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FORMATION OF AN AMP-PROTEIN COMPLEX IN HELA CELLS

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SUMMARY: A small basic protein with bound 5'-AMP has been detected in HeLa cells pulsed with [2-^{3}H]-adenosine. This protein-nucleotide complex is extractable from cells in cold 5% perchloric acid from which it is precipitated by 18% trichloroacetic acid. The labelled complex electrophoreses as a sharply defined peak on acid-urea acrylamide gels. It is stable under acid conditions, but readily dissociates at neutral pH. In lysates the AMP-protein complex is formed almost instantaneously and equally at 4^{o} or 25^{o}C with either 5'-AMP or 5'-AMP derived from 5'-ATP, as substrate.

INTRODUCTION

In an attempt to detect the formation of HI histone-poly(adenosine diphosphate ribose) complex in intact cells as reported (1), cells were pulsed with [2-^{3}H]-adenosine and then directly extracted with cold perchloric acid in which HI is soluble while most cellular proteins are insoluble. The extracted proteins were precipitated with 18% TCA and the precipitate electrophoresed on acid-urea gels. Small peaks of radioactivity corresponding to poly(ADP-ribosylated) HI monomer (2) and a specific Histone 1-poly(ADP-ribose) complex which has been shown to comprise of two HI molecules and a single chain of poly(ADP-ribose) (3), were detected on the gels, but a much more prominent peak of radioactivity was observed which migrated faster than the modified HI. This present report deals with a preliminary characterization of this major labelled species.

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MATERIALS AND METHODS

[2,8-^{3}H]-AMP (22 Ci/mmole) and [^{3}H]-ATP (96% pure) (13 and 26 Ci/mmole, labelled in the adenine moiety) were obtained from Schwarz-Mann. [2-^{3}H]-Adenosine (21 Ci/mmole) was from the Radiochemical Centre, Amersham. HeLa cells obtained from Flow Laboratories were maintained in shake culture as previously described (4).

Preparation of Cell Lysates: Cells were centrifuged at 500 x g for 5 min, washed once and swollen for 10 min in hypotonic buffer (A) (2 mM mercaptoethanol, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 5 mM EDTA, 10 mM Tris, pH 8.0), and finally disrupted by 6 strokes of a hand homogenizer or by sonicating.

Formation of AMP-Protein Complex: 500 µl aliquots of the cell lysate preparations (in buffer A) were incubated for either 10 sec at 4°C or 5 min at 25°C with 1.0 ml buffer (3 mM DTT, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Tris, pH 8.0) plus either 0.15 µmoles 5'-AMP and 50 µl [^{3}H]-AMP or 0.15µmoles 5'-ATP and 50 µl [^{3}H]-ATP. At the end of the incubation 500 µl 20% perchloric acid 0.2 M $NaHSO_3$ was added and the samples sonicated.

Isolation of AMP-Protein Complex: The sonicated perchloric acid suspension (5% final acid concentration) was centrifuged at 36,000 x g for 10 min and the supernatant precipitated with a final concentration of 18% TCA and the precipitated sample treated as described by Johns for the preparation of HI (5).

Acid-Urea Polyacrylamide Gel Electrophoresis: The final AMP-protein complex (HI) preparation was dissolved in 0.2 ml 0.9 N acetic-acid-15% sucrose and 90 µl aliquots applied to each of two acetic acid-urea polyacrylamide gels (7.5 x 0.5 cm tubes) and electrophoresed for 2 1/4 hours at 2 mA/tube as described by Panyim and Chalkley (6). At the end of the electrophoresis one gel was stained in 0.25% Coomassie Blue G250-9.2% acetic acid (v/v)-45.4% methanol (v/v) overnight, destained in a Canalco electrophoretic destainer, and scanned at 650 nm in a Clifford gel scanner. The second gel was cut into 1 mm slices which were dried overnight at 55°C before oxidizing in a Packard 306 sample oxidizer and the radioactivity in each slice determined in a Beckman scintillation counter.

RESULTS AND DISCUSSION

Acid-urea polyacrylamide gel electrophoresis of the 5% perchloric acid extractable, 18% TCA precipitable material from intact HeLa cells pulsed with [^{3}H]-adenosine is shown in Fig. 1A. A small peak of radioactivity is seen at fraction 12 and this corresponds to the position of the histone HI poly(ADP-ribose) complex which we have previously identified in HeLa cell nuclei incubated with NAD, the precursor for poly(ADP-ribose) (3). This complex has been shown to be composed of two molecules of HI and a single chain of poly(ADP-ribose) (3). A second small peak of radioactivity is seen at fraction 20 which corresponds to histone HI monomer bearing short poly(ADP-ribose) chains as previously reported (2). However, the

