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Phosphorylation of human serum amyloid A protein by protein kinase C

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Monokine-induced hepatic secretion of serum amyloid A protein (apo-SAA), an acute-phase reactant, is followed by rapid association with high-density lipoprotein (HDL) in plasma. Plasma clearance of apo-SAA is more rapid than any of the other HDL apolipoproteins. It has been shown that, of the acute-phase HDL_3 apolipoproteins, apo-SAA preferentially associates with neutrophil membranes. HDL apolipoproteins have been shown to activate protein kinase C in endothelial cells. We therefore investigated potential phosphorylation of HDL_3 apolipoproteins by protein kinase C. Apo-SAA was the only apolipoprotein phosphorylated (K_m = 12 mM). Phosphorylation of the apo-SAA-containing HDL_3 particle was selective for the more basic isoforms of apo-SAA (pI 7.0, 7.4, 7.5 and 8.0), with more acidic isoforms being phosphorylated when delipidated acute-phase apolipoproteins were used as substrate. However, phosphorylation was not in itself responsible for the establishment of the apo-SAA isoforms.

INTRODUCTION

Serum amyloid A protein (apo-SAA) is an acute-phase reactant in humans with levels increasing up to a 1000-fold after inflammation, infection or necrosis [1]. Apo-SAA is synthesized and secreted by hepatocytes, whereupon it rapidly associates with circulating high-density-lipoprotein (HDL) particles [2,3]. The bulk of apo-SAA is found in association with HDL_3 particles, wherein it may become the major apolipoprotein, exceeding the molar quantity of apolipoprotein A-I in certain individuals [4].

Certain disease states can give rise to persistent elevation of apo-SAA levels, and this may culminate in amyloidosis in susceptible individuals [1]. In the pathogenesis of amyloidosis the 11.5 kDa apo-SAA protein is proteolytically converted into an 8 kDa fragment, amyloid A protein (protein AA), which constitutes the bulk of the protein material of amyloid fibrils [5,6].

Apo-SAA is a polymorphic protein, with up to six major isoforms having been identified [7]. It has recently been established that individual variations in isoform patterns occur: three patterns have been described [8]. The basis for the variation in isoform patterns has not been established. Evidence for multiple alleles has been presented, but it is not known how many human apo-SAA genes are transcribed during the acute-phase response or to what extent post-translational modifications (such as phosphorylation) may be responsible for the phenotypic expression. Although apolipoproteins participate in a large number of metabolic processes, the function of apo-SAA is completely unknown. The exact mechanism of association and uptake of HDL particles by the extrahepatic cell is unknown, but it is interesting to note that apo-SAA is the predominant AP (acute-phase)-HDL_3 apolipoprotein associating with neutrophil membranes [9]. It is also known that the apolipoprotein moiety of normal HDL_3 may activate the Ca^{2+}/phospholipid-dependent kinase (C-kinase) in endothelial cells, giving rise to specific membrane protein phosphorylation [10]. We were therefore interested to see whether AP HDL_3 could serve as a substrate for C-kinase *in vitro*. C-kinase plays an important role in cell-surface signal transduction in a variety of mammalian tissues [11,12]. Activity of C-kinase results from receptor-mediated stimulation of membrane lipid turnover with resultant accumulation of 1,2-diacylglycerol [12,13]. Phosphorylation of cellular proteins by activated C-kinase plays an important regulatory role in cell proliferation and differentiation.

We found the apo-SAA moiety of HDL_3 to be a substrate for C-kinase. Cyclic AMP-dependent protein kinase (A-kinase) failed to phosphorylate apo-SAA. Phosphorylation was selective among the isoforms, but not in itself responsible for their existence. Only a small percentage of plasma apo-SAA is phosphorylated under physiological circumstances. The potential physiological relevance of these findings is discussed.

