elimination extracts were combined with the corresponding detergent/gel mixtures, and analyzed by scintillation counting.

Immunoprecipitation of Metabolically Labeled Cytosolic αB-Crystallin. Immunoprecipitations were performed at 0−4 °C. Cell lysates were precleared by incubation with 25 μL of protein A-Sepharose 6MB (Pharmacia) per mL of lysate for 30 min, and the immunoprecipitation was initiated by the addition of anti-α-crystallin antiserum in 3% bovine serum albumin. After the supernatants were incubated with the antiserum for 18 h, 40 μL of protein A-Sepharose beads was added, and the mixture was allowed to incubate for an additional 1.5 h. The immune complexes were then pelleted by microcentrifugation for 10 s. The beads were washed successively for 10 min each time with 1 mL of each of the following solutions: (1) RIPA; (2) 0.5 M lithium chloride, 50 mM Tris, pH 6.8; (3) 50 mM Tris, pH 6.8; (4) RIPA; (5) 50 mM Tris, pH 6.8. The washed pellets were resuspended in 60 mL of sample buffer (2% β-mercaptoethanol, 20% glycerol, 4% SDS, 0.2% bromphenol blue, 100 mM Tris, pH 6.8) and boiled 3 min. Released proteins were analyzed on 15% polyacrylamide gels.

Pulse−Chase Analysis of Cytosolic αB-Crystallin. As described above for metabolic labeling, confluent cultures of U373-MG astroglioma cells were preincubated for 30 min in glucose-free or leucine-free medium and then metabolically labeled for 10 h in the presence of 100 μCi/mL ^{3}H-glucosamine or 100 μCi/mL [^{3}H]leucine. The labeling medium was then replaced with complete DMEM, and the incubation was continued for 0−40 h. At each time point, cells from a single dish were rinsed twice with PBS and frozen at −87 °C. Later, cells were harvested by lysis in RIPA, and αB was immunoprecipitated and analyzed as described.

RESULTS

Rhesus Monkey Lens and Rat Lens αB-Cystallins Are Modified by O-GlcNAc. Previously we demonstrated that lens α-crystallins from a variety of vertebrate species, including rat and rhesus monkey, bear terminal GlcNAc residues that can be detected by the enzymatic addition of a [^{3}H]galactose molecule to accessible GlcNAc moieties (Roquemore et al., 1992). We further showed that, on all four bovine lens α-crystallin subunits, GlcNAc is present as a monosaccharide attached to the protein backbone through the hydroxyl groups of serine or threonine residues. These data also suggested that rat and rhesus monkey lens αB-crystallins are similarly modified. The fluorograph in Figure 1 shows the products of *in vitro* galactosyltransferase labeling

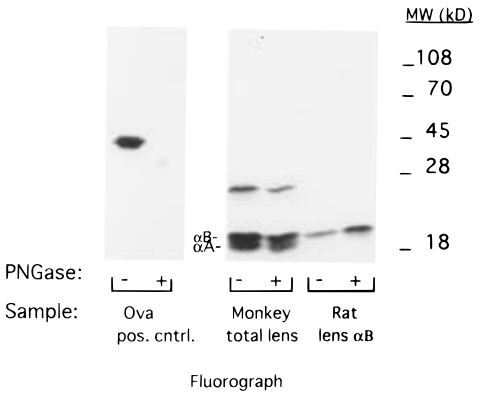

FIGURE 1: PNGase F treatment of [^{3}H]galactose-labeled monkey and rat lens crystallins. Fluorograph of [^{3}H]galactose-labeled rhesus monkey total lens proteins and [^{3}H]galactose-labeled rat lens αB-crystallin resolved by 15% SDS−PAGE. Samples were treated in the absence (−) or presence (+) of PNGase F. [^{3}H]Galactose-labeled ovalbumin (Ova) was used as a positive control for the enzyme PNGase F. The fluorograph was exposed to X-ray film for 2 months.

of rat lens αB-crystallins and rhesus monkey lens total soluble proteins using UDP-[^{3}H]galactose as the donor substrate. It is evident from this figure that the $M_r = 22\,000$ αB-crystallins from both rat and rhesus monkey lenses bear terminal GlcNAc moieties that are substrates for *in vitro* galactosylation. The identity of this 22K M_r band as αB-crystallin has been confirmed by chromatographic analysis, immunoblot, and immunopreciptation (Roquemore et al., 1992; data not shown) as well as by sequence analysis of the protein from rhesus monkey (see below).

The O-linkage of GlcNAc to proteins through serine and threonine hydroxyls can be cleaved by mild base-catalyzed β-elimination (Torres & Hart, 1984) but resists cleavage by peptide-N-glycosidase F (PNGase F), which is specific for N-linked oligosaccharides (Plummer & Tarentino, 1981; Tarentino et al., 1974). To test for resistance to PNGase F, crystallins from rat and rhesus monkey lenses were enzymically labeled with [^{3}H]galactose, incubated in the presence or absence of PNGase F, and applied to a G-50 column to remove any released carbohydrates. Proteins eluting in the void volume of the column were resolved on a 15% polyacrylamide gel. As shown in Figure 1, PNGase F was able to remove more than 98% of N-linked carbohydrates from a positive control ([^{3}H]galactose-labeled ovalbumin) but was not able to remove radiolabeled carbohydrate from the 22 kDa rat lens or monkey lens αB-crystallins. In contrast, Figure 2 demonstrates that [^{3}H]galactose-labeled GlcNAc on the crystallins was sensitive to β-elimination. The β-eliminated radiolabeled products removed from rhesus monkey and rat lens α-crystallins migrated on a TSK sizing column as the expected disaccharide (Figure 3A) and comigrated on high pH anion exchange chromatography (HPAEC) with authentic Gal-β1,4-GlcNAcitol standard (Figure 3B). Taken together, these data confirm that αB-crystallins from rhesus monkey lens and rat lens are modified with O-GlcNAc.