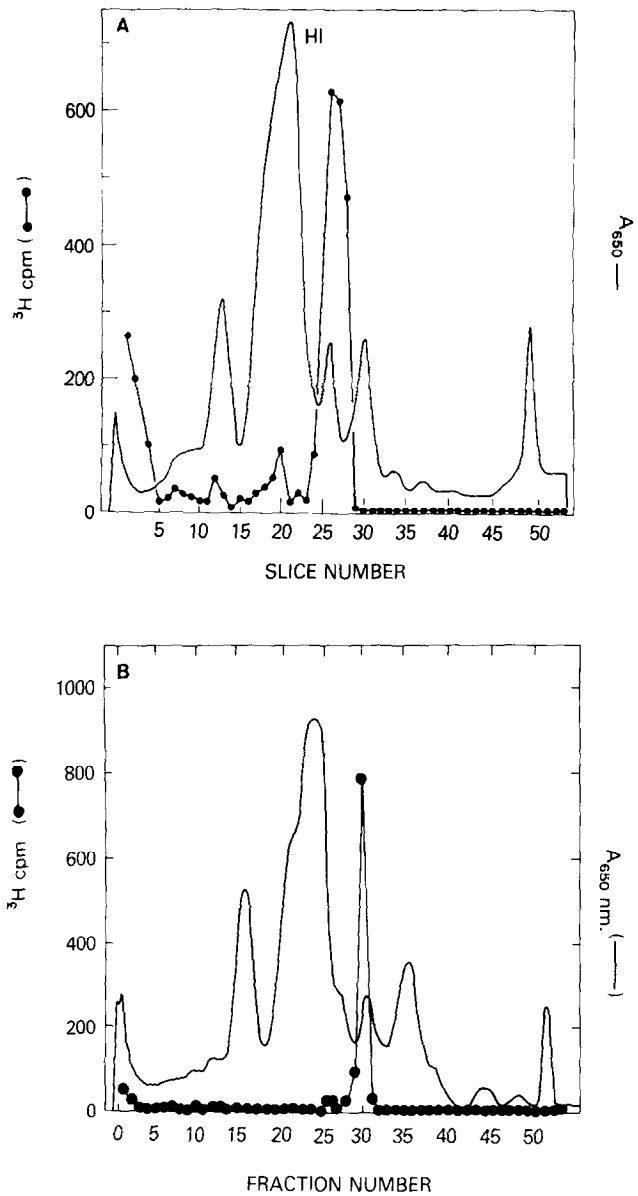

Fig. 1A. 750 ml cells (4 x 10^5 cells/ml) were pulsed for 2 hours with 1 mCi [2-^{3}H]-adenosine, before spinning down and washing with 50ml cold phosphate-buffered saline. The cell pellet was immediately extracted with cold 5% PCA-0.05 M $NaHSO_3$, sonicated, and the sample treated as described in Methods for the isolation of AMP-protein complex. The final preparation was dissolved in 0.5 ml 15% sucrose - 0.9 N acetic acid and 50 µl aliquots electrophoresed on acid urea gels and treated as described in Methods. ●—● ^{3}H-cpm; ——A_{650}.

Fig. 1B. 500µl cell lysate (total volume 3ml) prepared from 400ml cells (3 x 10^5 cells/ml) was incubated with [^{3}H]-ATP, extracted with PCA and the final preparation electrophoresed exactly as described in Methods. ●—● ^{3}H-cpm; ——A_{650}.

major labelled species electrophoreses faster than the major protein component (HI). This same species was labelled *in vitro* in cell lysates incubated with [^{3}H]-ATP or [^{3}H]-AMP as indicated in Fig. 1B. Using these nucleotide substrates no radioactivity was seen in areas of the gel corresponding to poly ADP-ribosylated HI. To test whether the radioactivity in the main peak on the gel was bound to protein, the TCA precipitated material was resuspended in 0.9 M acetic acid and 50 mg pepsin added to one-half of the sample. After one hour digestion at room temperature the electrophoretic mobility of the label was compared on acid-urea gels. In the sample without pepsin the radioactivity was seen at the usual position while that in the pepsin-treated sample was localized at the dye front, indicating protein-bound radioactivity. Digestion with a proteolytic enzyme under acid conditions was necessary because we have observed that the protein-bound radioactivity is irreversibly converted to a form not precipitated by 18% TCA upon a brief incubation of the preparation at neutral pH. When the radioactivity in the *in vivo* labelled preparation was dissociated from protein by incubation at neutral pH and analysed by PEI-cellulose thin layer chromatography, 79% of the radioactivity migrated as authentic 5'-AMP (Fig. 2). Treatment with alkaline phosphatase converted the radioactivity to a product that chromatographed as adenosine (Fig. 2). Analysis of the radioactivity by high pressure liquid chromatography on a μ-Bond C18 column (Waters) developed with 0.05 M sodium phosphate pH 5.0 or with a gradient of 0-20% methanol containing 0.32% tributylammonium hydroxide pH 3.2 also indicated 90% of the radioactivity was 5'-AMP. A similar analysis of the complex formed in cell lysates also indicated 5'-AMP to be the bound nucleotide when either [^{3}H]-ATP or [^{3}H]-AMP was used as substrate in the incubation.

