Identification of the adipocyte acid phosphatase as a PAO-sensitive tyrosyl phosphatase

L. L. SHEKELS, A. J. SMITH, R. L. VAN-ETTEN and D. A. BERNLOHR

Protein Sci. 1992 1: 710-721

Protein Science (1992), *1*, 710–721. Cambridge University Press. Printed in the USA.

Identification of the adipocyte acid phosphatase as a PAO-sensitive tyrosyl phosphatase

LAURIE L. SHEKELS,[1] ANNE J. SMITH,[1] ROBERT L. VAN ETTEN,[2] AND DAVID A. BERNLOHR[1]

[1] Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
[2] Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

(RECEIVED November 20, 1991; REVISED MANUSCRIPT RECEIVED February 5, 1992)

Abstract

We have partially purified an 18-kDa cytoplasmic protein from 3T3-L1 cells, which dephosphorylates pNPP and the phosphorylated adipocyte lipid binding protein (ALBP), and have identified it by virtue of kinetic and immunological criteria as an acid phosphatase (EC 3.1.3.2). The cytoplasmic acid phosphatase was inactivated by phenylarsine oxide (PAO) (K_{inact} = 10 μM), and the inactivation could be reversed by the dithiol, 2,3-dimercaptopropanol (K_{react} = 23 μM), but not the monothiol, 2-mercaptoethanol. Cloning of the human adipocyte acid phosphatase revealed that two isoforms exist, termed HAAPα and HAAPβ (human adipocyte acid phosphatase), which are distinguished by a 34-amino acid isoform-specific domain. Sequence analysis shows HAAPα and HAAPβ share 74% and 90% identity with the bovine liver acid phosphatase, respectively, and 99% identity with both isoenzymes of the human red cell acid phosphatase but no sequence similarity to the protein tyrosine phosphatases (EC 3.1.3.48). HAAPβ has been cloned into *Escherichia coli*, expressed, and purified as a glutathione *S*-transferase fusion protein. Recombinant HAAPβ was shown to dephosphorylate pNPP and phosphoALBP and to be inactivated by PAO and inhibited by vanadate (K_i = 17 μM). These results describe the adipocyte acid phosphatase as a cytoplasmic enzyme containing conformationally vicinal cysteine residues with properties that suggest it may dephosphorylate tyrosyl phosphorylated cellular proteins.

Keywords: acid phosphatase; phenylarsine oxide; tyrosine phosphatases

Although it has been appreciated for several years that the insulin receptor functions as a ligand-activated protein tyrosyl kinase, the exact steps in the insulin signaling pathway have remained elusive. The insulin signaling pathway initiates with the specific binding of insulin to the extracellular α subunit of the insulin receptor and the subsequent activation of the insulin receptor tyrosyl kinase through autophosphorylation of one or more tyrosyl residues on the cytoplasmic domain of the β subunit (Ellis et al., 1986; Rosen, 1987). Following activation of the receptor, a cascade of regulatory phosphorylation events occurs that culminates in alterations in cellular amino acid, protein, carbohydrate, and lipid metabolism. Efforts to dissect the individual steps in this cascade have been concentrated on the identification of physiologically relevant substrates for the receptor kinase and their corresponding phosphatases.

In 3T3-L1 adipocytes, PAO inhibits insulin-stimulated but not basal hexose transport (Frost & Lane, 1985). Concomitant with insulin addition to PAO-treated cells, a cytoplasmic 15-kDa phosphorylated protein accumulates (Bernier et al., 1987). Reversal of PAO action with 2,3-DMP results in the loss of the phosphorylated 15-kDa protein and the reacquisition of insulin sensitivity. The 15-kDa protein has been identified as the ALBP (also referred to as 422 and aP2) (Hresko et al., 1988), a cytoplasmic fatty acid binding protein belonging to a multi-

Reprint requests to: David A. Bernlohr, Department of Biochemistry, University of Minnesota, 1479 Gortner Avenue, St. Paul, Minnesota 55108.

Abbreviations: PAO, phenylarsine oxide; HAAP, human adipocyte acid phosphatase; PTPase, protein tyrosine phosphatase; 2,3-DMP, 2,3-dimercaptopropanol; ALBP, adipocyte lipid binding protein; WGA, wheat germ agglutinin; IRK, insulin receptor kinase; BHAP, bovine heart acid phosphatase; BLAP, bovine liver acid phosphatase; pNPP, *p*-nitrophenyl phosphate; GST, glutathione *S*-transferase; GST-K-PTPU323, GST–rat brain PTPase fusion protein; BLOTTO, bovine lacto transfer technique optimizer; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SSC, standard saline citrate (0.15 M NaCl, 0.0015 M sodium citrate).

gene family of hydrophobic ligand binding proteins. The phosphorylation of ALBP occurs with identical properties both in situ and in vitro (Chinander & Bernlohr, 1989; Buelt et al., 1991) suggesting that ALBP is a primary substrate of the insulin receptor kinase. Together the in situ and in vitro observations suggest that following insulin addition to cultured 3T3-L1 adipocytes, ALBP is phosphorylated by the insulin receptor and that its subsequent dephosphorylation proceeds via the action of a PAO-sensitive tyrosyl phosphatase.

Approximately 90% of the phosphoALBP phosphatase activity found in adipocytes is membrane associated (Liao et al., 1991). However, the existence of a soluble tyrosine phosphatase that is responsible for phosphoALBP dephosphorylation cannot be ruled out based on several lines of evidence. Firstly, in situ, phosphoALBP from 3T3-L1 cells resides cytoplasmically, and, in vitro, purified ALBP has no measurable affinity for membranes or artificial vesicles. Secondly, the bulk of the phosphatase activity in these cells is PAO insensitive, suggesting that if phosphoALBP were to associate with membranes it would be dephosphorylated readily. Lastly, Frost and Schwalbe (1990) have shown that in 3T3-L1 cells approximately half of the proteins modified by a tritium-labeled derivative of PAO were cytoplasmic. In light of these observations, we considered whether any known cytoplasmic phosphatases exhibited properties (i.e., active site sulfhydryl[s] and sensitivity to PAO) that would suggest that it may function as a phosphoALBP phosphatase.

Currently, there are two rapidly growing classes of protein tyrosyl phosphatases. The first class (to be referred to here as the PTPases; for review see Hunter [1989]) shares sequence similarity within a 25-kDa catalytic domain. They are sulfhydryl enzymes and include both receptorlike and nonreceptor PTPases. The receptorlike PTPases such as CD45 (Tonks et al., 1990) are membrane spanning proteins and often possess two catalytic regions on the intracellular domain. The nonreceptor PTPases frequently reside in the cytosol and contain only a single catalytic domain. Recently, Guan et al. (1990) have reported the cloning of a rat brain PTPase and the characterization of its catalytic properties. The expression of the catalytic domain in *Escherichia coli* as a soluble GST fusion protein, GST-K-PTPU323, has greatly facilitated its molecular analysis (Guan & Dixon, 1991).