MATERIALS AND METHODS

Preparation and iodination of HDL_3

Blood was collected (after informed consent and Ethical Committee approval) from patients exhibiting an acute-phase response and who had a variety of diseases. The pooled plasma was obtained as previously described [3]. HDL_3 (density 1.13–1.18 g/ml) fractions were prepared by discontinuous gradient ultracentrifugation [3,14]. Lipoproteins were iodinated by a modified ICl

Abbreviations used: protein AA, amyloid A protein; apo-SAA, serum amyloid A protein; AP-HDL_3, acute-phase high-density lipoprotein; BSA, bovine serum albumin; N-HDL_3, normal high-density lipoprotein; PBS, phosphate buffered saline (2.7 mM-KCl/6.5 mM-KH_2PO_4/Na_2HPO_4/136.9 mM-NaCl, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; H_7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SAC, *Staphylococcus aureus* Cowan strain I.

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method using $Na^{125}I$ (Amersham International, Amersham, Bucks., U.K.) [15].

Purification of apo-SAA

Apo-SAA was purified from AP-HDL_3 by using molecular-sieve chromatography as previously described [14].

Delipidation of HDL_3

Two procedures for the delipidation of HDL_3 were followed. Firstly, delipidation of HDL_3 before phosphorylation was done essentially as described previously [16]. A portion (500 μg) of AP-HDL_3 was treated with 5 ml of ethanol/diethyl ether (3:2, v/v) for 4 h at −20 °C and then centrifuged at 2750 rev./min (1000 *g*) in a SM-24 rotor (du Pont–Sorvall) for 20 min at −20 °C. The pellet was resuspended in 5 ml of ether, re-centrifuged for 30 min (see above) and the pellet resuspended in 300 μl of ether before layering on to 350 μl of 10 mM-Tris/1 mM-$CaCl_2$/10 mM-$MgCl_2$/0.25 mM-EGTA, pH 7.4. The ether was then evaporated off at 4 °C. In the second procedure, samples for electrofocusing were delipidated by the Folch method [17]. Briefly, 500 μl of chloroform/methanol (2:1, v/v) was added to the freeze-dried sample, centrifuged for 5 min in a Microfuge (8000 *g*), evaporated to dryness under N_2 gas and taken up in 200 mM-Tris/7 M-urea/5% 2-mercaptoethanol, pH 8.0.

Electrofocusing to detect apo-SAA isoforms

An LKB Ultromould gel-casting unit and a 0.3 mm spacer were used to cast gels on to Gelbond PAG plates (FMC, Rockland, Maine, U.S.A.). The gel mixture (7.7 ml) contained 1.28 ml of acrylamide (28.8% acrylamide/1.2% bisacrylamide), 7 M-urea and 128, 257 and 257 μl respectively of ampholytes in the pH range 3.5–10, 4–6.5 and 6–8. The gels were polymerized with 2.5 μl *NNN'N'*-tetramethylethylenediamine and 7.7 μl of 40% (w/v) ammonium persulphate solution. The pH range was developed with 1 M-NaOH and 0.33 M-H_3PO_4 electrode wicks. Gels were prefocused at 300 V for 30 min before sample application and then focused to equilibrium at 3–5 W for 3 h at 10 °C. Samples (see above) were applied at the anode. After this the pH gradient was determined from 1 cm × 0.5 cm gel strips soaked for 2 h in 2 ml of distilled water.

Gels were fixed, stained with Coomassie Brilliant Blue and autoradiographed on Kodak XAR film where appropriate.

SDS/PAGE

SDS/PAGE was performed by using a 5–20% (w/v) polyacrylamide gradient gel with a 3% stacking gel according to the method of Laemmli [18]. For two-dimensional electrophoresis the desired track was excised from the electrofocusing gel, lowered through 1% low-gelling-temperature agarose (Sigma) on to the stacking gel and electrophoresed until the dye front had left the gel. Gels were stained, dried, and then autoradiographed with Kodak XAR film where necessary.

Purification of C-kinase

C-kinase was purified to near homogeneity by using sequential chromatography over DEAE-Sephacel (Sigma), phenyl-Sepharose (Pharmacia) and protamine–agarose (Sigma) columns as previously described [19].