Lens αB-Crystallin Is Glycosylated at Threonine 170. To determine the site of O-GlcNAcylation, rhesus monkey lens αB-crystallin was separated from αA-crystallin by reverse-phase HPLC on a C-4 column (Figure 4) and enzymically labeled with [^{3}H]galactose. Radiolabeled proteins were then reduced and alkylated, digested with trypsin, and resolved

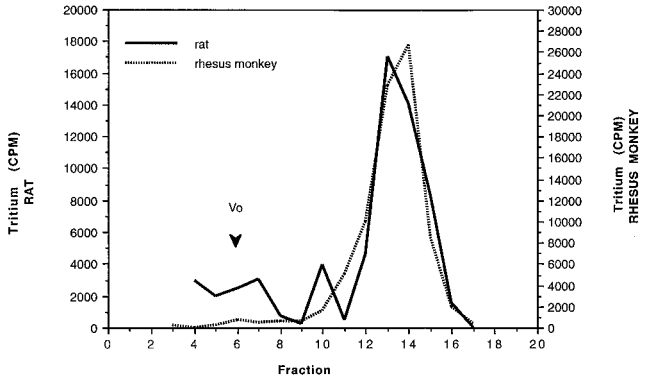

FIGURE 2: β-Elimination of [^{3}H]galactose-labeled αB-crystallin from rat and monkey lenses. [^{3}H]Galactose-labeled HPLC-purified αB-crystallins from rat and monkey lenses were analyzed by Sephadex G-50 Chromatography following base-catalyzed β-elimination.

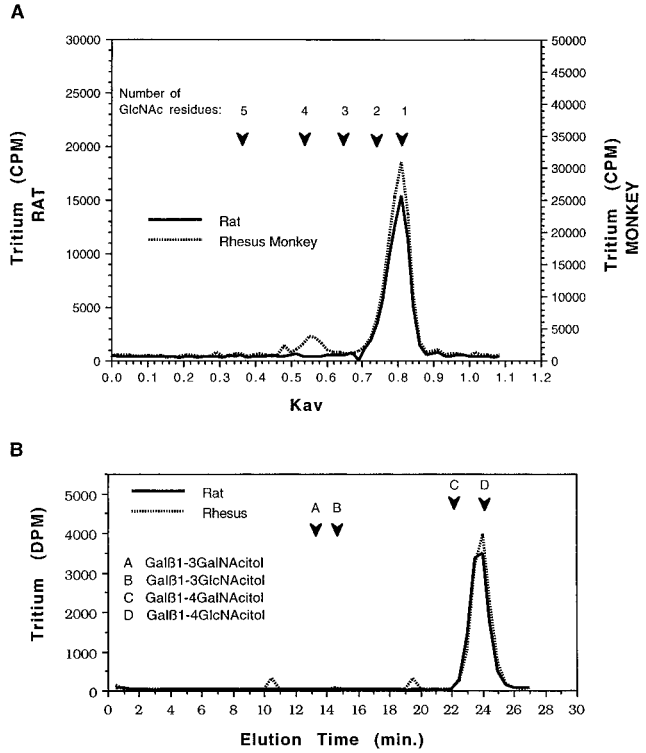

FIGURE 3: Characterization of β-eliminated saccharide. (A) TSK chromatography of tritiated saccharide released from rat and monkey lens αB-crystallins by base-catalyzed β-elimination. (Arrows) Elution positions of [^{3}H]galactose-labeled GlcNAc polymers [prepared as described by Haltiwanger *et al.* 1990]; the number of GlcNAc residues are indicated above each arrow. (B) high pH anion exchange chromatography of β-eliminated material. (Arrows) Elution positions of disaccharide standards.

by HPLC on an Alltech Econosphere C-18 column using an 80-min linear gradient of 0−40% acetonitrile in 0.1% trifluoroacetic acid. Figure 5 shows that the majority of the radiolabel was incorporated into a peptide eluting at 22 min from the HPLC column. A second radiolabeled peptide eluted at 20 min. The major radiolabeled peptide was further purified by two successive applications to a Dynamax C-18 column using a 70-min linear gradient of 0−20% acetonitrile in 10 mM triethylamine, pH 5.5 (Figure 6A,B). Sequence analysis identified the peptide as ^{164}EEKPAVTAAPK^{174} (Figure 6C), with Thr-170 being the only possible site of O-GlcNAcylation. The identity of this peptide was further confirmed by laser desorption mass spectrometry (Figure 7),

