Cross-Linking of the Skeletal Myosin Subfragment 1 Heavy Chain to the N-Terminal Actin Segment of Residues 40-113†

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ABSTRACT: Glutaraldehyde (GA) and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), a hydrophobic, carboxyl group directed, zero-length protein cross-linker, were employed for the chemical cross-linking of the rigor complex between F-actin and the skeletal myosin S-1. The enzymatic properties and structure of the new covalent complexes obtained with both reagents were determined and compared to those known for the EDC–acto–S-1 complex. The GA- or EEDQ-catalyzed covalent attachment of F-actin to the S-1 heavy chain induced an elevated Mg^{2+}-ATPase activity. The turnover rates of the isolated cross-linked complexes were similar to those for EDC–acto–S-1 (30 s^{-1}). The solution stability of the new complexes is also comparable to that exhibited by EDC–acto–S-1. The proteolytic digestion of the isolated AEDANS-labeled covalent complexes and direct cross-linking experiments between actin and various preformed proteolytic S-1 derivatives indicated that, as observed with EDC, the COOH-terminal 20K and the central 50K heavy chain fragments are involved in the cross-linking reactions of GA and EEDQ. KI-depolymerized acto–S-1 complexes cross-linked by EDC, GA, or EEDQ were digested by thrombin which cuts only actin, releasing S-1 heavy chain–actin peptide cross-linked complexes migrating on acrylamide gels with M_r 100K (EDC), 110K and 105K (GA), and 102K (EEDQ); these were fluorescent only when fluorescent S-1 was used. They were identified by immunostaining with specific antibodies directed against selected parts of the NH_2-terminal actin segment of residues 1–113. Only the peptide of residues 1–28 was cross-linked by EDC. In contrast, the actin peptides including residues 1–28 and 40–113 were cross-linked after GA or EEDQ treatment of acto–S-1. These studies extend further our knowledge on the interaction sites between actin and the myosin head heavy chain.

The myosin head or S-1[1] seems to be the unique domain of the protein sufficient for the expression of active movement during muscle contraction (Toyoshima et al., 1987). Information on the structure and conformation of the interface between actin and the myosin S-1 heavy chain is, therefore, important for understanding the molecular mechanism of energy transduction in muscle by the acto–S-1–ATP complex. Chemical cross-linking approaches, employing the water-soluble, zero-length cross-linker EDC, have been applied previously to the skeletal rigor acto–S-1 complex. They led to the identification of actin-binding sites on either side of the connector segment joining the central 50K region to the COOH-terminal 20K segment of the S-1 heavy chain (Mornet et al., 1981b; Sutoh, 1983). Similar sites are apparently also present within the S-1's from smooth, molluscan, and non-muscle myosins (Marianne-Pēpin et al., 1985; Labbē et al., 1986; Atkinson et al., 1987). These sites would be located within the distal third of the head where the attachment of actin to S-1 is thought to occur (Moore et al., 1970; Craig et al., 1980, 1985; Taylor & Amos, 1981; Vibert & Craig, 1982; Toyoshima & Wakabayashi, 1985). The acto–S-1 cross-linking has also revealed the proximity of the NH_2-terminal 12-residue segment of actin to the heavy chain binding region on the actin molecule (Sutoh, 1982). Immunochemical and ^1H NMR studies have further pointed out the involvement of the N-terminal actin segments of residues 18–28 and 21–40, respectively, in the recognition of the S-1 heavy chain (Mējean et al., 1986, 1987; Moir et al., 1987). Moreover, the contact interface for the heavy chain on the N-terminal actin domain seems to be still active during the interaction of actin with the S-1–ATP complex (Chen et al., 1985; Moir et al., 1987), but suggestions were made that distinct primary sequences could be cross-linked depending on the presence or absence of ATP (Arata, 1986; King & Green, 1987). On the other hand, the EDC-cross-linked acto–S-1 complex is, at present, a valuable tool for investigating by biochemical and physical methods the nucleotide-dependent structural states of the actin-attached crossbridges (Craig et al., 1985; Arata, 1986; King & Greene, 1987; Applegate & Flicker, 1987; Sevensson & Thomas, 1987). In particular, it has permitted identification of the weakly bound conformation associated with the binding of Mg-ATP to S-1. However, it remains to be clearly demonstrated whether the ATP effect is resulting from a specific

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[1] Abbreviations: S-1, myosin subfragment 1; acto-S-1, actomyosin subfragment 1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GA, glutaraldehyde; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; ATPase, adenosine-5′-triphosphatase; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G.

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change in structure of the actin-bound S-1 or from a dynamic change in the nature of the interface between actin and S-1.

To assess further the extent of the binding area between actin and S-1 and to approach the understanding of the mechanisms of the transitions within the actin-bound S-1, we describe, in this work, the preparation and characterization of two new enzymatically active covalent acto–S-1 complexes. They were formed by cross-linking actin to the S-1 heavy chain with glutaraldehyde or *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline, a hydrophobic, carboxyl group specific, zero-length cross-linker. In both complexes the 50K or 20K regions of the heavy chain were cross-linked to actin as in EDC–acto–S-1. But in contrast to the latter complex, the cross-linking of actin by both reagents involved not only the N-terminal segment of residues 1–12 but also the peptide spanning residues 40–113. For identification of the cross-linked actin regions, use was made of a rapid and direct approach combining the selective release of the cross-linked actin peptides by thrombin digestion (Labbé et al., 1986) and their immunodetection by a set of three antibodies directed against distinct epitopes within the N-terminal sequence of residues 1–113. These results have been previously reported in abstract form (Bertrand et al., 1987).

Materials and Methods

Reagents. Trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone], *Staphylococcus aureus* V8 protease, thrombin from human plasma, and endoproteinase Arg-C from mouse submaxillaris glands were purchased respectively from Worthington Biochemical Corp., Miles, Sigma Chemical Co., and Boehringer Mannheim. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), glutaraldehyde (25% aqueous solution) (GA), and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) were obtained from Sigma Chemical Co., Serva-Heidelberg, and Aldrich Chemical Co., respectively. Anti-rabbit IgG antibody and anti-sheep IgG antibody labeled with peroxidase or alkaline phosphatase were from Biosys (Compiègne, France). All other chemicals were of analytical grade.

Preparation of Proteins and S-1 Derivatives. Myosin was isolated from rabbit back and hind leg muscles according to Offer et al. (1973). S-1 was prepared by chymotryptic digestion of myosin filaments (Weeds & Taylor, 1975) and purified over Sephacryl S-200 eluted with 50 mM Tris-HCl buffer, pH 7.5.