A second class of phosphatases consists of low molecular mass enzymes termed acid phosphatases. Although these 18-kDa enzymes have an acidic pH optimum, particularly when measured with the acidic substrate pNPP, they are reasonably active on phosphotyrosyl protein substrates at pH 6–7 (Chernoff & Li, 1985; Boivin et al., 1987; Waheed et al., 1988). The acid phosphatases are also sulfhydryl enzymes (Laidler et al., 1982; Zhang & Van Etten, 1990; Wo et al., 1992); however, the sequences of the bovine liver protein (Camici et al., 1989) and the human red cell enzymes (Dissing et al., 1991) show no similarity to the catalytic domain of the PTPases (Charbonneau et al., 1989). The acid phosphatases contain eight cysteine residues all of which exist in the free thiol form despite the small size of the protein. The abundance of free sulfhydryls suggests that these enzymes may be sensitive to PAO modification. Additionally, the cytosolic location of the acid phosphatases may also allow these enzymes to act as soluble phosphoALBP phosphatases.

The purpose of this study was to determine if adipocytes express a low molecular mass cytoplasmic acid phosphatase, to investigate its inactivation with PAO, and to examine its phosphoALBP dephosphorylation activity. We characterize here the cytoplasmic low molecular weight acid phosphatase from 3T3-L1 adipocytes and demonstrate that it is a PAO-sensitive phosphatase. Furthermore, the cloning and sequencing of the human adipocyte acid phosphatase (HAAP) has revealed the presence of two isoforms, HAAPα and HAAPβ, both of which possess numerous cysteine residues similar to the liver, red cell, and heart enzymes.

Results

To test the hypothesis that adipocytes express an acid phosphatase capable of dephosphorylating phosphoALBP, we have partially purified a soluble low molecular weight phosphatase from 3T3-L1 adipocytes using a modification of the procedures described by Taga and Van Etten (1982). In the purification, a soluble protein extract was collected following digitonin permeabilization of the adipocyte plasma membranes. Previous studies have shown that the acid phosphatase is most stable at pH 5–6; consequently, a pH 5.0 soluble extract was obtained and used throughout the isolation. As shown in Figure 1, the phosphatase activity largely partitions with the low molecular weight fraction of the soluble adipocyte extract. The 3T3-L1 phosphatase was then examined for its ability to utilize phosphoALBP as a substrate and for its sensitivity to PAO. When the partially purified 3T3-L1 phosphatase (specific activity, 5 units/mg) was incubated with phosphoALBP, the substrate was dephosphorylated (Fig. 2A). To compare the dephosphorylation properties of the 3T3-L1 phosphatase to those of a member of the PTPase family of enzymes, the catalytic fragment of the rat brain phosphatase (Guan & Dixon, 1991) was utilized. The rat brain PTPase identified by Dixon and colleagues had previously been subcloned, expressed, and purified as a soluble GST fusion protein termed GST-K-PTPU323. As shown, when homogeneous GST-K-PTPU323 (specific activity, 29 units/mg) was assayed for phosphatase activity, it was also capable of dephosphorylating phosphoALBP (Fig. 2A). These results indicated that in vitro little specificity for dephosphorylation was evident. Consistent with this was our observation that a variety of membrane extracts contained active phosphoALBP phosphatase activity (results not shown and Liao et al. [1991]).

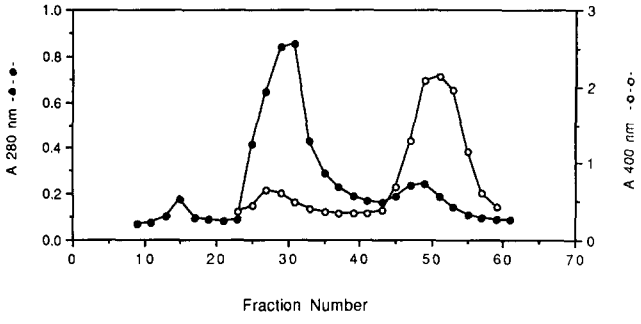


Fig. 1. Purification of the 3T3-L1 acid phosphatase. Cytosolic extract from adipocyte monolayers was prepared by treating the monolayers with 50 μg/mL digitonin. The digitonin releasate was acidified to pH 5.0 and the soluble protein recovered by centrifugation. Following delipidation, the soluble protein was fractionated over a Sephadex G-75 column. Column fractions were assayed for phosphatase activity using pNPP (o). Protein content was estimated by the absorbance at 280 nm (●).

To determine if the 3T3-L1 tyrosyl phosphatase was subject to PAO inactivation, the enzyme was incubated with 100 μM PAO and then assessed for phosphatase activity using pNPP. The 3T3-L1 acid phosphatase could be inactivated by 100 μM PAO (Fig. 2B).

To further characterize the 3T3-L1 enzyme preparation and its relatedness to the low molecular weight acid phosphatases, immunological and kinetic properties of the enzyme were assessed. To examine the immunological cross-reactivity of the 3T3-L1 enzyme to the acid phosphatases, antisera to the bovine heart acid phosphatase were used in western blot analysis. As shown in Figure 3, a single 18-kDa band was observed when anti-BHAP was used as a probe. Control sera did not react with the 3T3-L1 enzyme preparation (results not shown). Examination of the pH profile for the 3T3-L1 phosphatase revealed an acidic pH profile with optimum rates being obtained between pH 4.5 and 7.5 (Fig. 4A). At pH 5.0, the K_m for *p*-nitrophenyl phosphate was 164 μM (Fig. 4B). Both the pH profile and the Michaelis constant for pNPP were similar to those reported for the human placental and liver low molecular weight acid phosphatases (Waheed et al., 1988; Zhang & Van Etten, 1990). To characterize the PAO inactivation more fully, the concentration dependence of modification was measured. As shown in Figure 5A, PAO inactivation of the 3T3-L1 acid phosphatase occurs with a K_{inact} value of 10 μM, very similar to the observed in situ value of 7 μM for PAO inhibition of insulin-activated hexose transport. Because PAO interacts with vicinal dithiols, its action can be reversed by addition of a competing dithiol. In vitro when the 3T3-L1 acid phosphatase was first inactivated by PAO and then incubated with either 2,3-DMP or 2-mercaptoethanol only the dithiol was observed to reverse the action of PAO (Fig. 5B). The K_{react} for reactivation was 23 μM, a value similar to that observed in situ. Therefore, the inhibitory properties of PAO on the 3T3-L1 phosphatase mirrored the results observed in situ. Moreover, the ability of the PAO-inhibited enzyme to be reactivated by 2,3-DMP is analogous to that observed in cultured cells.