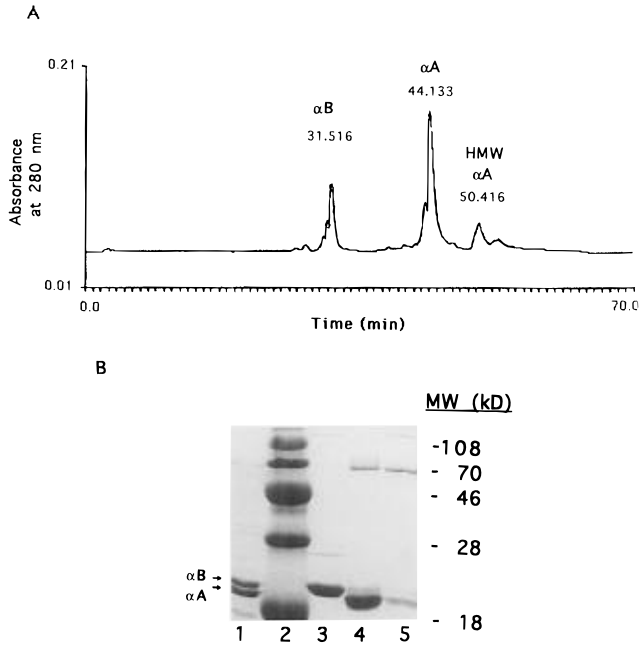

FIGURE 4: Separation of αB and αA crystallins by reverse-phase HPLC. Rhesus monkey α-crystallins were applied to a Dynamax C-4 HPLC column. The column was developed at a flow rate of 1.5 mL/min with a discontinuous gradient of 0 to 40% acetonitrile in 1% trifluoroacetic acid according to the method described by Swamy and Abraham (1991). (A) UV profile of eluted proteins. (B) Coomassie Blue-stained 15% polyacrylamide gel of proteins eluted from the HPLC column. (Lane 1) Bovine α-crystallin standards; (lane 2) molecular weight standards (BRL); (lane 3) HPLC peak 1 (αB-crystallin); (lane 4) HPLC peak 2 (αA-crystallin); (lane 5) HPLC peak 3 (high molecular weight αA-crystallin).

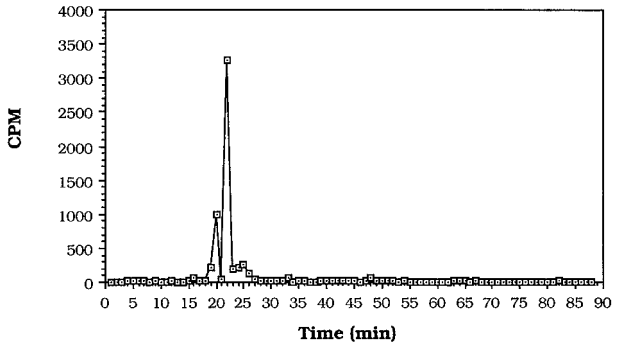

FIGURE 5: Reverse-phase HPLC analysis of trypsin-generated peptides from [^{3}H]galactose-labeled rhesus monkey αB-crystallin. [^{3}H]Galactose-labeled rhesus monkey αB-crystallin tryptic peptides were applied to an Alltech Econosphere C-18 HPLC column. The column was developed with an 80-min linear gradient of 0−40% acetonitrile in 0.1% trifluoroacetic acid.

which resolved molecular ions at *m*/*z* 1140.47, 1162.91, and 1179.76, corresponding to the hydrogen, sodium, and potassium adducts of the unglycosylated peptide, respectively, as well as molecular ions at *m*/*z* 1505.78, 1526.39, and 1544.29, corresponding to the same adducts of the peptide modified with a Gal-GlcNAc. The mass accuracy of this instrument is 0.1%. The radiolabeled peptide eluting at 20 min did not contain enough mass for sequence determination.

Rat Heart αB-Crystallin Is Modified by O-GlcNAc. To determine whether O-GlcNAc modification of αB is lens-specific or also occurs on αB-crystallin expressed in nonlens tissues, we examined αB-crystallin from rat heart tissue. Rat heart contains more αB per mg of protein than any other rat tissue besides the lens and the soleus muscle (Kato et al., 1991). Rat heart proteins were precipitated from homogenates with ammonium sulfate, dialyzed, and applied to a DEAE-Sepharose column. Proteins were eluted from the column with a linear gradient of potassium phosphate, and an aliquot of every third fraction was analyzed by SDS−PAGE (data not shown). Fractions 25−49, which contained a 22K M_r Coomassie Blue-staining band, were pooled, precipitated with ammonium sulfate, and applied to a Sephacryl S-200 sizing column. Since α-crystallins exist as large aggregates *in vivo*, they would be expected to run in the void volume of this column. Proteins eluting from the column were monitored by measuring absorbance at 280 nm, and an aliquot of every third fraction was analyzed by SDS−PAGE (data not shown). A 22K M_r Coomassie Blue-staining protein was observed in the void volume of the S-200 column (fractions 65−74).

Material eluting in the void volume of the S-200 column was then enzymically labeled with [^{3}H]galactose using galactosyltransferase to probe for terminal GlcNAc molecules. The radiolabeled proteins were resolved by electrophoresis in two dimensions (Figure 8A). A Western blot of the 2-D gel, probed with antiserum to α-crystallins, revealed