F-Actin was prepared according to Eisenberg and Kielly (1974). The protein pellet was resuspended in 10 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl, pH 8.0, containing 1 mM NaN_3.

F-Actin and S-1 were labeled with the fluorescent dye *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) as reported by Mornet et al. (1981a).

The trypsin cut (27K–50K–20K)-S-1 was obtained according to Mornet et al. (1980). The preparation of the split S-1 (22K–50K/45K–20K) formed by digestion with trypsin in the presence of 5 mM Mg^{2+}-ATP was carried out as described by Mornet et al. (1985). The derivative (75K–21K)-S-1 was produced by digestion of S-1 with the endoproteinase Arg-C at an enzyme:S-1 weight ratio of 1:25, at 25 °C, for 120 min, in 50 mM Tris-HCl, pH 8.0 (Bertrand et al., 1987b).

Protein concentrations were determined spectrophotometrically with extinction coefficients of $A^{1\%}_{280nm} = 7.5$ cm^{-1} for S-1 and 11 cm^{-1} for actin. The concentration of the proteolytic S-1 derivatives was determined according to Bradford (1976) with S-1 as a standard. The following molecular weights were used for calculating molar concentrations: 42000 for actin (Collins & Elzinga, 1975) and 115000 for S-1 (Margossian & Lowey, 1982).

Cross-Linking Reactions. F-Actin (2 mg/mL) and S-1 or S-1 derivatives (3 mg/mL) were incubated with 10 mM EDC essentially as described by Mornet et al. (1981b). The acto–S-1 complexes were cross-linked with 1 mM EEDQ (freshly dissolved in absolute ethanol) in 100 mM MES, pH 6.0, at 25 °C. The cross-linking reactions were allowed to proceed for 20 (EDC) or 45 min (EEDQ) and then quenched by the addition of a 10 molar excess of 2-mercaptoethanol. The cross-linking experiments with glutaraldehyde were conducted as follows: F-Actin (2 mg/mL) and S-1 (3 mg/mL) in 100 mM HEPES, pH 8.0, were reacted with 0.01% glutaraldehyde (1 mM) at 25 °C, for 0–30 min. The reaction was terminated by the addition of Laemmli's sample buffer (Laemmli, 1970). The cross-linked species were separated and analyzed by gel electrophoresis.

Proteolytic Cleavages of Cross-Linked Complexes. The protein solutions containing the covalent acto–S-1 complexes formed in the presence of EDC, glutaraldehyde, or EEDQ were brought to 100 mM Tris-HCl–5 mM Mg^{2+}-ATP, pH 8.0, and were digested with *S. aureus* V8 protease at a protease to S-1 weight ratio of 1:25, at 25 °C for 300 min (Chaussepied et al., 1983).

The same covalent acto–S-1 complexes were separated from un-cross-linked S-1 by centrifugation at 140000*g* for 1.5 h at 4 °C, after mixing with an equal volume of a dissociating solution containing 100 mM Tris-HCl, pH 8.0, 200 mM KCl, 10 mM $MgCl_2$, and 20 mM sodium pyrophosphate. The protein pellets were then submitted to a depolymerization process employing 0.6 M KI (Rouayrenc et al., 1985; Labbé et al., 1986). After incubation for 20 min at 0 °C in 0.6 M KI, the solutions were clarified by centrifugation at 140000*g* for 1 h at 4 °C and then exhaustively dialyzed against 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM dithiothreitol, and 0.1 mM $CaCl_2$ at 4 °C. After centrifugation the clear supernatants were brought to 1 mM EDTA and digested with thrombin at a protease to actin weight ratio of 1:10 at 25 °C for 300 min. Suitable protein aliquots were removed from these proteolytic reactions mixtures and analyzed by gel electrophoresis.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out in 5–18% polyacrylamide slab gels and in 7.5% (w/v) acrylamide gels (Laemmli, 1970), with a 50 mM Tris–100 mM boric acid buffer, pH 8.6 (Mornet et al., 1981a). Fluorescent bands were located in the gels by illumination with a long-wave ultraviolet light before staining with Coomassie blue (Weber & Osborn, 1969). The densitometric scanning of the gels was carried out with a Shimadzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator. The following proteins were used as molecular weight markers: myosin heavy chain (220K), RNA polymerase β subunit (155K) and β' subunit (165K), β-galactosidase (130K), actin (42K), S-1 heavy chain (95K), light chain 1 (LC_1, 25K), light chain 3 (LC_3, 17K), and the heavy chain fragments obtained by cleavage of S-1 with trypsin (27K, 50K, and 20K) and with Arg-C protease (75K and 21K).

Immunological Techniques. Polyclonal antibodies specifically directed against the N-terminal actin segment of residues 1–28 were isolated from sheep immune sera raised against glutaraldehyde-treated F-actin (Benyamin et al., 1986). As it will be illustrated in Figure 6, these antibodies did not recognize the actin segment containing residues 29–39. They

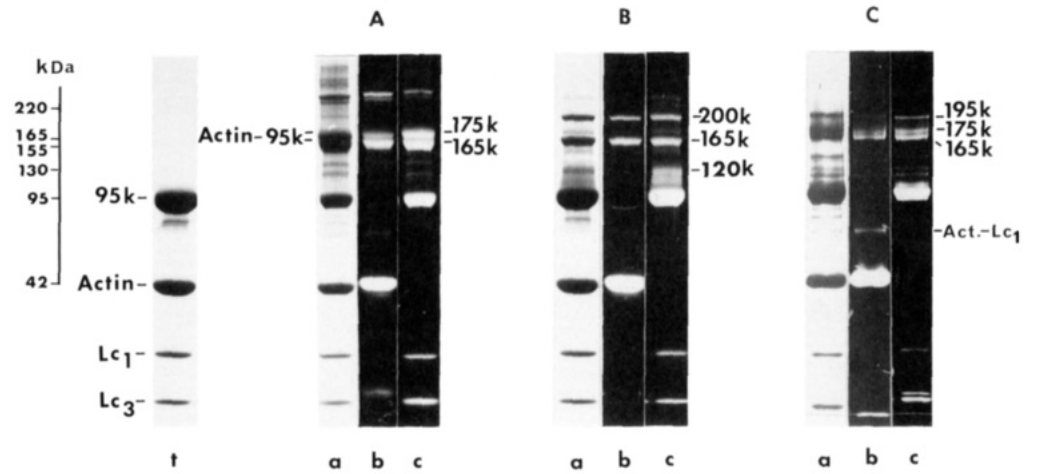

FIGURE 1: Comparative cross-linking patterns of acto–S-1 with EDC (A), glutaraldehyde (B), and EEDQ (C). The cross-linking reactions were carried out as specified under Materials and Methods. $NaDodSO_4$ gel electrophoresis was performed on a 5–18% gradient acrylamide. Complexes including either fluorescent actin (lanes b) or fluorescent S-1 (lanes c) were employed; t = acto–S-1 mixture before cross-linking. Protein bands were viewed under UV light (b and c) and then stained with Coomassie blue (a).

are directed against two epitopes (Roustan et al., 1986; Méjean et al., 1986); one comprises residues 1–7, and the other residues in the sequence 18–28. The antibody specific for the N-terminal seven residues was isolated as described by Roustan et al. (1986).