The mobility of this AMP-protein complex on the acid-urea polyacrylamide gels is consistent with it being a small basic protein having a molecular weight of approximately 10,000 daltons. Using this value and

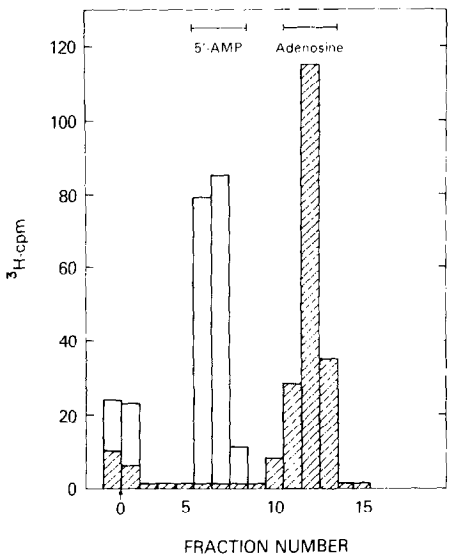

Fig. 2. 200ml cells (3 x 10^5/ml) were incubated for 1 hour with 400μCi [2-^{3}H]-adenosine, washed with phosphate-buffered saline, extracted with 5% PCA, and the final sample dissolved in 0.5ml water. 60μl aliquots were incubated for 30 min at 37°C with 10μl 50 mM $MgCl_2$, 20μl 0.5 M Tris, pH 8.0, and either 10μl H_2O (□) or 10μl alkaline phosphatase (▨) (400 U/mg, 1mg/ml, Boehringer). 20μl aliquots of this reaction mixture were applied to PEI-cellulose sheets together with 10μl adenosine (1mg/ml) and 10μl 5'-AMP (5mg/ml) and the chromatogram developed in methanol and then 1 N acetic acid before cutting into 1 cm strips and determining the radioactivity in each strip.

a value for the amount of [^{3}H]-AMP incorporated, we have calculated there to be approximately 6 x 10^4 acceptor protein molecules/cell if we make the assumption that there is one AMP bound per one protein molecule.

A comparison was made of the time and temperature dependency of [^{3}H]-ATP and [^{3}H]-AMP labelling of this protein complex. We observed that in the absence of EDTA an equal amount of label was incorporated into the complex on the gel at 4°C and 10 seconds or 25°C for 5 minutes whether [^{3}H]-ATP or [^{3}H]-AMP was the substrate (Table 1). However, in the presence of 10mM EDTA no radioactivity was seen on the gel when [^{3}H]-ATP was used as substrate, whereas production of this labelled complex from [^{3}H]-AMP was unaffected by EDTA (Table 1). As shown in Table 2, ATP is rapidly converted to AMP and ADP upon incubation with cell lysates in the absence of EDTA and under these conditions the labelled complex is seen on the gels

Table 1

Synthesis of Major Labelled Species In Vitro

Incubation	Major Peak, ^3H cpm
[^3H]-ATP, 4°C, 10 sec	1,397
[^3H]-ATP, 4°C, 10 sec, 10 mM EDTA	0
[^3H]-ATP, 25°C, 5 min	1,439
[^3H]-ATP, 25°C, 5 min, 10 mM EDTA	0
[^3H]-AMP, 4°C, 10 sec	1,425
[^3H]-AMP, 4°C, 10 sec, 10 mM EDTA	1,528
[^3H]-AMP, 25°C, 5 min	1,514
[^3H]-AMP, 25°C, 5 min, 10 mM EDTA	1,478

500µl cell lysate (total volume = 6ml) prepared from 1000 ml cells (3.5×10^5 cells/ml) was incubated as shown in the table and then the AMP-protein complex isolated, electrophoresed and analyzed as described in Methods.

Table 2

Metabolism of [^3H]-ATP by Cell Lysates

Incubation	% cpm			
	IMP	AMP	ADP	ATP
[^3H]-ATP, 4°C, 10 sec, EDTA	1	7	31	61
[^3H]-ATP, 4°C, 10 sec	2	24	47	27
[^3H]-ATP, 25°C, 5 min, EDTA	1	8	53	38
[^3H]-ATP, 25°C, 5 min	5	53	33	9

500µl cell lysate (total volume 2.5ml) prepared from 1000 ml cells was incubated with [^3H]-ATP in the presence and absence of 10 mM EDTA and processed as described in Methods. The 18% TCA supernatant was extracted 4-times with water-saturated ether, neutralized with KOH and the insoluble $KClO_4$ centrifuged down. 10µl of the supernatant were applied to PEI-cellulose thin layer sheets together with 1 mg/ml standard solutions of ATP, ADP, AMP and IMP and the chromatogram developed in methanol and then 1.6 M LiCl. The marker compounds were located under UV light, cut out and the cpm determined.