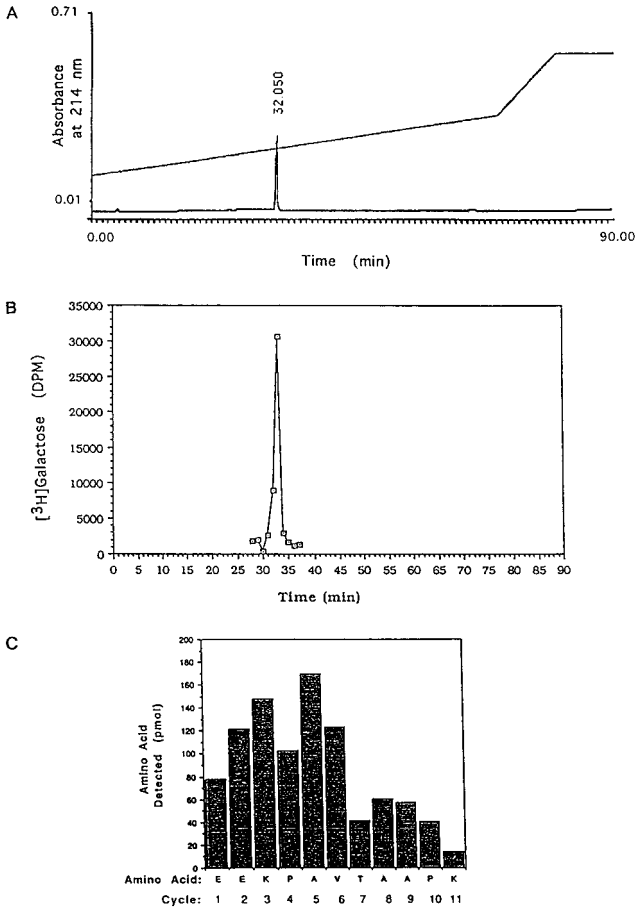

FIGURE 6: Purification and sequence analysis of the major radiolabeled rhesus monkey αB-crystallin tryptic peptide. The major radiolabeled rhesus αB-crystallin tryptic peptide eluting from an Alltech C-18 column at 22 min (see Figure 5) was further purified by two successive applications to a Dynamax C-18 column using a 70-min linear gradient of 0−20% acetonitrile in 10 mM triethylamine, pH 5.5. (A) UV profile of peptides eluted during the final HPLC purification step. (B) tritium profile of the eluted peptide. The major peak of radiolabeled material eluted in two 0.5-mL fractions. The fraction eluting at 33 min was subjected to sequence analysis. (C) Sequence analysis of the purified tryptic peptide.

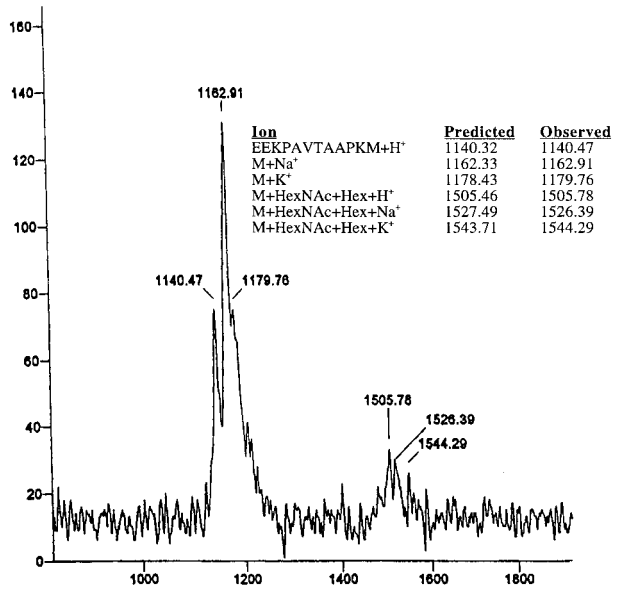

FIGURE 7: Laser-desorption mass spectrometry of HPLC-purified, radiolabeled, rhesus monkey αB-crystallin tryptic peptide. [^{3}H]-Galactose-labeled, HPLC-purified, tryptic peptide from rhesus monkey αB-crystallin was analyzed by laser-desorption mass spectrometry.

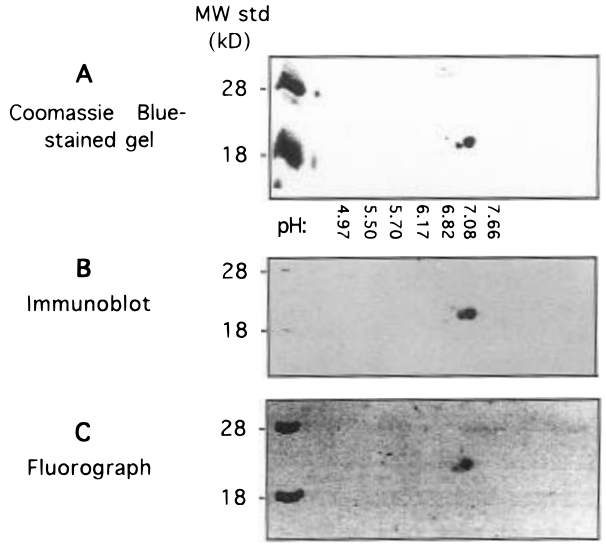

FIGURE 8: Two-dimensional analysis and immunoblotting of partially purified rat heart α-crystallins. [^{3}H]Galactose-labeled rat heart proteins were resolved by nonequilibrium pH gradient electrophoresis in the first dimension followed by electrophoresis on a 15% SDS−polyacrylamide gel. (A) Coomassie Blue-stained gel. (B) Immunoblot of a companion gel run under the same conditions probed with antiserum to α-crystallins. (C) Fluorograph of gel shown in panel A.