The antibodies directed against the actin segment of residues 40–113 were obtained from rabbit antisera induced by S-carboxymethylated actin (Benyamin et al., 1986). They were purified by adsorption to an affinity column (Beyamin et al., 1986), prepared by coupling Sepharose 4B to the 10K peptide generated by thrombic cleavage of G-actin and corresponding to residues 40–113 (Muszbeck et al., 1975). This peptide was isolated by gel filtration of a thrombic digest of actin over a Sephadex G-75 superfine column (2 × 150 cm) eluted with 2 mM Tris-HCl, pH 8.0 (Muszbeck et al., 1975). Its purity and the absence of the C-terminal 27K thrombic fragment (residues 114–375) were assessed by gel electrophoresis (Figure 7) and amino acid analyses; in addition, the ELISA assay indicated no contamination with the thrombic actino peptide of residues 1–28 (Figure 6).

Western blotting of peptides and proteins was carried out as previously described (Roustan et al., 1982). The competitive ELISA assay, using F-actin coated in the bottom of microtiter plates (Luxlon) and alkaline phosphatase labeled second antibody, was conducted as described elsewhere (Roustan et al., 1986; Méjean et al., 1986).

ATPase Measurements. The Mg^{2+}-dependent ATPase activity of the covalent acto–S-1 complexes was measured at 25 °C in a medium (1 mL) containing 50 mM Tris-HCl, 5 mM ATP, and 10 mM $MgCl_2$, pH 8.0, S-1 was added at 0.05 mg/mL. The Mg^{2+}-ATPase of S-1 was determined under similar conditions with 1 mg/mL S-1. Inorganic P_i was measured colorimetrically by an automated phosphate system as previously described (Mornet et al., 1979).

RESULTS

Characterization of the Covalent Acto–S-1 Complexes Formed by Cross-Linking with Glutaraldehyde or N-(Ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline. When the rigor acto–S-1 (A1 + A2) complex, including either fluorescent F-actin or fluorescent S-1, was treated with glutaraldehyde or *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) under the optimal conditions described and analyzed by gel electrophoresis, typical protein band patterns were obtained as illustrated in panels B and C of Figure 1, respectively. The distribution of the fluorescence facilitated identification of the component proteins of the observed new fluorescent bands. The glutaraldehyde treatment yielded two major covalent actin–heavy chain products with apparent molecular weights of 165K and 200K whereas three cross-linked actin–heavy chain species, with molecular weight values 165K, 175K, and 195K, resulted from the incubation of acto–S-1 with EEDQ. During the latter reaction there was also formation of an additional entity with a mass of 67K which was not observed with glutaraldehyde and which incorporated the fluorescence of actin only. The identification of this particular product as the adduct of actin with the A1 light chain was recently described in detail (Labbé et al., 1986). The electrophoretic mobility of the glutaraldehyde—or EEDQ—generated 165K and 175K bands is quite similar to that displayed by the major protein doublet formed on joining actin with S-1 and with EDC as a control (Figure 1A). In contrast, the production of the new 200K or 195K species is characteristic of the reactions catalyzed by the two former reagents. The glutaraldehyde treatment was conducted under mild conditions with, in particular, a very low concentration of reagent which caused no apparent intermolecular union between actin or S-1 molecules but only a significant cross-linking of actin with the S-1 heavy chain. The limited amount of doublet bands migrating at the 120K position and incorporating the fluorescence of S-1 only would represent cross-linked heavy chain–light chain species, the formation of which was also observed during the reaction of glutaraldehyde with S-1 alone (Hiratsuka, 1986).

The extent of acto–S-1 cross-linking with glutaraldehyde or EEDQ was determined by densitometric measurements of the 95K heavy chain band present on the gels before and after each cross-linking process. The yeilds, shown in Table I, were 20–25% and 10–12% for the glutaraldehyde and the EEDQ

Table I: Yield and Enzymatic Activity of the Covalent Acto–S-1 Complexes[a]

	% of cross-linking	turnover rate (s^{-1})
EDC	30–35	25–30
glutaraldehyde	20–25	22–27
EEDQ	10–12	26–30

[a] The cross-linked acto–S-1 complexes were isolated following dissociation of residual S-1 as indicated under Material and Methods. After spectrophotometric determination of the amount of cross-linked S-1 (Mornet et al., 1981b), the Mg-ATPase activity was measured on appropriate protein aliquots. The mean values of at least five preparations of each covalent complex are given.