A common property of tyrosyl phosphatases is inhibition by micromolar concentrations of vanadate. Consequently, the effect of sodium orthovanadate on the 3T3-L1 phosphatase was examined in vitro. Vanadate

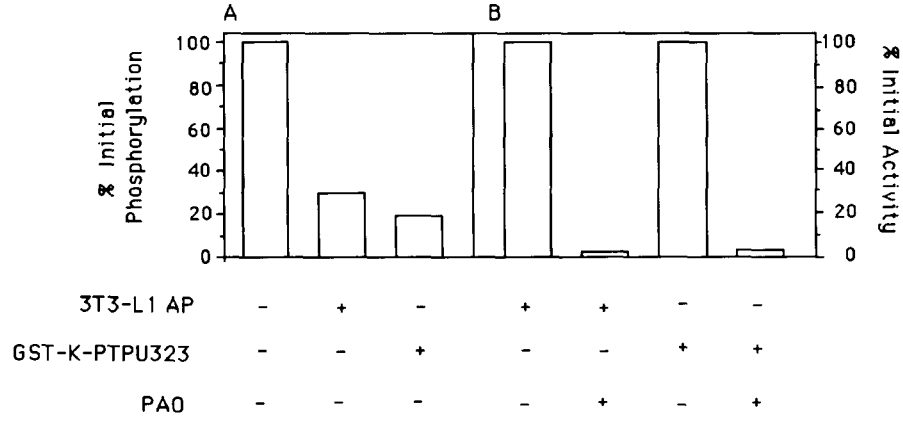


Fig. 2. Dephosphorylation of phosphoALBP and PAO inactivation of the acid phosphatase. **A**: ALBP was phosphorylated by the soluble insulin receptor kinase, and ATP was removed by gel filtration. The phosphorylated proteins were then incubated in the presence or absence of 1 μg of the 3T3-L1 acid phosphatase (specific activity = 5 units/mg) for 60 min or 1 μg of GST-K-PTPU323 (specific activity = 29 units/mg) for 40 min. The reactions were quenched by addition of Laemmli sample buffer, and the proteins were separated by SDS-PAGE. PhosphoALBP was visualized by autoradiography and the phosphorylation level was measured by densitometry. **B**: One microgram of the 3T3-L1 acid phosphatase or 1 μg of the GST-K-PTPU323 was incubated with 100 μM PAO at pH 8.8 in 10 mM Tris for 30 min at room temperature. The reaction was then adjusted to pH 5.0 as described in Materials and methods and the phosphatase activity was then measured using pNPP as a substrate.

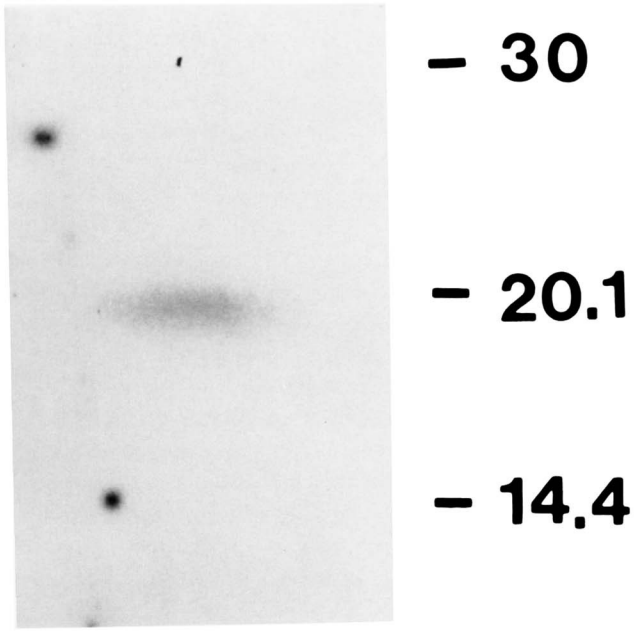


Fig. 3. Immunological identification of the 3T3-L1 phosphatase. The partially purified 3T3-L1 acid phosphatase was subjected to SDS-PAGE and the proteins were transferred to nitrocellulose. The filter was then probed with anti-BHAP sera followed by [^{125}I]Protein A as described and exposed to Kodak X-ray film at −70 °C.

markedly inhibited the phosphatase with a K_i value of 4 μM at pH 5.0 (Table 1). The human placental acid phosphatase has been shown to have a histidine residue at or near its active site (Waheed et al., 1988). To investigate whether the 3T3-L1 enzyme may be mechanistically (or structurally) similar, the histidine modification reagent diethyl pyrocarbonate was utilized. Incubation of DEP with partially purified 3T3-L1 phosphatase inhibited the enzyme activity in a concentration-dependent manner with a $K_{0.5}$ of 6 mM (results not shown). Because of the chemical instability of DEP, these reactions were not further characterized.

To further ascertain that the adipocyte acid phosphatase is related to the low molecular weight acid phosphatases from placenta, heart, and liver, the adipocyte acid

Table 1. *The kinetic parameters for partially purified 3T3-L1 acid phosphatase and homogeneous GST-HAAPβ were measured as described in Materials and methods*[a]

Protein	K_m (μM)	V_{max} (μmol/min/mg)	K_i (μM)
353-L1 AP	164	2	4
GST-HAAPβ	182	10	17

[a] For both enzymes, phosphatase activity and inhibition by vanadate were measured using pNPP as substrate in a buffer containing 50 mM sodium acetate, pH 5.0, and 5 mM EDTA at 37 °C.