the presence of two 22K M_r immunoreactive spots which migrated on nonequilibrium pH gradient electrophoresis with pH values of 7.1 and 7.0 (Figure 8B). The larger protein spot, migrating at a pH of 7.1, is most likely the unphosphorylated form of αB-crystallin, which has a published pI of 7.1 (Spector et al., 1985). The identity of the protein at pH 7.0 is not known. A companion 2-D gel was stained with Coomassie Blue, impregnated with En^{3}Hance, dried, and exposed to film. The resulting fluorograph revealed the presence of two radiolabeled proteins, corresponding to the two 22K M_r proteins migrating at pH 7.1 and 7.0 (Figure 8C). These data indicate that rat heart αB-crystallins are modified with terminal GlcNAc. The radiolabeled carbohydrate on the 22K M_r protein subsequently was found to be resistant to removal by PNGase F (data not shown), consistent with its attachment to the protein through an O-linkage. To further confirm that the 22K M_r rat heart protein was in fact αB-crystallin, the radiolabeled protein was excised from a 15% polyacrylamide gel, digested with trypsin, and analyzed by reverse-phase HPLC. In parallel, authentic αB-crystallin from rat lens was similarly labeled, trypsinized, and analyzed. As shown in Figure 9, the major radiolabeled peptides from both the 22K M_r protein and authentic αB-crystallin eluted at 21.5 (±0.2) min in an 80-min linear gradient of 0−40% acetonitrile in 0.1% trifluoroacetic acid. This migration position is characteristic of the peptide ^{164}EEKPAVTAAPK^{174} (Figure 5), which was shown above to be glycosylated in the rhesus monkey αB, and which is exactly conserved in the rat (Iwaki et al., 1990a; Atomi et al., 1991a,b; Hendriks et al., 1990). These data indicate that both rat lens and rat heart αB-crystallin are glycosylated on peptide 164−174 at Thr 170. Since this site is conserved in human, bovine, mouse, and hamster αB-crystallins (Kramps et al., 1977; van der Ouderaa et al., 1974; Klemenz et al., 1991a; Quax-Jeuken et al., 1985), it is likely that nonlens αB-crystallins in these species are also modified with O-GlcNAc at Thr 170. HPLC analysis of rat heart αB-crystallin tryptic peptides also indicated the presence of another glycosylated peptide eluting at 25.5 min.

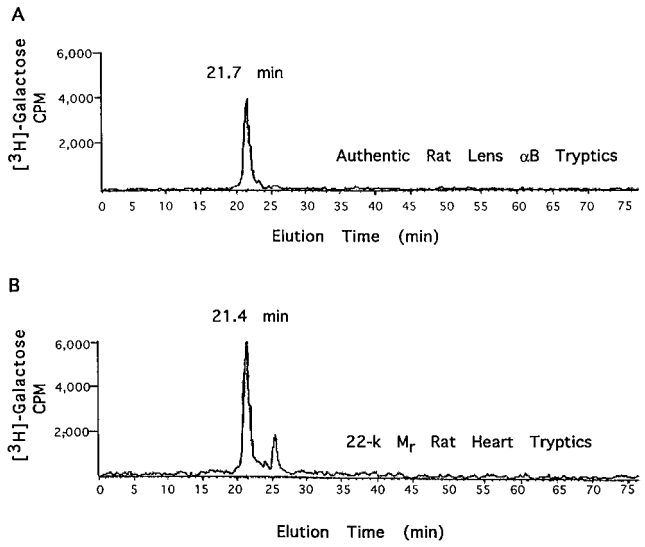

FIGURE 9: Comparative mapping of tryptic peptides from rat heart 22K M_r protein and rat lens αB-crystallin. Tryptic peptides generated from [^{3}H]galactose-labeled proteins were resolved by reverse-phase HPLC on a C-18 column using an 80-min linear gradient of 0−40% acetonitrile in 0.1% trifluoracetic acid. (A) tritium profile of rat lens αB-crystallin tryptic peptides. (B) Tritium profile of tryptic peptides from rat heart 22K M_r protein.

Turnover of O-GlcNAc on αB-Crystallin Is Much Higher Than the Polypeptide. Human U373-MG astroglioma cells constitutively express high levels of cytosolic αB-crystallin. The dynamics of O-GlcNAc addition to αB-crystallin were examined in this cell line by comparing rates of biosynthesis and degradation of both the carbohydrate and the protein. Turnover of the carbohydrate moiety of αB was monitored by following metabolically incorporated [^{3}H]glucosamine, while turnover of the protein backbone was monitored by measuring metabolically incorporated [^{3}H]leucine. After metabolic labeling, αB was recovered by immunoprecipitation and analyzed by SDS−PAGE. Radiolabel incorpo-

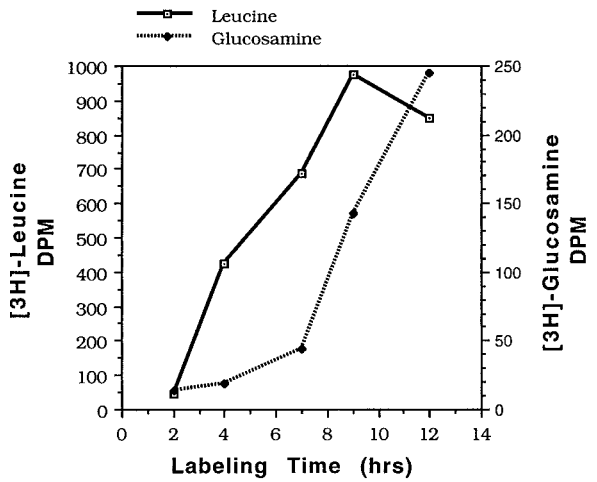

FIGURE 10: Biosynthetic rate of the carbohydrate and protein portions of cytosolic αB-crystallin from U373-MG astroglioma cells. U373-MG astroglioma cells were metabolically labeled for 2−12 h with [^{3}H]glucosamine or [^{3}H]leucine and immunoprecipitated with antibody to α-crystallins. Tritium CPM were recovered from immunoprecipitated proteins excised from a 15% polyacrylamide gel. Each sample was counted for 10 min. Preimmune serum (PIS) was used as a negative control for the immunoprecipitations.