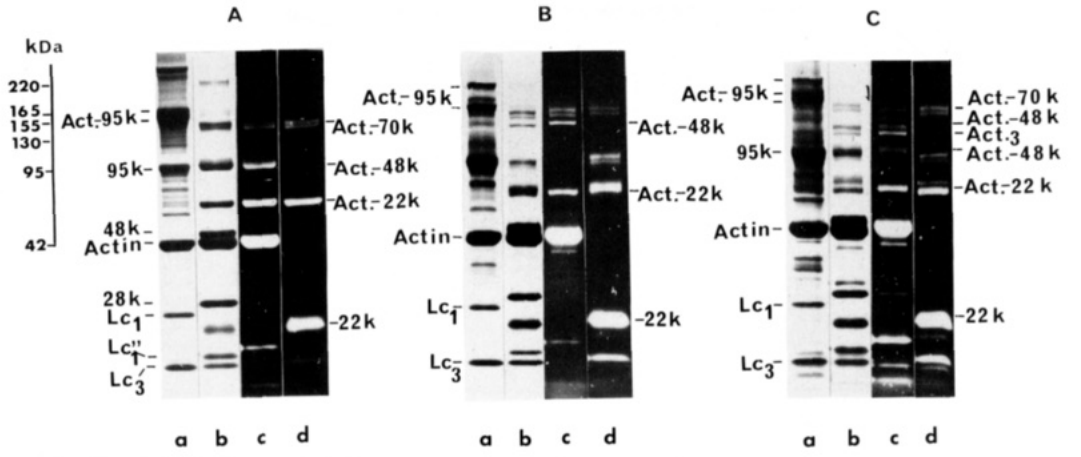

FIGURE 2: Characterization of the S-1 heavy chain fragments cross-linked to actin with EDC (A), glutaraldehyde (B), and EEDQ (C). The covalent complexes including either fluorescent actin (lanes c) or fluorescent S-1 (lanes d) were digested with *S. aureus* protease as reported under Materials and Methods. The distribution of the fluorescence in the digests (c and d) was compared with the corresponding patterns of the gels stained with Coomassie blue (b); (lanes a) covalent complexes before proteolysis. The minor 150K species labeled acto–70K would represent actin cross-linked to the uncleaved C-terminal (48K + 22K) segment.

reactions, respectively, as compared to 30–35% for the EDC-mediated cross-linking. Furthermore, the corresponding covalent complexes formed were isolated and were found to exhibit the elevated Mg^{2+}-ATPase activity that is characteristic of the permanent attachment of actin to S-1 (Mornet et al., 1981b). The turnover rates were in the range 22–30 s^{-1}, as compared to 25–30 s^{-1} for the EDC–acto–S-1 complex used as a control (Table I).

Identification of the S-1 Heavy Chain Regions Cross-Linked to Actin. Two experimental approaches were employed for identification of the heavy chain segments to which actin was cross-linked by glutaraldehyde or EEDQ. They involve the electrophoretic analysis of the cross-linked products formed either on digestion of the covalent acto–S-1 complexes with *S. aureus* protease which cuts only the heavy chain or on cross-linking of actin to various fragmented S-1 derivatives. The cross-linked species related to the EDC–acto–S-1 complex were used as controls. The overall results are presented in Figures 2 and 3. The fluorescent (AEDANS)acto–S-1 and (AEDANS)S-1–actin complexes were digested with *S. aureus* protease in the presence of Mg^{2+}-ATP. This protease cuts the S-1 heavy chain into 28K, 48K, and 22K fragments (Chaussepied et al., 1983). The added nucleotide suppresses the protective influence of actin on the cleavage of the 48K–22K junction without changing the pattern of the proteolysis. As shown in panels B and C of Figure 2, respectively, the glutaraldehyde digest contained two new species with apparent masses of 138K (acto–48K) and 64K (acto–22K) whereas three main products with molecular weight values 125K and 90K (both acto–48K) and 64K (acto–22K) were detected in the EEDQ digest. The control EDC digest yielded the two expected distinct entities with molecular weight values of 90K and 64K which are known to correspond to acto–48K and acto–22K peptides, respectively (Figure 2A and Figure 3A, lanes a–d). The 64K species present in the two former digests incorporated the fluorescence of both actin and S-1; it should, therefore, correspond to actin cross-linked to the COOH-terminal 22K heavy chain fragment. The 138K, 125K, and 90K species included the fluorescence of actin only. Their composition was deduced on the basis of the results obtained on cross-linking actin to the preformed proteolytic S-1 derivatives (Figure 3B,C). They would correspond to actin cross-linked to the central 50K heavy chain segment. Thus, the glutaraldehyde-induced covalent complex between actin and the tryptic (27K–50K–20K)-S-1 produced a single 140K band containing the actin fluorescence only (Figure 3B, lanes a–d). However, when the derivative employed is the tryptic (22K–50K/45K–20K)-S-1, this fluorescence was associated with a doublet band of 140K–135K (acto–50K and acto–45K) (Figure 3B, lanes e–h). Obviously, the latter protein bands should represent actin cross-linked to the 50K and 45K heavy chain segments, respectively. Consequently, the single band of molecular weight about 170K (acto–75K) produced on reaction of glutaraldehyde with the complex of actin and (75K–21K)-S-1 (a derivative formed by digestion with Arg-C protease) was regarded as the adduct of actin and the NH_2-terminal 75K heavy chain fragment (Figure 3B, lanes i and j). For all the proteolytic S-1 derivatives, glutaraldehyde-catalyzed cross-linking of actin to heavy chain segments including the 50K region resulted in the production of species with higher apparent masses than expected from their protein composition. Furthermore glutaraldehyde failed to cross-link actin to the 20K peptide present in the three S-1 derivatives employed. The glutaraldehyde-mediated union between actin and the COOH-terminal heavy chain region is observed only with intact S-1. In contrast, EEDQ cross-linking between actin and (27K–50K–20K)-S-1 led to the production of an acto–20K entity together with two new bands at the 130K and 90K positions (both acto–50K) which were associated with the actin fluorescence only (Figure 3C, lanes a–d). These were assumed to represent two complexes of actin and 50K fragment migrating on the gel with different electrophoretic mobilities. They would correspond to the two slower 195K and 175K bands initially observed on cross-linking of native acto–S-1 with EEDQ. Similarly, besides the production of an acto–21K band, the treatment of acto–(75K–21K)-S-1 with EEDQ generated also two new sepecies with apparent molecular weight values of 160K and 125K (both acto–75K) (Figure 3C, lanes i and j). The latters could be only cross-linked adducts of actin and the 75K fragment as suggested by their ability to incorporate the actin fluorescence.

In conclusion, like cross-linking studies of acto–S-1 with EDC, glutaraldehyde or EEDQ treatment of acto–S-1 in this study resulted in a covalent union between actin and either the 20K or 50K segment of the S-1 heavy chain. The overall cross-linking reactions between actin and the various proteolytic S-1's are summarized in Figure 4.