Cell-free phosphorylation reactions

Phosphorylation reaction mixtures contained 200 μg of AP-HDL_3 or delipidated AP-HDL_3 and 2.5 μg of purified C-kinase in a buffer consisting of 10 mM-Tris/HCl, pH 7.4, 10 mM-$MgCl_2$, 250 μM-EGTA, in the absence or presence of 1 mM-$CaCl_2$, 10 μg of phosphatidylserine and 2 μg of diolein (dioleoylglycerol) in a final volume of 200 μl. Reactions were initiated by the addition of 2 nmol of [γ-^{32}P]ATP containing 1×10^7 c.p.m. at 30 °C for 10 min and terminated by boiling in SDS sample buffer for SDS/PAGE or by precipitation with ice-cold 20% (w/v) trichloracetic acid for electrofocusing. Before electrofocusing, trichloroacetic acid was extracted twice with diethyl ether. The specificity of the reactions were tested by omission of C-kinase or its allosteric effectors. The ability of cyclic AMP-dependent kinase to phosphorylate the apoproteins of HDL_3 was tested in a similar regime using an extract of cAMP-dependent kinase in the absence or presence of cyclic AMP [20].

For the determination of the K_m for apo-SAA phosphorylation, various concentrations of AP-HDL_3 were phosphorylated, as described above, for time periods ranging between 2 and 30 min. The amount of radiolabel incorporated was determined by slicing phosphorylated 11.5 kDa bands from the gels, followed by Čerenkov counting. The K_m was calculated by Lineweaver–Burk-plot analysis.

Intact-cell phosphorylation reactions

Jurkat cells were depleted of phosphate for 1 h in phosphate-free Earle's balanced salt solution. Cells were then labelled with 0.5 mCi [^{32}P]P_i (specific radioactivity 3700 Ci/mmol)/ml for 2 h 30 min in the same medium containing phosphate-free 5% (v/v) fetal-calf serum. The cells were washed twice, and 10^7 cells were incubated in borosilicate glass tubes for 1 h at 37 °C with 100 μg of AP-HDL_3 in a final volume of 1 ml of phosphate-free medium. Incubations were stopped by centrifugation, washing and lysing the cell pellets in 300 μl of buffer containing 0.5% sodium deoxycholate, 0.5% Triton X-100, 1 mM-ATP, 10 mM-NaF, 100 μM-vanadate, 2 mM-PMSF, aprotinin (100 kallikrein-inhibitory units/ml), 300 mM-NaCl, 20 nM-*p*-nitrophenyl phosphate and 50 mM-Tris/HCl, pH 8.0. Triton-soluble material was collected after centrifugation and analysed by Western blotting, revealed with an avidin–biotin system (Dakopatts, Copenhagen, Denmark), followed by autoradiography.

Phosphoamino acid analysis

Phosphorylated apo-SAA bands were cut from gels and hydrolysed in 5.7 M-HCl. Phosphoamino acid analysis was performed as previously described [21].

Tryptic-peptide mapping

Phosphorylated apo-SAA bands were excised from gels, electroeluted [22], precipitated in 20% trichloroacetic acid, washed with diethyl ether and resuspended in 20 mM-Tris/HCl, pH 7.4. Digestion of the eluted protein was performed essentially as described previously [22], by using Trypsin (Difco Laboratories) at 75 μg/ml for

1 h at 37 °C. The reaction was terminated by freeze-drying.

Two-dimensional mapping of tryptic peptides of phosphorylated apo-SAA was carried out as described previously [23]. High-voltage (1000 V) electrophoresis, performed for 1 h on cellulose plates in acetic acid/formic acid/water (3:1:16, by vol.), was used as the first dimension. The second dimension was by ascending chromatography in butan-1-ol/pyridine/acetic acid/water (13:10:2:8, by vol.). Plates were dried and autoradiographed.

Removal of phosphate from AP-HDL_3

Delipidated or non-delipidated AP-HDL_3 (150 μg/ml) was incubated with 10 μg (16 units) of calf intestinal alkaline phosphatase (EC 3.1.3.1) in 200 μl of 20 mM-Tris/150 mM-NaCl/10 mM-$MgCl_2$ buffer, pH 7.6, at 37 °C for 4 h. Reactions were terminated by precipitation with trichloracetic acid, and samples were prepared for electrofocusing as described above.