(Table 1). In the presence of EDTA considerably less AMP is produced from ATP (Table 2) and under these conditions no labelled complex is produced (Table 1). Thus it seems clear that AMP binds to the protein directly in the complex formation and that ATP must first be converted to AMP.

The specificity of the AMP-protein complex formation was assessed in the presence and absence of 10mM EDTA with a 20-fold molar excess of a variety of adenine nucleotides. As shown in Table 3, only 5'-AMP in the presence and absence of EDTA and ATP and ADP in the absence of EDTA competed with labelled 5'-AMP for complex formation. Adenosine, 3', 5'-cyclic AMP,

Table 3

Adenine Nucleotide Specificity for the Synthesis of the Major Labelled Species

Competing Nucleotide	^{3}H cpm Peak	% Inhibition of Complex Formation
None, 4°C, 10 sec	575	-
None, 25°C, 5 min	568	-
ATP, 4°C, 10 sec	0	100
ATP, 4°C, 10 sec + EDTA	560	1.4
ATP, 25°C, 5 min	0	100
ADP, 4°C, 10 sec	0	100
ADP, 4°C, 10 sec + EDTA	600	0
ADP, 25°C, 5 min	0	100
5'-AMP, 4°C, 10 sec	0	100
5'-AMP, 4°C, 10 sec + EDTA	0	100
5'-AMP, 25°C, 5 min	0	100
Adenosine, 25°C, 5 min	554	2.5
ADP-ribose, 25°C, 5 min	522	8
Cyclic AMP, 25°C, 5 min	524	8
2', 3',-AMP, 25°C, 5 min	595	0

500µl cell lysate (total volume 6 ml) prepared from 400ml cells (3.5×10^5/ml) was incubated in the standard reaction mixture in the presence and absence of 10 mM EDTA and 2 mM of the appropriate nucleotide, extracted with PCA and the sample processed and electrophoresed as described in Methods.

a mixture of 2' and 3'-AMP and ADP-ribose were without effect. The ability of ATP and ADP to compete out the label from the complex in the absence of EDTA arises from the production of AMP from these compounds which is the true substrate. A variety of other purine and pyrimidine nucleotides have also been tested (TMP, UMP, XMP, IMP, GMP and CMP) and none of these affect the production of the AMP-protein complex.

The addition of 5mM NaF or Oubain (5mM) to inhibit the Na^+/K^+ activated ATPase (7, 8) had no effect on the formation of the protein-AMP complex. The stability of the AMP-protein complex is unlike that of adenylylated glutamine synthetase (9) or DNA ligase from E. coli (10). In terms of the kinetics and temperature dependency it resembles the atractyloside-sensitive (11) bongkrekic acid-sensitive (12) adenine nucleotide transport system of rat liver mitochondria. However, this system involves ATP and ADP and not AMP.

All of the complex-forming activity is in a 105,000 x g supernatant fraction of HeLa cells or rat liver. Nevertheless, 70% of the AMP-protein complex formed in vivo from an [^{3}H]-adenosine pulse is localized in the nuclear pellet following low speed centrifugation. This AMP-protein complex is formed in vivo in cultured cells as well as in the livers and mammary tumors of perfused rats. Although the significance of these findings is at present unknown, further effort is under way to characterize this complex.

REFERENCES

1. Ueda, K., Omachi, A., Kawaichi, M., and Hayaishi, O. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 205-209.
2. Lorimer III, W.S., Stone, P.R., and Kidwell, W.R. (1977). Exp. Cell Res. 106, 261-266.
3. Stone, P.R., Lorimer III, W.S., and Kidwell, W.R. (1977). Eur. J. Biochem. 81, 9-18.
4. Kidwell, W.R., and Burdette, K.E. (1974) Biochem. Biophys. Res. Commun. 61, 766-773.

5. Johns, E.W. (1964) Biochem. J. 92, 55-59.
6. Panyim, S., and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346.
7. Glyn, I.M. (1957) J. Physiol. 136, 148-173.
8. Kirschener, L.B. (1964) Arch. Biochem. Biophys. 106, 57-64.
9. Shapiro, B.M., Kingdon, H.S., and Stadtman, E.R. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 642-649.
10. Little, J.W., Zimmerman, S.B., Oshinsky, C.K., and Gellert, M. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 2004-2011.
11. Winkler, H.H., Bygrave, L.F., and Lehninger, A.L. (1968) J. Biol. Chem. 243, 20-28.
12. Henderson, P.J.F., and Lardy, H.A. (1970) J. Biol. Chem. 245, 1319-1326.