Identification of the Glutaraldehyde and EEDQ Cross-Linking Sites on Actin. To characterize the actin segments cross-linked to the S-1 heavy chain, we employed a rapid and

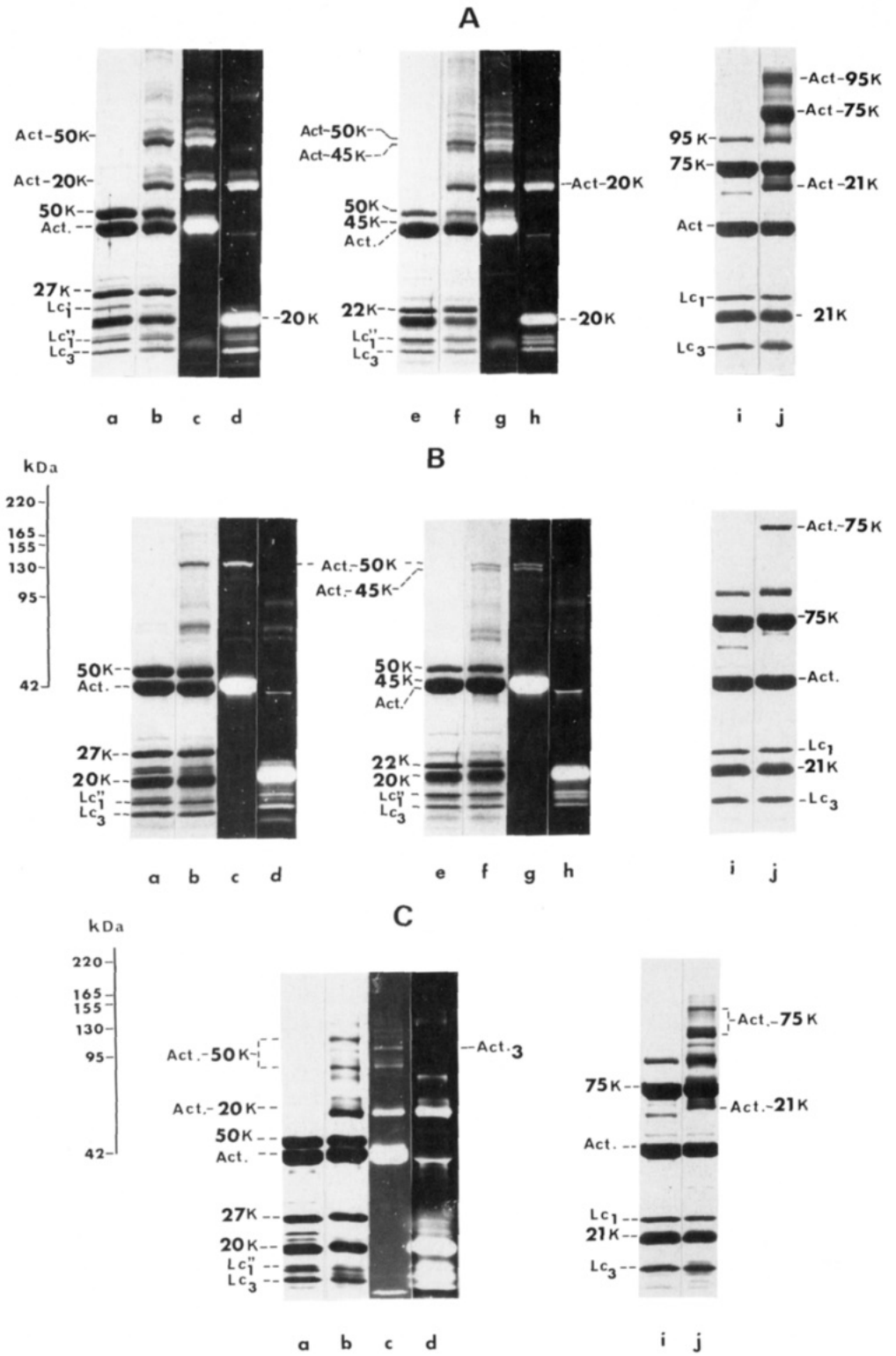

FIGURE 3: Comparative analyses of the cross-linking between actin and various proteolytic S-1 derivatives with EDC (A), glutaraldehyde (B), and EEDQ (C). Complexes of actin and (27K–50K–20K)-S-1 (lanes a–d), (22K–50K/45K–20K)-S-1 (lanes e–h), or (75K–21K)-S-1 (lanes i and j) were cross-linked as described under Materials and Methods. They included either fluorescent actin (lanes c and g) or fluorescent S-1 (lanes d and h). Coomassie-stained 5–18% acrylamide gels (a, b, e, f, i, and j) were compared with corresponding fluorescent gels (c, d, g, and h) viewed under UV light.

direct approach combining selective proteolytic and immunochemical methods. The various fluorescent covalent acto–S-1 complexes were isolated, depolymerized, and digested with thrombin which cuts only actin and not S-1. After gel electrophoresis of the digests, the actin peptides cross-linked to the intact 95K heavy chain were identified by immunoblotting with specific antibodies directed against different regions spanning the actin N-terminal residues 1–113. The fragmentation patterns and distribution of the fluorescence of either IAEDANS-actin or IAEDANS-S-1 are presented in Figure 5. Thrombin is known to split EDTA-treated G-actin into four fragments corresponding to residues 1–28, 29–39, 40–113, and 114–375; the two latter regions are termed M and L peptides with molecular weight values of about 10K and 27K, respectively (Muszbeck et al., 1975). Following the reactions with either cross-linker, the actin material was de-

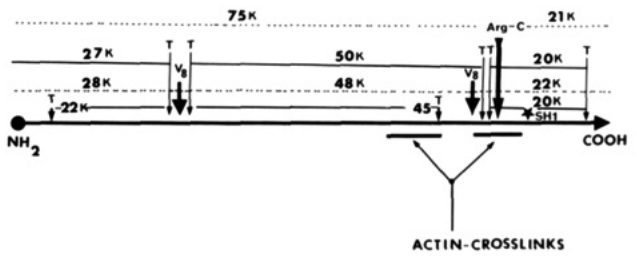

FIGURE 4: Schematic diagram illustrating the localization of the S-1 heavy chain region cross-linked to F-actin with glutaraldehyde and EEDQ as deduced from the cleavage of the cross-linked heavy chain with *S. aureus* protease and from the patterns of cross-linking between actin and the proteolytic S-1 derivatives. These include the indicated major peptides generated by trypsin (T), *S. aureus* protease (V8), and the arginine-specific endoproteinase (Arg-C). The star refers to the SH1 group labeled by 1,5-IAEDANS in fluorescent S-1; for other details, see text.