P_i determinations

Twice-delipidated apo-SAA was used to determine the P_i content by the Malachite Green method of Itaya & Ui [24].

RESULTS

Phosphorylation of apo-SAA by C-kinase

When AP-HDL_3 was incubated with C-kinase in the presence of its agonists and [γ-^{32}P]ATP, only one protein of apparent molecular mass 11.5 kDa was seen to be phosphorylated (Fig. 1). This was confirmed as apo-SAA by immunoprecipitation (Fig. 1, track 14).

The specificity of the kinase was confirmed in several ways. No phosphorylation of apo-SAA could be revealed if the phosphorylation reactions were carried out in the absence of $CaCl_2$, phosphatidylserine and diolein (Fig. 1, track 2). Maximal labelling was seen if all three allosteric effectors were present (Fig. 1, track 5). However, Ca^{2+} by itself induced limited phosphorylation of the 11.5 kDa substrate (Fig. 1, track 3). Addition only of phosphatidylserine did not result in phosphorylation (Fig. 1, track 4). The potent C-kinase inhibitor H_7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride] substantially decreased the labelling of this substrate (Fig. 1, tracks 6 and 7). The HDL_3 preparation was free of any inherent kinase activity (results not shown). Cyclic AMP-dependent protein kinase failed to phosphorylate apo-SAA or any of the other apolipoproteins of AP-HDL_3 (results not shown).

The K_m value of apo-SAA in the phosphorylation reaction, as determined by Lineweaver–Burk analysis, was shown to be 12 μM.

Phosphoamino acid analysis and peptide mapping

Phosphoamino acid analysis performed on ^{32}P-labelled apo-SAA cut from SDS-containing gels revealed serine phosphorylation (Fig. 2). Two-dimensional tryptic phosphopeptide maps revealed two labelled fragments of unequal intensity, as shown in Fig. 3.

Phosphorylation of various isoform patterns

Recently three different human apo-SAA isoform patterns were described [8]. Pattern 1 (heterozygotes) exhibits six major isoforms with pI values of 6.0, 6.4, 7.0, 7.4, 7.5 and 8.0, whereas patterns 2 and 3 (homozygotes) lack the pI-7.4/8.0 and pI-7.0/7.5 isoforms respectively (Fig. 4*a*). Therefore all three phenotypes share the two acidic isoforms of pI 6.0 and 6.4, but differ in their content of the relatively more basic isoforms in the pI 7.0–8.0 range. It was thus of interest to see whether C-kinase had a predilection for any of these isoforms. Fig. 4(*a*) shows that the enzyme specifically phosphorylated isoforms with pI values greater than 7.0, resulting in anodal migration. The autoradiogram of apo-SAA isoforms of pattern 1 after phosphorylation shows four distinct radioactive bands with pI values of 6.4, 6.5, 6.75 and 6.9. The autoradiogram of phosphorylated protein patterns 2 and 3 showed distinct radioactive bands at pI 6.4/6.75 and 6.5/6.9 respectively (Fig. 4*a*). Note that the autoradiogram of pattern 1 is a composite of patterns 2 and 3.

Confirmation that the phosphorylated bands at pI 6.4, 6.5, 6.75 and 6.9 were indeed apo-SAA was obtained from two-dimensional electrofocusing and SDS/5–20%-