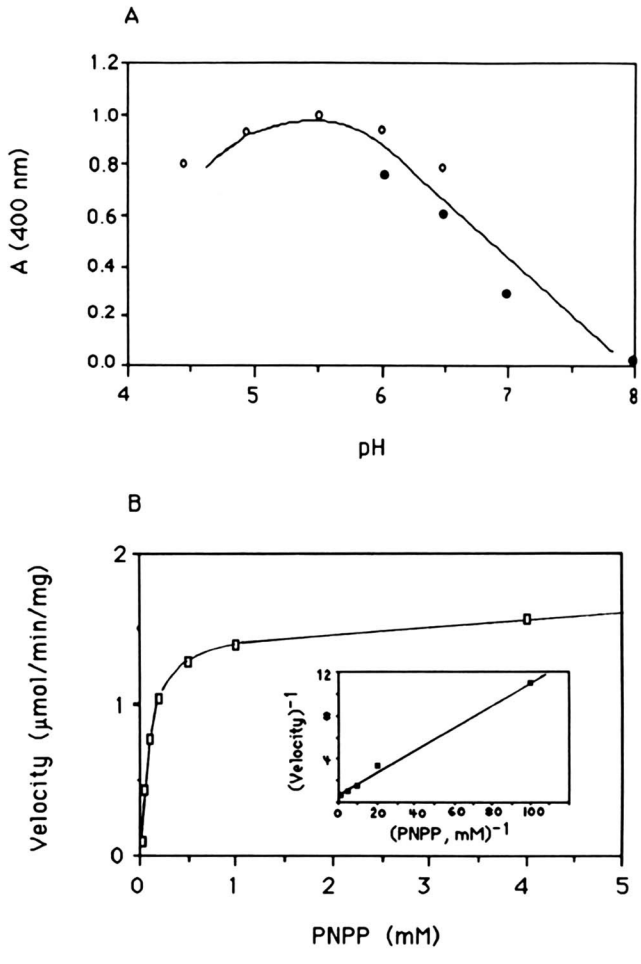


Fig. 4. Characterization of the 3T3-L1 acid phosphatase activity. **A**: The pH profile. The phosphatase activity of the 3T3-L1 enzyme was measured by the absorbance at 400 nm using pNPP as substrate at the indicated pH values in either (○) 50 mM sodium acetate or (●) 50 mM Tris-acetate. **B**: K_m and V_{max} determination. The phosphatase activity of the 3T3-L1 enzyme was measured at the indicated concentrations of pNPP as described in Materials and methods. **Inset**: Lineweaver-Burk analysis.

phosphatase from fat cells was cloned and sequenced. From an initial screening of a human adipocyte cDNA library using an EcoR1-Sal1 fragment of the BHAP cDNA as the hybridization probe, a 968-bp cDNA insert, termed HAAPα, was obtained. Sequence analysis showed that HAAPα lacked the codons corresponding to amino acids 1-11 of the bovine liver acid phosphatase. Using the HAAPα cDNA as the hybridization probe, a longer clone was subsequently isolated and found to contain a 471-nucleotide open reading frame and a 991-nucleotide 3′-untranslated region, which includes a polyadenylylation recognition sequence (Proudfoot et al., 1976) at nucleotide 1,404 (Fig. 6). The two clones differ in the 3′-untranslated region by only 2 nucleotides and a 4-nucleotide insertion in HAAPα. Interestingly, transla-

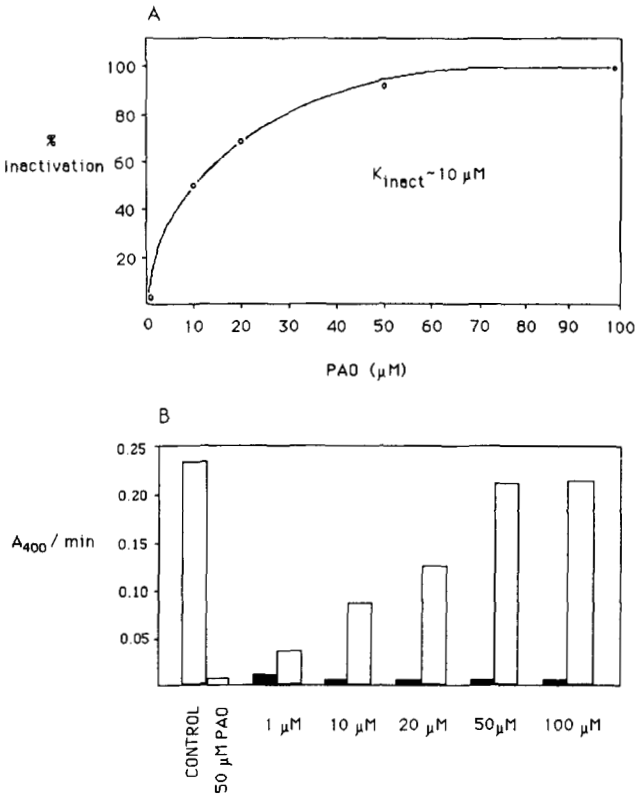


Fig. 5. PAO inactivation and DMP reactivation of the 3T3-L1 acid phosphatase. **A**: PAO inactivation. The 3T3-L1 acid phosphatase was modified with the given concentrations of PAO at pH 8.8 for 30 min at room temperature. Following modification, the phosphatase activity was measured at pH 5.0, using pNPP as described in Materials and methods. **B**: Reactivation of the 3T3-L1 phosphatase. After modification with 50 μM PAO, the 3T3-L1 acid phosphatase was incubated with the indicated concentrations of either 2-mercaptoethanol (filled bars) or 2,3-DMP (open bars) for 60 min. The phosphatase activity on pNPP was then assayed at pH 5.0.

tion of the open reading frame of the second clone, termed HAAPβ, revealed that it encoded an isoform of HAAPα (Fig. 7). Overall the protein coding region of HAAPα shares 81% sequence similarity to HAAPβ. A region of 34 amino acids constitutes the isoform-specific domain in which HAAPα and HAAPβ share only 43% sequence similarity.

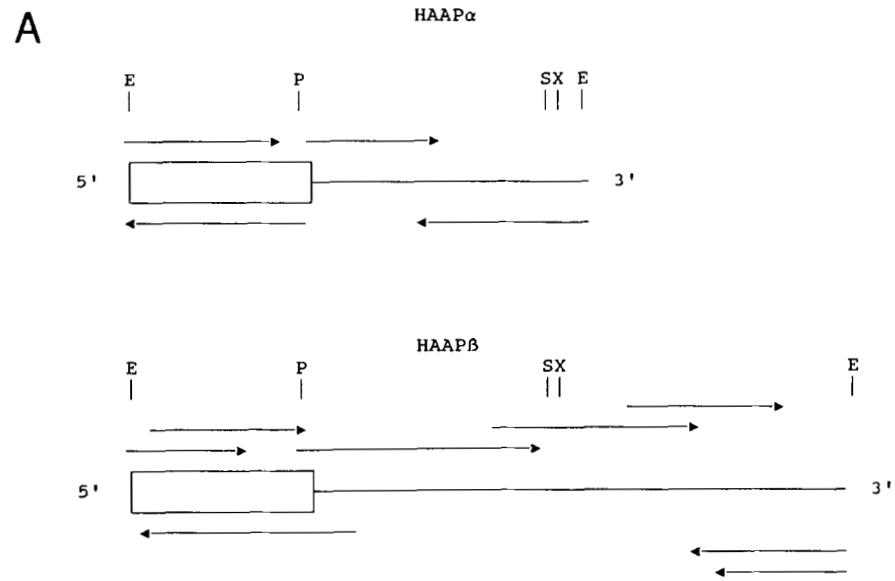
To examine the adipocyte acid phosphatase gene structure, Southern analysis of human DNA with the cDNA of HAAPα (Fig. 8) was performed. When HAAPα was used as the hybridization probe, multiple bands were obtained in every restriction digest. Identical results were obtained using HAAPβ as the hybridization probe. Multiple bands in Southern analysis typically suggest multiple genes. However, given the virtually identical 3′-untranslated region of HAAPα and HAAPβ, it is reasonable to suggest that the isoenzymes arise from differential splicing of a single gene and that there may be pseudogenes present in the human genome.