graded by thrombin into the same fragments (Figure 5). In addition, new bands with apparent molecular weights in the range 100K–110K were generated (Figure 5, lanes b). They incorporated the fluorescence of the S-1 heavy chain only (Figure 5, lanes c and d). Thus, they did not include the fluorescent C-terminal actin fragment of residues 114–375. We conclude that they represent cross-linked species of the 95K heavy chain and the N-terminal nonfluorescent thrombic peptides of actin (residues 1–113). The nature of these peptides was probed by the use of three distinct actin antibodies. The first one (referred to as A1) is directed against two epitopes located within the sequences of residues 1–7 and 18–28, respectively (Roustan et al., 1986; Méjean et al., 1986). Its specificity was quantitatively assessed by the ELISA assays reported in Figure 6. The second antibody (referred to as A2) is specifically directed against the epitope of residues 1–7 (Roustan et al., 1986). The third new antibody (referred to as A3) was directed against antigenic determinants within the actin segment of residues 40–113 (Boyer et al., 1987; Bertrand et al., 1987a). The Western blotting assay, depicted in Figure 7, lanes d–f, indicates that only the thrombic peptide which spans this segment was heavily stained by this antibody. The immunostaining analyses of each cross-linked complex with these antibodies are presented in Figure 8. The single 100K heavy chain–actin peptide band present in the digest of EDC–acto–S-1 was stained by A1 but not by A2 or A3 (Figure 8A, lanes b–e). This result is consistent with the expected carbodiimide-induced cross-linking of the actin thrombic fragment of residues 1–28 (Sutoh, 1982). The failure of A2 to recognize its epitope within residues 1–7 could result from a steric hindrance or the chemical modification afforded by the direct covalent coupling of the heavy chain to some of the carboxyl groups clustered in this epitope. Thus the observed reaction of A1 was made possible by the available second epitope within residues 18–28. In contrast, the pair of bands with molecular weight values of approximately 105K and 110K released by thrombin from glutaraldehyde-treated acto–S-1 were recognized by each of the three antibodies (Figure 8B, lanes b–e). Consequently, the two N-terminal segments of actin corresponding to residues 1–28 and 40–113, respectively, were concomitantly cross-linked by glutaraldehyde to the S-1 heavy chain. It is noteworthy that, unlike the EDC cross-linking, the glutaraldehyde-mediated reaction did not suppress the binding of A2 to its epitope. The difference in mobility displayed by the two protein bands could be caused by the heterogeneity of the cross-linking sites on actin and/or the S-1 heavy chain. Finally, the single protein entity with apparent mass 102K formed in the digest of EEDQ cross-linked acto–S-1 was recognized by both A1 and A3 but not by A2 (Figure 8C, lanes b–e). This result reveals also that the actin segments of residues 1–28 and 40–113 were simultaneously cross-linked by EEDQ to the S-1 heavy chain. In the digests of glutaraldehyde- and EEDQ-treated acto–S-1, a small amount of residual nondissociated 95K heavy chain is still observable, serving as an intrinsic marker. Its presence readily eliminates any cleavage of S-1 with thrombin. On the other hand, the specificity of the three actin antibodies is further illustrated by their inability to react with this 95K band. The diagram shown in Figure 9 summarizes the positioning of the S-1 cross-links on the N-terminal region of actin.

DISCUSSION

This work illustrates the ability of glutaraldehyde, a general protein cross-linker (Peters & Richards, 1977), to induce a selective cross-linking between actin and the myosin S-1 heavy chain. The reaction involves mainly the covalent union of the N-terminal domain of actin with the 50K and 20K heavy chain regions which are known to contain the putative actin binding sites of S-1 (Mornet et al., 1981b; Sutoh, 1983). For the 50K

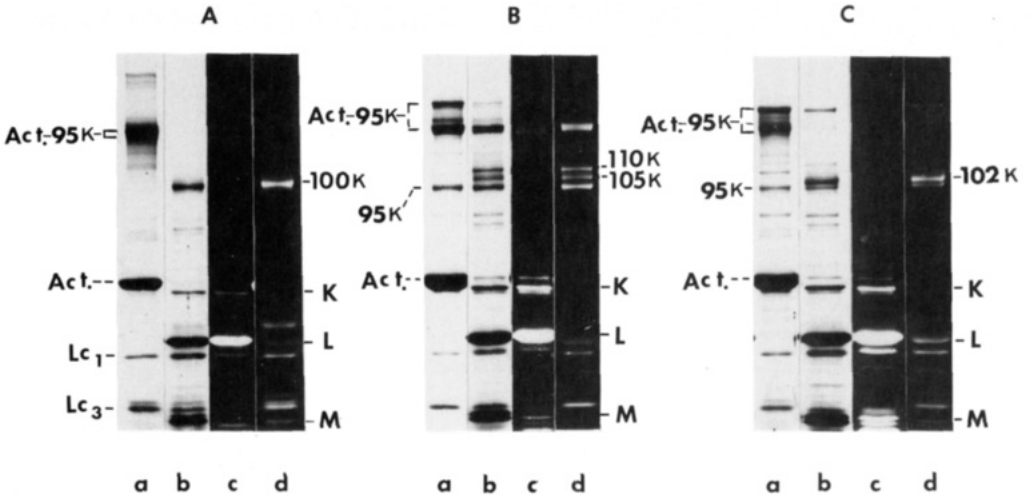

FIGURE 5: Thrombic digestion of the covalent acto–S-1 complexes formed with EDC (A), glutaraldehyde (B), and EEDQ (C). The various complexes (lanes a) were isolated almost free of un-cross-linked S-1, depolymerized by 0.6 M KI, and digested with thrombin as reported under Materials and Methods. The digests containing either fluorescent actin (lanes c) or fluorescent S-1 (lanes d) were electrophoresed on 5–18% gradient acrylamide gels. The fluorescence patterns of the gels (lanes c and d) were compared with the corresponding Coomassie blue stained gels (lanes b). Note the extensive but specific release by thrombin of the actin M and L peptides together with their precursor, the faint K band.

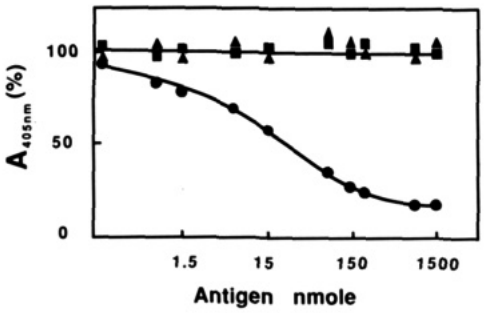

FIGURE 6: Specificity of the antibodies directed against the N-terminal actin segment of residues 1–28 determined by competitive ELISA assay. Coated F-actin was incubated with 75 μL of the antibody solution (24 μg/mL) in the presence of the indicated concentrations of the following antigens: F-actin (●) or the thrombic peptide of residues 40–113 (■) or of residues 29–39 (▲). The two latter fragments were isolated by gel filtration of a thrombic digest of actin as specified under Materials and Methods. The percent of antibody binding was measured at 405 nm in the presence of *p*-nitrophenyl phosphate (1 mg/mL) with a rabbit anti-sheep IgG antibody labeled with alkaline phosphatase as the secondary antibody.