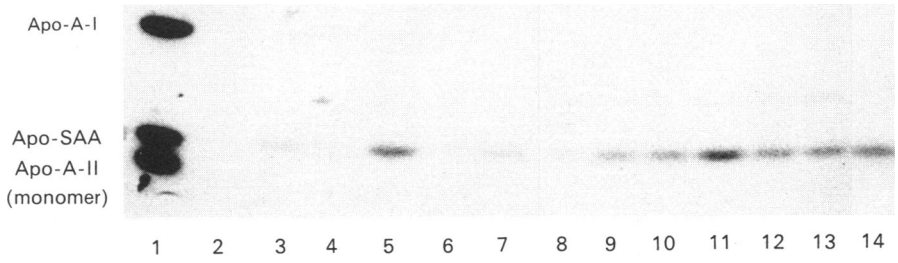

Fig. 1. Autoradiogram of AP-HDL_3 phosphorylated by C-kinase

AP-HDL_3 was incubated in the presence of C-kinase and [γ-^{32}P]ATP in the standard assay mixture described in the Materials and methods section. Reaction mixtures were analysed by SDS/PAGE and the gel was stained and autoradiographed. Track 1, ^{125}I-labelled AP-HDL_3; track 2, omitting Ca^{2+}, phosphatidylserine and diolein from reaction mixture; track 3, Ca^{2+} (1.5 mM) present, no phosphatidylserine or diolein; track 4, phosphatidylserine (5 μg/ml) and diolein (2.5 μg/ml) present, no Ca^{2+}; track 5, phosphatidylserine (5 μg/ml) and diolein (2.5 μg/ml) and Ca^{2+} (1.5 mM) present; track 6, reaction mixture as in track 3, in the presence of 20 μM-H_7; track 7, reaction mixture as in track 5, in the presence of 20 μM-H_7; tracks 8–11, reaction mixture containing phosphatidylserine (5 μg/ml), diolein (2.6 μg/ml) and, in respective tracks, 0.5, 0.6, 0.75 and 1.5 mM-Ca^{2+}; tracks 12 and 13, Ca^{2+} (1.5 mM), diolein (2.5 μg/ml) and, respectively, 10 and 20 μg of phosphatidylserine/ml; track 14, ^{32}P-labelled precipitate obtained with anti-apo-SAA. Abbreviations: Apo-A-I and -II, apolipoproteins A-I and A-II.

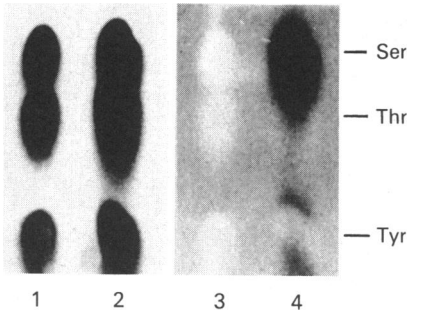

Fig. 2. Phosphoamino acid analysis of phosphorylated Apo-SAA

Phosphorylated amino acids were analysed as described in the Materials and methods section. Track 1, ninhydrin stain of non-labelled phosphoamino acid standards; track 2, ninhydrin stain of ^{32}P-labelled apo-SAA mixed with phosphoamino acid standards; track 3, autoradiogram of track 1; track 4, autoradiogram of track 2.

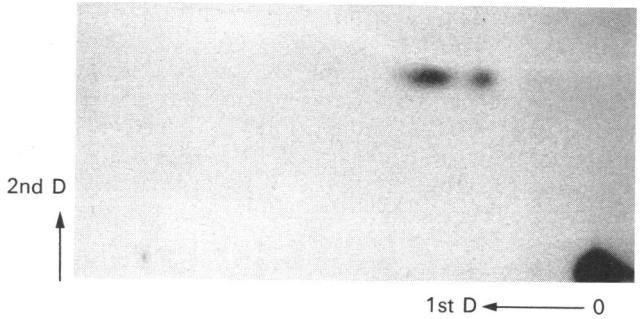

Fig. 3. Autoradiogram of Apo-SAA tryptic phosphopeptides

AP-HDL$_3$ was phosphorylated by C-kinase and the ^{32}P-labelled apo-SAA analysed by tryptic-peptide mapping as described in the Materials and methods section. The autoradiogram displays two tryptic phosphopeptides. The origin is indicated by 'O'. Abbreviations: 1st D, first dimension; 2nd D, second dimension.

(w/v)-PAGE. All four labelled isoforms migrated at an apparent molecular mass of 11.5 kDa, as revealed by the autoradiogram of the second-dimension SDS/polyacrylamide gel (Fig. 5).

Extension of the pH range to pH 3.8 failed to show phosphorylation of more acidic isoforms (results not shown). Although alkaline phosphatase was effective in stripping the ^{32}P label from apo-SAA (Fig. 4*b*, track j), this did not result in a shift in position of the major isoforms as detected by Coomassie Blue staining (Fig. 4*b*, track h).