To definitively demonstrate that the human adipocyte acid phosphatases were capable of phosphoALBP dephosphorylation and that our protein preparations were not contaminated with another tyrosine phosphatase, the phosphatases were expressed in *E. coli* as fusion proteins with GST. The GST-HAAPα fusion protein was found to be insoluble when expressed in *E. coli* and refractory to analysis, consequently no further characterization was performed. In contrast, the HAAPβ fusion was expressed as a soluble protein and readily purified from *E. coli* by glutathione agarose affinity chromatography. The purified phosphatase exhibited a single 44-kDa Coomassie staining band after SDS-PAGE (Fig. 9) corresponding to 26 kDa of GST and 18 kDa of HAAPβ. The fusion protein had a specific activity of 20 units/mg when assayed with pNPP. The K_m for pNPP was 182 μM, which is similar to the value measured for the 3T3-L1 acid phosphatase (Table 1). Vanadate inhibited the GST-HAAPβ fusion with a K_i of 17 μM, a value similar to that for vanadate inhibition of the bovine heart acid phosphatase (K_i = 29 μM) (Zhang & Van Etten, 1990). When assessed for its PAO sensitivity, the phosphatase activity of GST-HAAPβ was completely abolished by 100 μM PAO. Finally, HAAPβ catalyzed the dephosphorylation of phosphoALBP and the phosphorylated insulin receptor as shown in Figure 10.

Discussion

The present investigation was undertaken to address the hypothesis that there exists in adipocytes a member of the acid phosphatase family, that it should possess tyrosine phosphatase activity, and by virtue of conformationally vicinal essential cysteine(s) that it should be inactivated by the arsenical PAO. Based on pH dependence, Michaelis constant, inhibition by orthovanadate and diethyl pyrocarbonate, and immunological cross-reactivity with anti-BHAP antiserum, we have shown that our partially pure protein preparation is indeed the adipocyte acid phosphatase. Definitive evidence that adipocytes express a low molecular weight acid phosphatase was provided by the cloning, sequencing, and expression of the adipocyte isoenzymes.

Comparison of the HAAP protein sequences with the amino acid sequence of the bovine liver acid phosphatase indicates that BLAP shares 90% similarity to HAAPβ and 74% similarity to HAAPα with no sequence similarity to the catalytic domain of the PTPases. The BLAP differs from the isoform-specific region of HAAPβ by only two amino acids. Additional differences in the amino-terminal sequences of the two adipocyte enzymes are also present. Due to the abundance of proline residues in the amino-terminus of HAAPα and the lack of similarity between the adipocyte enzymes and BLAP in this region, it is possible that the differences in the sequences



B

```
HAAPα:                                                  G AAT TCC CCC GCG CGC AGA GAG GCC GCG AGA CCC      66
HAAPß:  GAA TTC CCG CGC AGA GGC CGC AAG TCC GTG CTG TTT GTG TGT CTG GGT AAC ATT TGT CGA TCA CCC

HAAPα:  ATT GCA GAA GCA GTT TTC AGG AAA CTT GTA ACC GAT CAA AAC ATC TCA GAG AAT TGG AGG GTA GAC     132
HAAPß:  ATT GCA GAA GCA GTT TTC AGG AAA CTT GTA ACC GAT CAA AAC ATC TCA GAG AAT TGG GTC ATT GAT

HAAPα:  AGC GCG GCA ACT TCC GGG TAT GAG ATA GGG AAC CCC CCT GAC TAC CGA GGG CAG AGC TGC ATG AAG     198
HAAPß:  AGC GGT GCT GTT TCT GAC TGG AAC GTG GGC CGG TCC CCA GAC CCA AGA GCT GTG AGC TGC CTA AGA

HAAPα:  AGG CAC GGC ATT CCC ATG AGC CAC GTT GCC CGG CAG ATT ACC AAA GAA GAT TTT GCC ACA TTT GAT     264
HAAPß:  AAT CAT GGC ATT CAC ACA GCC CAT AAA GCA AGA CAG ATT ACC AAA GAA GAT TTT GCC ACA TTT GAT

HAAPα:  TAT ATA CTA TGT ATG GAT GAA AGC AAT CTG AGA GAT TTG AAT AGA AAA AGT AAT CGA GTT AAA ACC     330
HAAPß:  TAT ATA CTA TGT ATG GAT GAA AGC AAT CTG AGA GAT TTG AAT AGA AAA AGT AAT CGA GTT AAA ACC

HAAPα:  TGC AAA GCT AAA ATT GAA CTA CTT GGG AGC TAT GAT CCA CAA AAA CAA CTT ATT ATT GAA GAT CCC     396
HAAPß:  TGC AAA GCT AAA ATT GAA CTA CTT GGG AGC TAT GAT CCA CAA AAA CAA CTT ATT ATT GAA GAT CCC

HAAPα:  TAT TAT GGG AAT GAC TCT GAC TTT GAG ACG GTG TAC CAG CAG TGT GTC AGG TGC TGC AGA GCG TTC     462
HAAPß:  TAT TAT GGG AAT GAC TCT GAC TTT GAG ACG GTG TAC CAG CAG TGT GTC AGG TGC TGC AGA GCG TTC

HAAPα:  TTG GAG AAG GCC CAC tgaggcaggttcgtgccctgctgcggccagcctgactagaccccaccctgaggtcctgcatttctcagtcggtgtgtaatc     553
HAAPß:  TTG GAG AAG GCC CAC tgaggcaggttcgtgccctgctgcggccagcctgactagaccccaccctgaggtcctgcatttctcagtcggtgtgtaatc

HAAPα:  acgttccagggcccaaagccagctctttgttcagttgacttactgtttcttaccttaaaaagtaattgtagatggaaatcagttgtgtttggcaggagaatcaata     659
HAAPß:  acgttccagggcccaaagccagctctttgttcagttgacttactgtttcttaccttaaaaagtaattgtagatggaaatcagttgtgtttggcaggagaatcaata

HAAPα:  aaaatctttgattcagacagcttatggggtattttaagcattcttagactagttgaacatctcactttgccccagttacaaaaatagtagaacaagcaacataaaa     765
HAAPß:  aaaatctttgattcagacagcttatggggtattttaagcattcttagactagttgaacatctcacttagctccagttacaaaaatagtagaacaagcaacataaaa

HAAPα:  caatgaaggaaaacctcacttgaaggcccaggtcaacatctaagcctgttgagacttagataatcgagtctacctcttcagtaggtttgtgtggatggcctggagg     871
HAAPß:  caatgaaggaaaacctcacttgaaggcccaggtcaacatctaagcctgttgagacttagataatcgagtctacctcttcagtaggtttgtgtggatggcctggagg

HAAPα:  caggtgccctctgctccccagtgctacctctctcttccctagggccttttgtggattgacagtagtcccctccgtagagctcacagtctagattagaagtgtttta     977
HAAPß:  caggtgc----tgctccccagtgctacctctctcttccctagggccttttgtggattgacagtagtcccctccgtagagctcacagtctagattagaagtgtttta

HAAPα:  atttctacacaccggaattc  1007
HAAPß:  atttctacacacccatagtgcacacttgtatattgaaaagatagggaagagagaaacatttatggaatcagtcgttggcaccttcaatacttcatgatttttgtcg     1083

HAAPß:  agtttacttcatgaggaggtcagcccattggctcccatctgaaccactttgcctctgaaacttaattacatccagaaagaaggacacttgtatgctagtctatggt     1189
        cagttgaggaatatgactgtttttatatgcacatgtaacccaaatgtccaatataaatttggcttatttttttaaaataatttttaaaagttgggaaaagtgttattat     1295
        ttggcatgcttaaatattgaataagtattcttcatcagcatttaataaatgtataggcagatgtaaggtaatttctgtgtattttgagataatgtcaaaatcatga     1401
        atatttcaaaataaactggggagttataaaaatacaactagagatataaaaaaaaaaaaaaaaaaaggaattc  1481
```