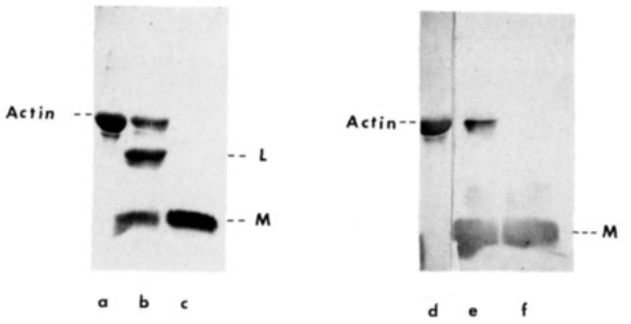

FIGURE 7: Characterization of the antibody to the actin segment of residues 40–113. Western blotting analysis of the intact actin and its thrombic digestion products. (Lanes a and d) Intact actin; (lanes b and e) thrombic digest of actin. The M and L bands represent the two large fragments of residues 40–113 and 114–375, respectively. (Lanes c and f) Isolated peptide of residues 40–113. (Left panel) 15% linear acrylamide gels stained with Coomassie blue; (Right panel) peptides transferred onto a nitrocellulose membrane stained with the antibody against the fragment of residues 40–113. Note that only actin and the M peptide were recognized by this antibody.

segment, in particular, the glutaraldehyde cross-linking is directed toward its NH_2-terminal 45K moiety essentially as observed previously with the zero-length cross-linker EDC (Labbé et al., 1984; Mornet & Ue, 1985). Recently, the treatment of S-1 or tryptic S-1 with glutaraldehyde, under mild conditions, was shown to promote a specific internal cross-linking between the 50K and 20K peptides (Hiratsuka, 1986). The establishment of the 50K–20K cross-linkages was accompanied by a marked enhancement of the Mg^{2+}-ATPase activity of S-1. However, our data indicate that the reaction of glutaraldehyde with the acto-S-1 complex, under nearly similar experimental conditions, did not cause a significant intra heavy chain cross-linking but mainly the covalent union between actin and the S-1 heavy chain. This was expected as the decrease or suppression of the intramolecular 50K–20K cross-linking upon actin binding has been observed previously (Mornet et al., 1981a; Labbé et al., 1982). Therefore, the elevated Mg^{2+}-ATPase displayed by the isolated glutaraldehyde-cross-linked acto–S-1 complex cannot be due to an interfragment cross-linking in S-1 but rather is due to the covalent attachment of actin to the S-1 heavy chain. This conclusion is supported by the recent finding that high Mg^{2+}-ATPase levels equivalent to the actin-activated myosin ATPase at infinite actin concentration were measured following cross-linking with glutaraldehyde of pure actomyosin or muscle myofibrils under rigor conditions (El-Saleh et al., 1986). Because glutaraldehyde appears valuable for the freezing of the "on" and "off" states of the myofibrils, our present study will help the understanding of its mode of action on the actin–myosin head system.

In the course of this investigation we have also analyzed the covalent acto–S-1 complex generated by the hydrophobic, carboxyl group directed cross-linker *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ). Previously, we have shown this zero-length cross-linking agent to elicit the covalent linkage of actin to the Al light chain and to the S-1 heavy chain as well (Labbé et al., 1986). In particular, EEDQ appeared more efficient than EDC in the cross-linking of the light chain to the COOH-terminal part of actin. However, unlike the EDC cross-linking, the reactions with either EEDQ or glutaraldehyde suggest for the first time the close proximity of the actin segment of residues 40–113 to the S-1 heavy chain in the rigor acto–S-1 complex. In this region there are 11 carboxyl side chains within positions 51–107. A hydrophobic environment around the EEDQ-activated carboxyl groups involved in cross-linking would favor their modification with this reagent rather than with water-soluble EDC. The latter cross-linker is, however, efficient in the activation of carboxyls located in the overlapping sequence of residues 87–119 since this particular segment can be cross-linked by EDC to α-actinin (Mimura & Asano, 1987). Like S-1, the latter protein was also cross-linked to the N-terminal actin segment of residues 1–12. For identification of the actin segment 40–113 within the cross-linked heavy chain–actin peptide species, use was made of an immunochemical approach previously described (Benyamin et al., 1986; Sutoh & Mabuchi, 1986). It is founded on the detection of the cross-linked N-terminal actin

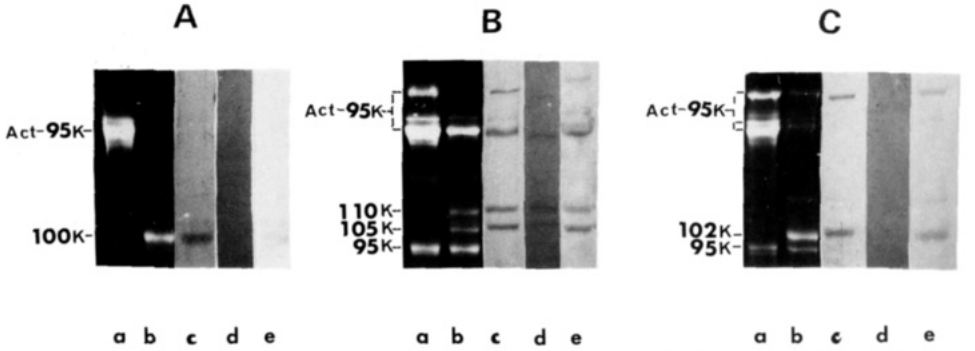

FIGURE 8: Identification of the actin peptides cross-linked to the S-1 heavy chain with EDC (A), glutaraldehyde (B), and EEDQ (C), using three specific antibodies directed against the N-terminal actin segment of residues 1–113. Covalent acto–S-1 complexes including fluorescent S-1 (lanes a) were digested with thrombin (lanes b). After electrophoresis on 7.5% acrylamide gels, the 100K–110K species containing the actin peptides cross-linked to the fluorescent heavy chain were visualized by immunoblotting with either of the following anti-actin antibodies: Al, directed against residues 1–28 (lanes c); A2, directed against residues 1–7 (lanes d); A3, directed against residues 40–113 (lanes e).