Effect of delipidation

Phosphorylation after delipidation of AP-HDL$_3$ resulted in an altered pattern of phosphorylation, as seen on the autoradiogram (Fig. 6). Not only were the relatively basic isoforms much more intensely phos-

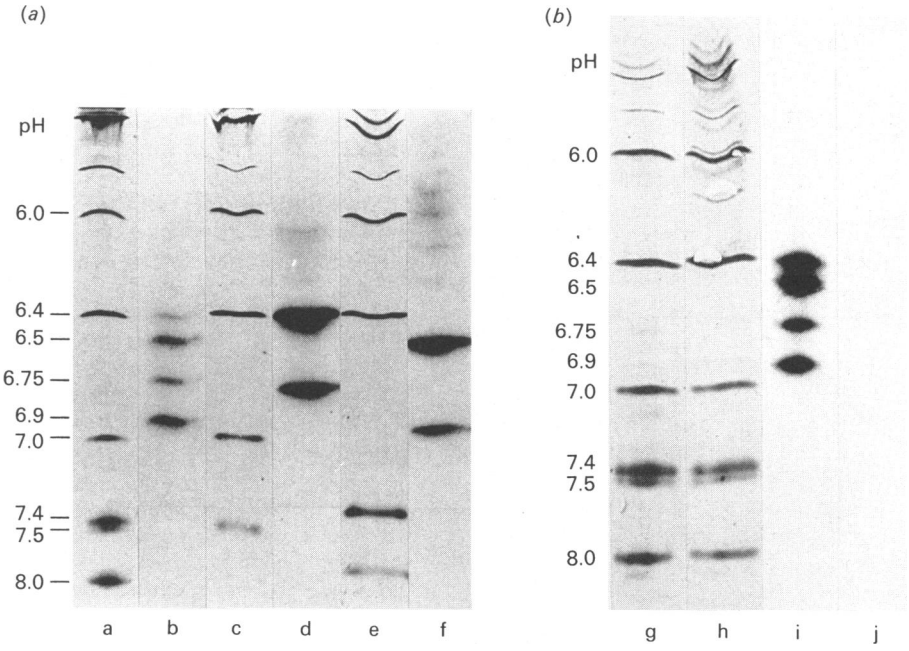

Fig. 4. Isoelectric focusing of phosphorylated AP-HDL$_3$

(*a*) AP-HDL$_3$ was phosphorylated *in vitro* by C-kinase and the reaction mixtures analysed by electrofocusing as described under 'Cell-free phosphorylation' in the Materials and methods section. Track a, Coomassie Blue stain of ^{32}P-labelled AP-HDL$_3$ displaying isoform pattern 1 (notice faint bands between pI 6.4 and 7.0, possibly representing phosphorylated minor isoforms); track b, autoradiogram of track a; track c, Coomassie Blue stain of ^{32}P-labelled AP-HDL$_3$ displaying isoform pattern 2; track d, autoradiogram of track c; track e, Coomassie Blue stain of ^{32}P-labelled AP-HDL$_3$ displaying isoform pattern 3; track f, autoradiogram of track e. (*b*) Material from apo-SAA isoform pattern 1 was treated with alkaline phosphatase as described in the text. Tracks g and h represents the Coomassie Blue stain before (track g) and after (track h) dephosphorylation, whereas tracks i and j represent the respective autoradiograms.

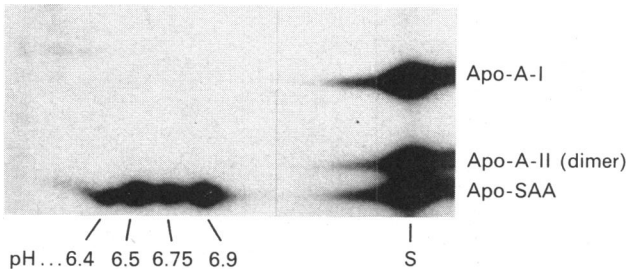

Fig. 5. Autoradiogram of two-dimensional electrophoresis of ^{32}P-labelled Apo-SAA

AP-HDL$_3$ (isoform pattern 1) was phosphorylated by C-kinase and the reaction mixture analysed by two-dimensional electrophoresis as described in the text. The autoradiogram of the second-dimension SDS/PAGE, using ^{125}I-labelled AP-HDL$_3$ as standard (S), displays ^{32}P-labelled isoforms corresponding to apo-SAA in apparent molecular mass. Apo-A-I and -II are defined in Fig. 1.