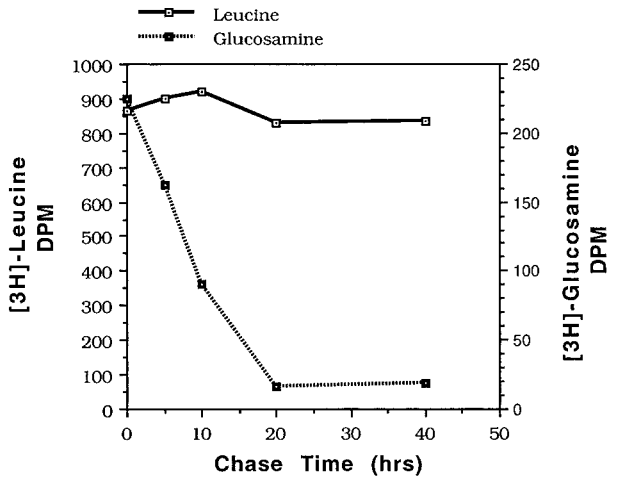

FIGURE 11: Degradation rate of the carbohydrate and protein portions of cytosolic αB-crystallin from U373-MG astroglioma cells. U373-MG cells were pulse-labeled for 10 h with [^{3}H]-glucosamine or [^{3}H]leucine and then chased for 0−40 h. α-Crystallins were then immunoprecipitated with antibody to α-crystallins. Tritium CPM were recovered from immunoprecipitated proteins excised from a 15% polyacrylamide gel. Preimmune serum (PIS) was used as a negative control for the immunoprecipitation.

rated into αB was either quantitated directly by counting material eluted from gel slices or visualized by fluorography after impregnation of the polyacrylamide gel with fluor. Both methods of analysis yielded comparable results. Figure 10 shows that [^{3}H]leucine was incorporated at a linear rate into cytosolic αB-crystallin over the period from 2 to 6 h. During the same time period, the rate of [^{3}H]glucosamine incorporation into αB-crystallin lagged behind the rate of [^{3}H]leucine incorporation, but then increased substantially from 6 to 12 h. The lag phase in carbohydrate incorporation into αB-crystallin is most likely due to a large intracellular pool of UDP-GlcNAc (known to be in the millimolar range). This is a phenomenon commonly observed in metabolic labeling of proteins with radiolabeled carbohydrates. Because intracellular concentrations of the carbohydrate precursors are unknown, the rate of biosynthetic incorporation of glucosamine during the lag phase cannot be determined from these data. Notably, during the chase, we observed a dramatic difference between the degradation rates of αB protein and carbohydrate (Figure 11). Incorporated [^{3}H]-leucine was degraded relatively slowly, exhibiting an apparent $t_{1/2}$ of 13.7 days, whereas incorporated [^{3}H]glucosamine turned over much more rapidly, with a $t_{1/2}$ of less-than 10 h.

DISCUSSION

αB-Crystallin from the rhesus monkey lens was found to be modified with O-GlcNAc on the tryptic peptide ^{164}-EEKPAVTAAPK-^{174}, which is located at the C-terminus of the αB chain. Since O-GlcNAc is added only to serine or threonine residues, the site of O-GlcNAcylation is Thr 170. This sequence is also conserved in human, bovine, rat, mouse, and hamster αB-crystallins. Interestingly, the αB-crystallin Thr 170−Ala 171 bond is the only site at which proteolytic degradation of αB chains has been observed *in vivo* and is highly labile to at least six different proteases *in vitro* (Siezen & Hoenders, 1979). It is well documented that in the lens the proteolytic fragment αB(1−170) increases with aging of α-crystallins (Siezen et al., 1979; van Kleef et al., 1974, 1976). Perhaps the presence of O-GlcNAc at Thr 170 plays some role in regulating the degradation of αB-crystallin. It is interesting to note that that the homologous αA-crystallin chain is sensitive to *in vitro* proteolysis in the region of amino acids 156−163 (Siezen & Hoenders, 1979), which encompass its major O-GlcNAcylation site, Ser 162.

Table 1: Selected Sites of O-GlcNAc Attachment *in Vivo*[a]

rat neurofilament (NF-M)[b]	...P-S-V-T...
rat neurofilament (NF-M)[b]	...P-S-T-V...
rat neurofilament (NF-M)[b]	...P-A-X-T-Q
rhesus monkey αB-crystallin	E-E-K-P-A-V-T-A-A-P-K
αA-crystallin (except monkey)[c]	AI-P-V-S-R-E-E-K
monkey αA-crystallin[d]	E-E-K-P-S-S-A-P-S-S
rat neurofilament (NF-L)[b]	...P-V-S-S-S...
human serum response transcription factor[e]	...P-V-S-A-S...
human cytomegalovirus tegument basic phosphoprotein (UL32)[f]	P-S-V-P-V-S-G-S-A-

[a] O-GlcNAcylation sites are underlined. [b] Dong et al. (1993). [c] Roquemore et al. (1992). [d] E. P. Roquemore and G. W. Hart, unpublished observations. [e] Reason et al. (1992). [f] Greis et al. (1994).

Comparison of the O-GlcNAcylation sites of several different proteins reveals certain commonalities. As shown in Table 1, many known sites of O-GlcNAc addition, including the αB site at Thr 170, contain a proline two or three amino acid residues N-terminal to the glycosylated residue. Another common feature of O-GlcNAc addition is the presence of a valine residue in close proximity to the O-GlcNAcylation site. In keeping with this pattern, Thr 170 is preceded by a valine residue. Interestingly, αB-crystallin also contains the sequence ^{158}TIPITR^{163}, which is homologous to the αA O-GlcNAcylation site (^{158}AIPVSR^{163}), yet Thr 162 has not been found to be O-GlcNAcylated, nor is the synthetic peptide ^{158}TIPITREEK^{166} a substrate for the cytosolic O-GlcNAc-transferase enzyme (L. Blomberg, L. Kreppel, and G. W. Hart, unpublished results). Perhaps the lack of αB O-GlcNAcylation at Thr 162 is due to the substitution of an isoleucine for a valine at position 161,

indicative of the extraordinary peptide sequence specificity of the O-GlcNAc transferase(s).