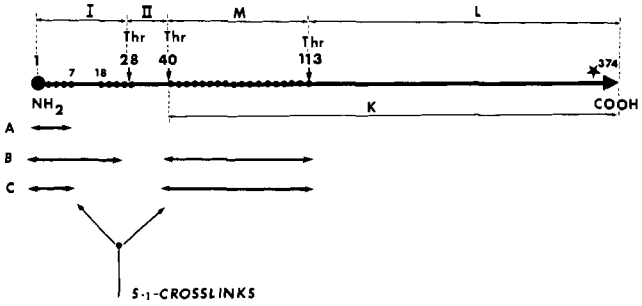

FIGURE 9: Schematic diagram showing the actin segments cross-linked to the S-1 heavy chain with EDC (A), glutaraldehyde (B), and EEDQ (C). Following thrombin digestion (Thr) of the isolated cross-linked acto–S-1 complexes, these segments were released covalently bound to the intact 95K heavy chain. After gel electrophoresis, they were identified by immunostaining with three specific antibodies directed against epitopes within the N-terminal sequence of residues 1–113. The star refers to cysteine-374 labeled by 1,5-IEADANS in fluorescent actin.

fragments by specific antibody populations. In this study we employed for the first time a polyclonal antibody population selectively directed against the actin peptide of residues 40–113 together with two other well-known antibodies which served for the characterization of the fragment of residues 1–28 (Mêjean et al., 1987). On the basis of the present cross-linking data and in agreement with immunochemical and ^{1}H NMR studies (Mêjean et al., 1986, 1987; Moir et al., 1987), at least part of the contact area of the S-1 heavy chain on actin would spread over multiple sites on the N-terminal third of actin. It is noteworthy that the differential behavior of smooth and striated muscle isoactins in myosin ATPase activation was correlated with amino acid substitutions at positions 17 and 89 of the actin sequence (Mossakowska & Strzelecka-Golaszewska, 1985). Also, the nitration of Tyr-69 in skeletal actin was reported to suppress the actin stimulation of the myosin ATPase without inhibiting its binding (Chantler & Gratzer, 1975). Similarly, a restricted proteolytic cleavage of the actin peptide at Val-43 or Met-47 decreases the affinity of the resulting nicked F-actin for the S-1–nucleotide complex and impairs the actin-activated ATPase of S-1 (Konno, 1987; Schwyter & Reisler, 1988). Finally, the integrity of Arg-95 is thought to be essential for tropomyosin binding to actin (Johnson & Blazyk, 1978). In this regard the reactivity of the antibody A3 toward F-actin is modified upon binding of tropomyosin or myosin S-1 (Boyer et al., 1987). The location of the tropomyosin site within the cross-linked 40–113 residues segment could be relevant to models of regulation which predict overlapping positions on actin for tropomyosin- and S-1–nucleotide intermediates during the actomyosin ATPase cycle (Lehrer & Morris, 1982; Hill et al., 1983).

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Expression and Stability of c-sis mRNA in Human Glioblastoma Cells†

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ABSTRACT: The production of platelet-derived growth factor like (PDGF-like) material by glioblastomas may be involved in the conversion of normal cells to tumor cells. In an investigation of this problem, we have examined some of the properties of the platelet-derived growth factor B-chain mRNA (c-sis mRNA) by a sensitive and quantitative RNA–RNA solution hybridization method. In 5 out of 8 human glioblastoma cell lines, c-sis mRNA was present, and in the line with the highest level, there were approximately 4–10 molecules per cell. The half-lives of the c-sis mRNA in two glioblastoma cell lines were 2.6 and 3.4 h, while in human umbilical vein endothelial (HUVE) and bladder carcinoma (T24) cells they were 1.6 and 2.5 h, respectively. Inhibiting protein synthesis produced no significant alteration of the c-sis mRNA half-lives in the glioblastoma or HUVE cells. The A-U-rich sequence at the 3′ end of the c-sis mRNA therefore does not appear to affect the mRNA stability in the presence of cycloheximide as it does in other transcripts. The similarity of the c-sis mRNA half-lives in normal and tumor cells suggests that regulation of stability of c-sis mRNA is not a major factor in tumorigenesis in the glioblastoma cell lines examined.

Mechanisms by which normal glial cells are converted to glioblastomas are still poorly understood. Some data are now available, however, suggesting the involvement of an autocrine growth loop (Sporn & Todaro, 1980) in glial cell tumorigenesis, whereby growth factor receptors are altered and/or growth factors themselves are abnormally produced. The epidermal growth factor (EGF) receptor gene, for instance, has been shown to be either amplified or structurally altered in a number of human glioblastomas (Libermann et al., 1985). Rat neuroglioblastomas induced by transplacental ethylnitrosourea (ENU) contain a point mutation in the transmembrane domain of the c-erb-B-2 (neu) transforming gene (Bargmann et al., 1986), the structure of which has considerable homology to the EGF receptor gene (Schechter et al., 1984). Long-term culture of neonatal rat brain cells similarly treated with ENU results in phenotypic transformation, the timing of which correlates with expression of the c-sis protooncogene (Lens et al., 1986), the gene encoding the B chain of platelet-derived growth factor (PDGF). These ENU-induced rat gliomas, as well as numerous cultured human glioblastomas, produce a PDGF-like mitogenic protein (Lens et al., 1986; Betsholtz et al., 1983; Nister et al., 1984; Pantazis et al., 1985), while normal human glial cells do not (Betsholtz et al., 1983). Because glial cells contain cell-surface receptors for PDGF (Heldin et al., 1981) and respond to exogenous PDGF by proliferating (Westermark & Wasteson, 1976; Besnard et al., 1987), the autocrine production of PDGF by glioblastomas may be a mechanism for constitutive stimulation of cellular growth. In support of this hypothesis, the growth rate in serum-free medium of various clonal glioblastoma cell lines correlates fairly well with their level of PDGF secretion (Nister et al., 1986). In addition, the in vivo growth of nude mouse tumors induced by v-sis-transformed fibroblasts correlates directly with their level of PDGF secretion (Huang et al., 1984). Although glioblastomas can express mRNA corresponding to both the A (Betscholtz et al., 1986a) and B (Pantazis et al., 1985; Betscholtz et al., 1986a; Eva et al., 1982) chain of PDGF, it is not clear whether glioblastoma-derived PDGF is composed of PDGF A chains, B chains, or both. The formation of glioblastomas in monkeys injected intracerebrally with simian sarcoma virus (SSV) (Swenberg, 1977), an acutely

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