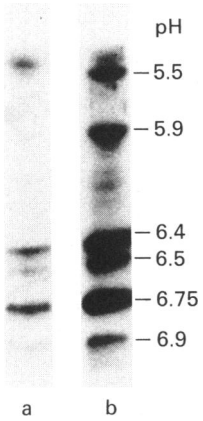

Fig. 6. Electrofocusing of phosphorylated AP-HDL$_3$ before and after delipidation

AP-HDL$_3$ displaying apo-SAA isoform pattern 1 was delipidated and phosphorylated by C-kinase, and the reaction mixture analysed by electrofocusing as described under in the Materials and methods section. The autoradiogram displays phosphorylation of isoforms before (track a) and after delipidation (track b) of the AP-HDL$_3$ Similar amounts of protein were used. Notice enhanced labelling of material at pI 6.4, 6.5, 6.75 and 6.9 as well as appearance of new bands at pI 5.5 and 5.9 after delipidation.

phorylated, but additional isoforms of pI 5.9 and 5.5 appeared for the first time under these conditions. Their identity as apo-SAA isoforms was confirmed by two-dimensional electrofocusing and SDS/5–20%-(w/v)-PAGE (results not shown).

P_i determinations

Triplicate measurement of the stoichiometric relationship between phosphate and various batches of 25 μg of apo-SAA purified from plasma followed by extensive delipidation showed 0.12 ± 0.03 mol of phosphate/mol of apo-SAA.

Intact-cell labelling studies

Incubation of the Jurkat T-cell line with AP-HDL$_3$

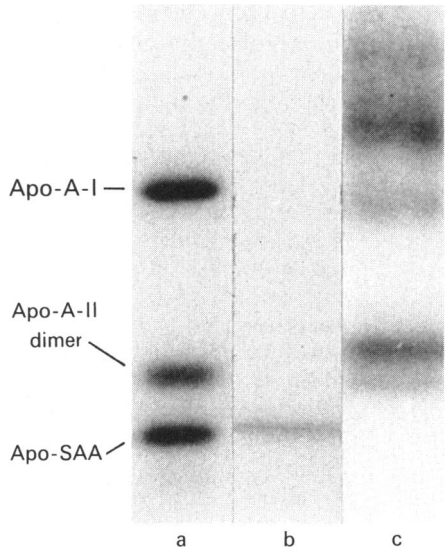

Fig. 7. Association of AP-HDL$_3$ with cells

AP-HDL$_3$ was incubated with ^{32}P-labelled lymphocytes, and the Triton-soluble material was analysed by Western blotting as described in the Materials and methods section. Control incubations were carried out with ^{125}I-labelled AP-HDL$_3$ and unlabelled lymphocytes. Track a, autoradiogram of ^{125}I-labelled AP-HDL$_3$; track b, transblot of apo-SAA present in Triton-soluble material of ^{32}P-labelled lymphocytes incubated with AP-HDL$_3$; track c, autoradiogram of track b. Apo-A-I and -II are defined in Fig. 1.

particles led to association of apo-SAA with the Triton-soluble material, as detected by Western blotting (Fig. 7, track b). This is in agreement with our recent findings indicating the association of apo-SAA with neutrophil cell membranes. Using phosphorylated Jurkat cells, we failed to show phosphorylation of the membrane-associated apo-SAA (Fig. 7, track c).

DISCUSSION

In the present study we investigated the possibility that apo-SAA-containing HDL$_3$ could act as a substrate for C-kinase *in vitro* as well as *in vivo*. Our results showed phosphorylation of apo-SAA *in vitro* selective for more basic isoforms with AP-HDL$_3$ as substrate. Phosphorylation *per se* was not responsible for the isoform phenomenon. Our results are interesting from a number of other viewpoints which require further comment.