Fig. 6. Nucleotide sequences of HAAPα and HAAPβ. **A**: Sequencing strategy and restriction sites. The coding regions of HAAPα and HAAPβ are depicted by the open boxes followed by the 3′-untranslated region. The sequencing strategy is illustrated by the arrows. Selected restriction sites are indicated; E, EcoR1; P, Pst1; S, Sac1; and X, Xbal. **B**: Nucleotide sequences of HAAPα and HAAPβ were determined by the dideoxy sequencing method. The EcoR1 cloning sites are underlined and the polyadenylation recognition site is marked by a double underline.

```
HAAPα:                                                  Pro Ala Arg Arg Glu Ala Ala Arg Pro Ile Ala  22
 BLAP:  Ala Glu Gln Val Thr Lys Ser Val Leu Phe Val Cys Leu Gly Asn Ile Cys Arg Ser Pro Ile Ala
HAAPß:  Pro Arg Arg Gly Arg Lys Ser Val Leu Phe Val Cys Leu Gly Asn Ile Cys Arg Ser Pro Ile Ala

HAAPα:  Glu Ala Val Phe Arg Lys Leu Val Thr Asp Gln Asn Ile Ser Glu Asn Trp Arg Val Asp Ser Ala  44
 BLAP:  Glu Ala Val Phe Arg Lys Leu Val Thr Asp Gln Asn Ile Ser Asp Asn Trp Val Ile Asp Ser Gly
HAAPß:  Glu Ala Val Phe Arg Lys Leu Val Thr Asp Gln Asn Ile Ser Glu Asn Trp Val Ile Asp Ser Gly

HAAPα:  Ala Thr Ser Gly Tyr Glu Ile Gly Asn Pro Pro Asp Tyr Arg Gly Gln Ser Cys Met Lys Arg His  66
 BLAP:  Ala Val Ser Asp Trp Asn Val Gly Arg Ser Pro Asn Pro Arg Ala Val Ser Cys Leu Arg Asn His
HAAPß:  Ala Val Ser Asp Trp Asn Val Gly Arg Ser Pro Asp Pro Arg Ala Val Ser Cys Leu Arg Asn His

HAAPα:  Gly Ile Pro Met Ser His Val Ala Arg Gln Ile Thr Lys Glu Asp Phe Ala Thr Phe Asp Tyr Ile  88
 BLAP:  Gly Ile Asn Thr Ala His Lys Ala Arg Gln Val Thr Lys Glu Asp Phe Val Thr Phe Asp Tyr Ile
HAAPß:  Gly Ile His Thr Ala His Lys Ala Arg Gln Ile Thr Lys Glu Asp Phe Ala Thr Phe Asp Tyr Ile

HAAPα:  leu Cys Met Asp Glu Ser Asn Leu Arg Asp Leu Asn Arg Lys Ser Asn Arg Val Lys Thr Cys Lys 110
 BLAP:  Leu Cys Met Asp Glu Ser Asn Leu Arg Asp Leu Asn Arg Lys Ser Asn Gln Val Lys Asn Cys Arg
HAAPß:  Leu Cys Met Asp Glu Ser Asn Leu Arg Asp Leu Asn Arg Lys Ser Asn Arg Val Lys Thr Cys Lys

HAAPα:  Ala Lys Ile Glu Leu Leu Gly Ser Tyr Asp Pro Gln Lys Gln Leu Ile Ile Glu Asp Pro Tyr Tyr 132
 BLAP:  Ala Lys Ile Glu Leu Leu Gly Ser Tyr Asp Pro Gln Lys Gln Leu Ile Ile Glu Asp Pro Tyr Tyr
HAAPß:  Ala Lys Ile Glu Leu Leu Gly Ser Tyr Asp Pro Gln Lys Gln Leu Ile Ile Glu Asp Pro Tyr Tyr

HAAPα:  Gly Asn Asp Ser Asp Phe Glu Thr Val Tyr Gln Gln Cys Val Arg Cys Cys Arg Ala Phe Leu Glu 154
 BLAP:  Gly Asn Asp Ala Asp Phe Glu Thr Val Tyr Gln Gln Cys Val Arg Cys Cys Arg Ala Phe Leu Glu
HAAPß:  Gly Asn Asp Ser Asp Phe Glu Thr Val Tyr Gln Gln Cys Val Arg Cys Cys Arg Ala Phe Leu Glu

HAAPα:  Lys Ala His 157
 BLAP:  Lys Val Arg
HAAPß:  Lys Ala His
```

Fig. 7. Amino acid sequences of the low molecular weight acid phosphatases. The nucleotide sequences of HAAPα and HAAPβ were translated into the amino acid sequence and are shown in comparison to the amino acid sequence of the bovine liver acid phosphatase. The region from amino acids 40 to 73 is the isoform-specific region.

at the amino-termini represent errors from the reverse transcriptase used in cloning rather than true sequence differences. Despite repeated attempts, longer cDNA clones containing additional 5′ sequences have not been obtained. Molecular cloning of the acid phosphatase gene(s) is in progress to clarify this issue.