Because αB-crystallin is known to act as a heat-shock protein in nonlens tissues, we were interested in determining whether O-GlcNAcylation of αB is a lens-specific phenomenon or can occur outside of the lens as well. A 22K M_r protein in rat heart was found to be glycosylated with O-GlcNAc, based on its ability to be labeled with [^{3}H]-galactose by the enzyme galactosyltransferase and by its resistance to cleavage by the enzyme PNGase F. The 22K M_r heart protein reacted positively with antibody to α-crystallins and was composed of two components with differing isoelectric points corresponding to the unphosphorylated form of αB-crystallin (αB2) and to an unidentified form of the protein. Mann and co-workers (Mann et al., 1991) have documented the existence of an unphosphorylated form of αB-crystallin that has a pI of 6.9, intermediate between the unphosphorylated αB2 and the phosphorylated αB1, which have published isoelectric points of 7.1 and 6.7, respectively (Spector et al., 1985). It is unclear whether this protein is the same as the form we observed, which migrates on a nonequilibrium pH gradient gel to a pH of 7.0. The apparent absence from our preparation of the phosphorylated form, αB1, may be due to the action of phosphatases released during the isolation procedure. Alternatively, αB1 may be a low-abundance protein in nonstressed nonlenticular tissues. Kato and co-workers, using a similar protocol for the isolation of αB-crystallin from skeletal muscle, also report the purification of the unphosphorylated form only (Kato et al., 1992).

Tryptic map comparison of [^{3}H]galactose-labeled rat heart 22K M_r protein and rat lens αB-crystallin further confirmed that the rat heart 22K M_r protein is αB-crystallin and suggest that both proteins are glycosylated on the same peptide, ^{164}EEKPAVTAAPK^{174}. The tryptic map of [^{3}H]galactose-labeled rat heart αB-crystallin contained a second radiolabeled peptide which migrated at 25.5 min on reverse-phase HPLC. This peptide may be the product of an additional cleavage of peptide 164−174 at Lys 166. The resulting radiolabeled peptide might be expected to elute at a later time due to the net loss of one negative charge. The high ratio of enzyme to substrate in this case may have forced this normally unfavorable cleavage reaction to occur. Alternatively, the peptide at 25.5 min may represent a second site of O-GlcNAc addition not found on αB-crystallin from the lens.

To investigate the dynamics of O-GlcNAc on nonlens αB-crystallin, we selected U373-MG astroglioma cells, since they constitutively express elevated cytosolic levels of this protein. Metabolic labeling of U373-MG cells with [^{3}H]glucosamine resulted in the production of radiolabeled αB-crystallin, consistent with O-GlcNAc modification of this protein. We observed a dramatic difference in the degradation rates of αB protein versus carbohydrate. [^{3}H]Leucine, metabolically incorporated into αB-crystallin, turned over minimally during a 40-h chase period, while radiolabeled O-GlcNAc incorporated into αB was completely turned over after the first 20 h of the chase period. These data are similar to those of Chou et al. (1992), who observed that turnover of O-GlcNAc on cytokeratins proceeds far more rapidly than turnover of the protein core. The results of these metabolic labeling experiments suggest that O-GlcNAc on cytosolic αB-crystallin is recycling much more rapidly than the polypeptide itself. These findings are consistent with a regulatory role for O-GlcNAc on nonlens αB-crystallin. It has been proposed that phosphorylation of small heat shock proteins may modulate their function as heat shock proteins (Crete & Landry, 1990). The finding that O-GlcNAc-modification of nonlens αB-crystallin occurs in a dynamic fashion raises the possibility that this posttranslational modification may constitute an additional level of regulatory control in the heat-shock response.

ACKNOWLEDGMENT

We thank members of the Hart laboratory for critical reading of the manuscript and J. S. Zigler for generously providing α-crystallin antiserum and rhesus monkey lenses.