Several observations confirm the specificity of the kinase–substrate interaction. Cyclic AMP-dependent protein kinase which, like C-kinase, phosphorylates proteins on serine residues, failed to phosphorylate any apolipoprotein moiety in AP-HDL$_3$. C-kinase, on the other hand, only phosphorylated the apo-SAA moiety of AP-HDL$_3$. This reaction was diminished in the presence of the potent and relatively specific C-kinase inhibitor H_7. Maximum phosphorylation required the presence of all three allosteric effectors (Ca^{2+}, phosphatidylserine and diolein) to optimally activate the kinase. It is of interest that the presence of Ca^{2+} by itself could result in limited substrate phosphorylation in the presence of AP-HDL$_3$. It is likely that these particles contain sufficient

endogenous phosphatidylserine to be able to activate the enzyme submaximally in the presence of Ca^{2+}.

Phosphorylation occurred at more than one serine site, as determined by phosphoamino acid analysis and tryptic-peptide mapping. The latter procedure yielded two labelled fragments of uneven intensity, which would suggest that the more intense spot represents either a multiple phosphorylated fragment or differential phosphorylation. The published amino acid sequence of an apo-SAA isoform [5] reveals seven serine residues. Of the potential tryptic fragments, only one fragment has two serine residues. Further studies need to be conducted to determine the exact sites of phosphorylation, since these could influence lipid binding and proteolytic cleavage.

The selectivity of isoform phosphorylation and submolar association of phosphate with total apo-SAA are both interesting and complex findings. Only the more basic isoforms with pI values greater than 7 could be shown to serve as substrates for C-kinase across our pH range of detection. Phosphorylation of pI isotypes outside the focusing range cannot be excluded. With both the Coomassie Blue-stained pattern as well as the autoradiogram of phosphorylated material it would seem as if phenotype 1 is a composite of patterns 2 and 3, which would suggest that pattern 1 represents the heterozygous state between homozygous patterns 2 and 3. An important question is whether phosphorylation could be responsible for establishing the major isoform patterns by post-translational modification of a more elementary precursor. We think this to be unlikely for the following reasons: Firstly, although phosphorylation *in vitro* results in an anodal shift of certain relatively basic isoforms, there was no shift in the position of the major isoform bands visible by Coomassie Blue staining. This would indicate that relatively few molecules in each basic isoform population were phosphorylated. Secondly, alkaline phosphatase treatment did not result in any rearrangement of the major isoforms (Fig. 4*b*). Thirdly, the association of phosphate with apo-SAA is of substoichiometric proportions. These observations do not exclude the existence of minor phosphorylated isoforms. By using Western blotting and sensitive immunogold staining, further minor bands in addition to the corresponding pI-6.4 isoform were detected, with pI values corresponding to those of the substrates phosphorylated *in vitro* (results not shown).

The observation that only the relatively more basic isoforms of apo-SAA were phosphorylated could be related to the selective access of C-kinase to more basic isoforms as they exist in relation to the lipid shell of AP-HDL_3, since delipidation before phosphorylation *in vitro* resulted in labelling of additional acidic isoforms (Fig. 6).

It is interesting that the acute-phase response often occurs under circumstances when C-kinase is also activated in several tissues and cells involved in the inflammatory response e.g. neutrophils and lymphocytes. It is therefore possible that apo-SAA, redistributed to selected cell membranes via AP-HDL_3 particles, could serve as substrate for C-kinase. It was shown that apo-SAA associates with HDL_3 particles to change their size and composition [3], and also that apo-SAA can be selectively distributed from the AP-HDL_3 particle to the plasma membrane of neutrophils [9] and lymphocytes. Our attempts to show that membrane-associated apo-SAA was phosphorylated in intact ^{32}P-labelled lymphocytes were inconclusive.

In conclusion we have shown that apo-SAA, and more specifically its basic isoforms, is a good substrate for C-kinase. The biological significance of this finding requires further elucidation.

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