Recently, the protein sequences of two isozymes of the human red cell acid phosphatase have been determined (Dissing et al., 1991). The human acid phosphatase locus is highly polymorphic with three common alleles in the population each encoding two isozymes, designated f and s for fast and slow electrophoretic migration. Interestingly, the sequences of the red cell isozymes, ACP1 Bf and ACP1 Bs, show that they differ from each other in a region of 34 amino acids analogous to that found in the adipocyte acid phosphatases. Comparing the human adipocyte sequences to the red cell acid phosphatase sequences indicates that HAAPα and the red cell Bf isozyme are identical in the isoform-specific region as are HAAPβ and the red cell Bs enzymes. Overall, the adipocyte acid phosphatases share 99% sequence similarity to the respective red cell isoenzymes. Outside the isoform-specific region the adipocyte enzymes differ from the red cell enzymes by only two amino acids at positions 4 and 105. Whereas residue 4 resides in a region of questionable sequence integrity as discussed above, the difference at position 105 of the red cell and adipocyte enzymes may reflect a polymorphism. The observation that a single allele gives rise to two isoforms has led Dissing et al. (1991) to suggest that the two isoenzymes are the products of differential splicing of a single primary transcript.

Further examination of the primary sequences of HAAPα and HAAPβ along with that of liver and red cell acid phosphatases reveals the presence of eight conserved cysteinyl residues. Studies utilizing sulfhydryl-directed reagents have indicated that the acid phosphatases possess at least one essential cysteine in the active site (Laidler et al., 1982; Zhang & Van Etten, 1990). Additionally, the observation that PAO inactivates the enzyme(s) indicates that at least two sulfhydryl groups are conformationally vicinal. Two cysteine residues, C145 and C148, are positioned in a Cys-X-X-Cys motif near the carboxyl-terminus with the potential to be conformationally vicinal by analogy to that motif in a variety of protein disulfide isomerases and prokaryotic thioredoxins (Pigiet & Conley, 1978). Alternatively, chemical modification with thiol-directed reagents (Camici et al., 1989) has pointed to cysteine residues near the amino-terminus as being essential for activity. Further structure–function analysis will be necessary to resolve the issue of essential cysteine residues.

Recently, Liao et al. (1991) have reported the identification of two PAO-sensitive membrane-associated tyrosyl phosphatases capable of dephosphorylating ALBP. The properties of these enzymes suggest that they are members of the PTPase family of protein tyrosyl phosphatases. Consistent with this we have noted that the activity of the GST-K-PTPU323 fusion protein is also

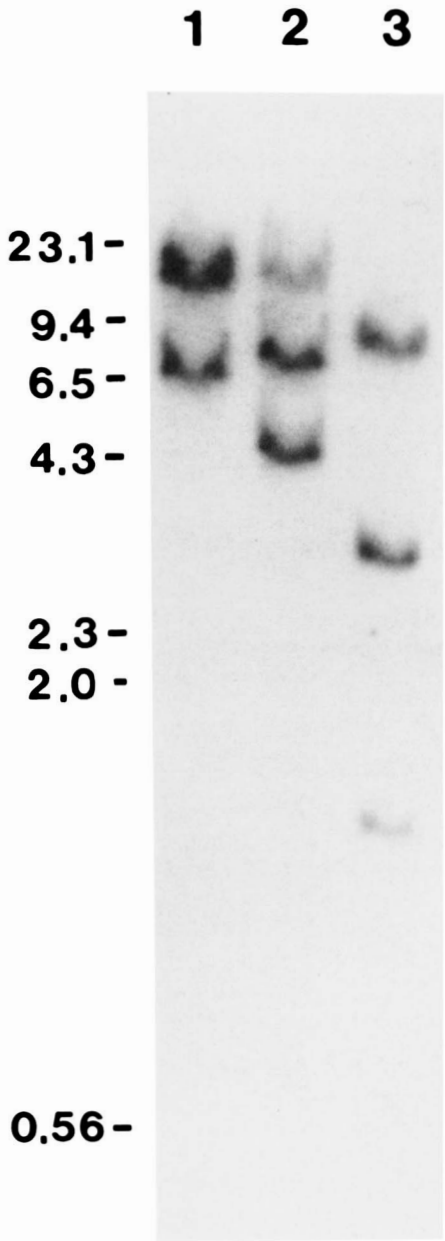


Fig. 8. Southern analysis. Ten micrograms of human DNA was digested with the indicated restriction enzyme and separated on a 1.2% agarose gel. The DNA fragments were transferred to nitrocellulose and probed with the EcoR1 fragment of HAAPα as described in Materials and methods. The restriction enzymes used are lane 1, EcoR1; lane 2, HinDIII; and lane 3, Pst1. The numbers indicate standards in kilobases.

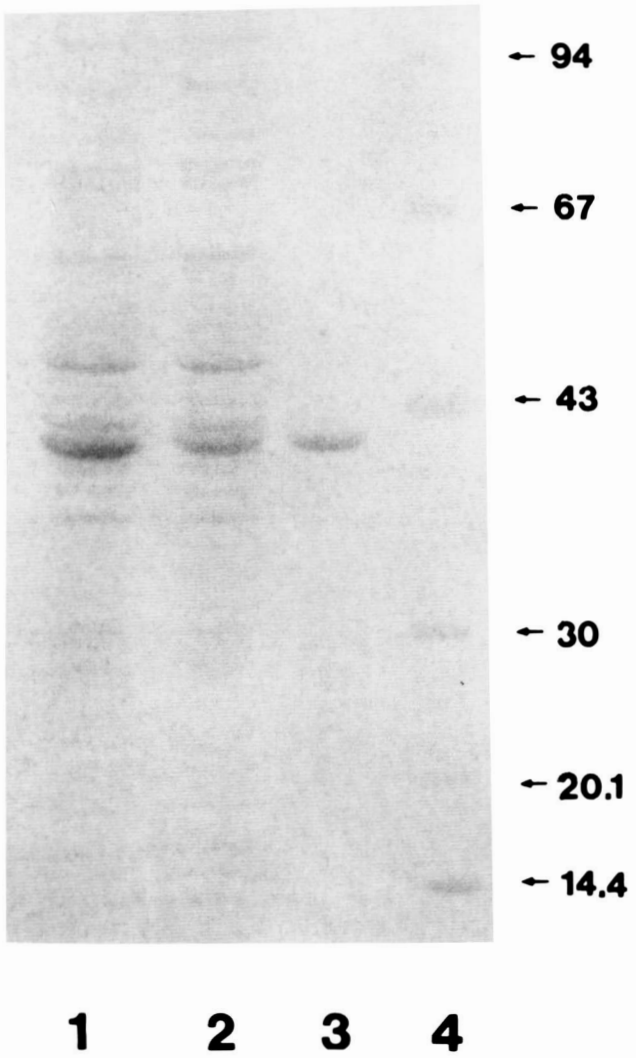


Fig. 9. Purification of recombinant HAAPβ. HAAPβ was expressed as a GST fusion protein as described under Experimental procedures. The soluble *E. coli* extract was incubated with glutathione agarose and the bound protein was eluted with 5 mM glutathione. The purity of the eluted fusion protein was assessed by SDS-PAGE. Lane 1, soluble *E. coli* extract; lane 2, flow through from glutathione agarose; lane 3, eluted GST-HAAPβ fusion protein; lane 4, molecular weight markers. Numbers indicate molecular mass in kilodaltons.