REFERENCES

Arrigo, A.-P., & Welch, W. J. (1987) *J. Biol. Chem. 262*, 15359.
Arrigo, A.-P., Suhan, J. P., & Welch, W. J. (1988) *Mol. Cell. Biol. 8*, 5059.
Atomi, Y., Yamada, S., & Nishida, T. (1991a) *Biochem. Biophys. Res. Commun. 181*, 1323.
Atomi, Y., Yamada, S., Strohman, R., & Nonomura, Y. (1991b) *J. Biochem. (Tokyo) 110*, 812.
Bhat, S. P., & Nagineni, C. N. (1989) *Biochem. Biophys. Res. Commun. 158*, 319.
Chevrier, M. R., & Cotter, R. J. (1991) *Rapid Commun. Mass. Spectrom. 5*, 611.
Chiesi, M., Longoni, S., & Limbruno, U. (1990) *Mol. Cell. Biochem. 97*, 129.
Chou, C.-F., & Omary, M. B. (1993) *J. Biol. Chem. 268*, 4465.
Chou, C.-F., Smith, A. J., & Omary, M. B. (1992) *J. Biol. Chem. 267*, 3901.
Chou, T.-Y., Dang, C. V., & Hart, G. W. (1995a) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4417.
Chou, T.-Y., Dang, C. V., & Hart, G. W. (1995b) *J. Biol. Chem. 270*, 18961.
Crete, P., & Landry, J. (1990) *Radiat. Res. 121*, 320.
Dong, D. L.-Y., Xu, Z.-S., Chevrier, M. R., Cleveland, D. W., Cotter, R. J., & Hart, G. W. (1993) *J. Biol. Chem. 268*, 16679.
Dubin, R. A., Wawrousek, E. F., & Piatigorsky, J. (1989) *Mol. Cell. Biol. 9*, 1083.
Duguid, J. R., Rohwer, R. G., & Seed, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5738.
Greis, K. D., Gibson, W., & Hart, G. W. (1994) *J. Virol. 68*, 8339.
Haltiwanger, R. S., Kelly, W. G., Blomberg, M. A., Roquemore, E. P., Dong, D. L.-Y., Kreppel, L., Chou, T.-Y., & Hart, G. W. (1992) *Biochem. Soc. Trans. 20*, 264.
Hendriks, W., Weetink, H., Voorter, C. E. M., Sanders, J., Bloemendal, H., & de Jong, W. W. (1990) *Biochim. Biophys. Acta 1037*, 58.
Holt, G. D., & Hart, G. W. (1986) *J. Biol. Chem. 261*, 8049.
Iwaki, T., Kume-Iwaki, A., Liem, R. K. H., & Goldman, J. E. (1989) *Cell 57*, 71.
Iwaki, A., Iwaki, T., Goldman, J. E., & Liem, R. K. H. (1990a) *J. Biol. Chem. 265*, 22197.
Iwaki, T., Kume-Iwaki, A., & Goldman, J. E. (1990b) *J. Histochem. Cytochem. 38*, 31.
Kato, K., Shinohara, H., Kurobe, N., Inaguma, Y., Shimizu, K., & Ohshima, K. (1991) *Biochim. Biophys. Acta 1074*, 201.
Kato, K., Shinohara, H., Goto, S., Inaguma, Y., Morishita, R., & Asano, T. (1992) *J. Biol. Chem. 267*, 7718.
Kato, K., Goto, S., Hasegawa, K., Shinohara, H., & Inaguma, Y. (1993) *Biochim. Biophys. Acta 1175*, 257.
Kelly, W. G., Dahmus, M. E., & Hart, G. W. (1992) *J. Biol. Chem. 268*, 10416.
Kearse, K. P., & Hart, G. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1701.
Klemenz, R., Frohli, E., Aoyama, A., Hoffmann, S., Simpson, R., Moritz, R. L., & Schafer, R. (1991a) *Mol. Cell. Biol. 11*, 808.
Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., & Aoyama, A. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3652.

Kramps, J. A., de Man, B. M., & de Jong, W. W. (1977) *FEBS Lett. 74*, 82.
Landry, J., Chretien, P., Laszlo, A., & Lambert, H. (1991) *J. Cell. Physiol. 147*, 93.
Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., & Anderson, C. W. (1992) *J. Biol. Chem. 267*, 794.
Lee, Y. J., Curetty, L., & Corry, P. M. (1990) *Biochem. Biophys. Res. Commun. 172*, 119.
Longoni, S., James, P., & Chiesi, M. (1990) *Mol. Cell. Biochem. 97*, 113.
Mann, E., McDermott, M. J., Goldman, J., Chiesa, R., & Spector, A. (1991) *FEBS Lett. 294*, 133.
Nagineni, C. N., & Bhat, S. P. (1989) *FEBS Lett. 249*, 89.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell 12*, 1133.
Pineda, R., 2d, Chan, C. C., Ni, M., Hayden, B. J., Johnson, M. A., Nickerson, J., & Chader, G. J. (1993) *Curr. Eye Res. 12*, 239.
Plummer, T. H., Jr., & Tarentino, A. L. (1981) *J. Biol. Chem. 256*, 10243.
Ponten, J., & Westermark, B. (1978) *Med. Biol. 56*, 184.
Quax-Jeuken, Y., Quax, W., Van Rens, G., Khan, M., & Bloemendal, H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5819.
Rollet, E., & Best-Belpomme, M. (1986) *Biochem. Biophys. Res. Commun. 141*, 426.
Roquemore, E. P., Dell, A., Morris, H. R., Panico, M., Reason, A. J., Savoy, L.-A., Wistow, G. J., Zigler, J. S., Jr., Earles, B. J., & Hart, G. W. (1992) *J. Biol. Chem. 267*, 555.
Roquemore, E. P., Chou, T.-Y., & Hart, G. W. (1994) *Methods Enzymol. 230*, 443−460.
Siezen, R. J., & Hoenders, H. J. (1979) *Eur. J. Biochem. 96*, 431.
Siezen, R. J., Bindels, J. G., & Hoenders, H. J. (1979) *Exp. Eye Res. 28*, 551.
Spector, A., Chiesa, R., Sredy, J., & Garner, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4712.
Swamy, M. S., & Abraham, E. C. (1991) *Curr. Eye Res. 10*, 213.
Swiedler, S. J., Hart, G. W., Tarentino, A. L., Plummer, T. H., Jr., & Freed, J. H. (1983) *J. Biol. Chem. 258*, 11515.
Tarentino, A. L., Plummer, T. H., Jr., & Maley, F. (1974) *J. Biol. Chem. 249*, 818.
Torres, C.-R., & Hart, G. W. (1984) *J. Biol. Chem. 259*, 3308.
Vincent, M., & Tanguay, R. (1982) *J. Mol. Biol. 162*, 365.
Welch, W. J. (1985) *J. Biol. Chem. 260*, 3058.
Whitehead, T. P., Kricka, L. J., Carter, T. J. N., & Thorpe, G. H. G. (1979) *Clin. Chem. 25*, 1531.
van Kleef, F. S. M., Nijzink-Maas, M. J. C. M., & Hoenders, H. J. (1974) *Eur. J. Biochem. 48*, 563.
van Kleef, S. M., Willems-Thijssen, W., & Hoenders, H. J. (1976) *Eur. J. Biochem. 66*, 477.
van der Ouderaa, F. J., de Jong, W. W., Hilderink, A., & Bloemendal, H. (1974) *Eur. J. Biochem. 49*, 157.

BI951918J