sensitive to PAO (L.S., D.A.B., unpubl.) suggesting that the criteria of PAO sensitivity in identification of a specific phosphoALBP phosphatase may not be as exacting as originally considered. Taken together it appears that several distinct proteins are capable of catalyzing the dephosphorylation of ALBP in vitro. The cycle of ALBP phosphorylation/dephosphorylation has been proposed to be involved in fatty acid trafficking (Waggoner & Bernlohr, 1990), antilipolysis (Hresko et al., 1990), and insulin signaling (Bernier et al., 1988), but in situ evidence to definitively support any particular model has not been forthcoming. It should be stressed that although the PTPases identified by Liao et al. (1991) and the acid phosphatase identified and reported here dephosphorylate phosphoALBP, there exists no direct evidence to demonstrate that any of these enzymes function analogously in situ. Clearly, the availability of the cDNAs for

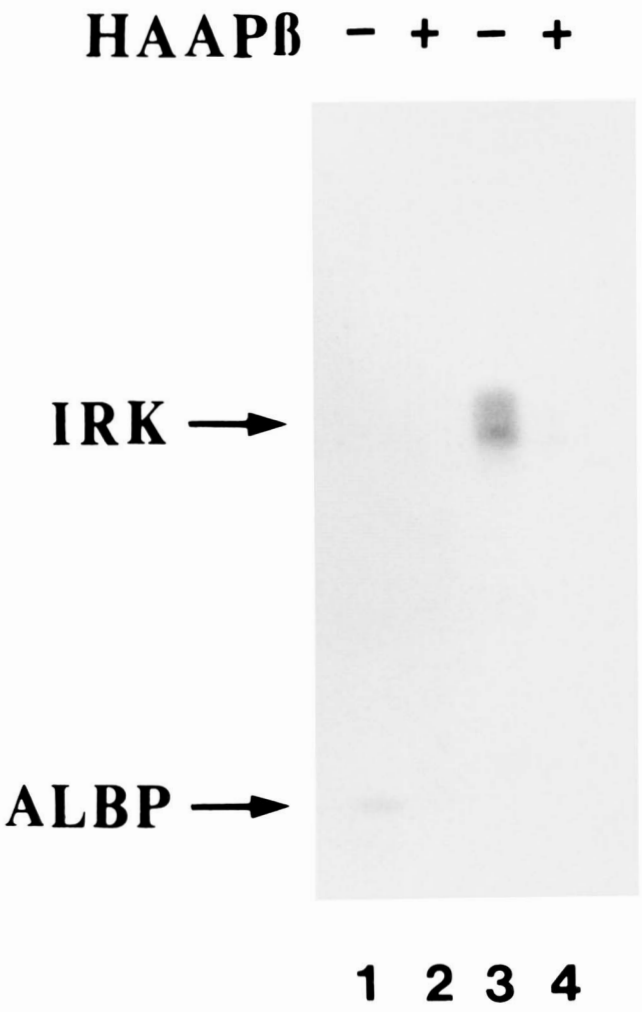


Fig. 10. Dephosphorylation of phosphoALBP and IRK by GST-HAAPβ. PhosphoALBP or phosphorylated insulin receptor kinase was incubated with 7 μg of fusion protein GST-HAAPβ for 2 h at 37 °C. The reactions were quenched by addition of Laemmli sample buffer and the proteins were separated by SDS-PAGE followed by autoradiography at −70 °C. Lane 1, phosphoALBP alone; lane 2, phosphoALBP in the presence of GST-HAAPβ.

the adipocyte acid phosphatases as well as the ability to easily purify active enzyme from expressing *E. coli* will aid us in the delineation of the metabolic role(s) of these enzymes.

Materials and methods

Materials

[γ^{32}P]ATP (3,000 Ci/mmol) was obtained from Amersham. [^{125}I]insulin (111 μCi/μg) was from DuPont-NEN. WGA-Sepharose and *N*-acetylglucosamine were purchased from Sigma. Insulin was purchased from GIBCO. The human abdominal fat cDNA library was prepared as previously described (Baxa et al., 1989). All other chemicals were reagent grade.

Isolation of ALBP and the 3T3-L1 insulin receptor

ALBP was isolated from either cultured 3T3-L1 adipocytes (Matarese & Bernlohr, 1988) or expressing *E. coli* cultures (Xu et al., 1991). Quantitation of ALBP was based upon an extinction coefficient of 1.55×10^4 M^{-1} cm^{-1}. Insulin receptor was partially purified from 3T3-L1 adipocytes as previously described (Chinander & Bernlohr, 1989). The yield of active insulin receptor was quantitated by measuring the specific binding of [^{125}I]insulin using the polyethylene glycol precipitation method as originally described by Cuatrecasas (1972) and modified by Kohanski and Lane (1983). The insulin receptor preparation was stored at 4 °C and used within 2 weeks. The soluble 48-kDa cytoplasmic domain of the human insulin receptor was obtained from Dr. Leland Ellis, Dallas, Texas.

In vitro phosphorylation assays and isolation of phosphoALBP

ALBP was phosphorylated with either partially purified 3T3-L1 insulin receptor or the cloned 48-kDa soluble kinase domain of the human insulin receptor (Cobb et al., 1989). When using the partially purified 3T3-L1 insulin receptor, phosphorylation of the insulin receptor β subunit and ALBP was conducted at room temperature with 2 μg of the WGA-Sepharose insulin receptor preparation in a buffer containing 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 4 mM $MnCl_2$, and 100 nM insulin. Following a 30-min preincubation of the insulin receptor with insulin, 3 mM $MgCl_2$, and 0.2 mM $MnCl_2$, autophosphorylation was initiated by the addition of 100 μM ATP. Insulin-stimulated autophosphorylation was rapid, reaching a maximum level within 30 min. ALBP was added 30 min after the initiation of autophosphorylation, and the reactions were allowed to proceed for the times indicated. In phosphorylation reactions utilizing the soluble insulin receptor kinase, the kinase (2 μg) was incubated with ALBP in a buffer containing 25 mM HEPES, pH 7.5, 5 mM $MnCl_2$, and 100 μM ATP. Homogeneous phosphoALBP was then isolated by a combination of anion exchange chromatography and affinity chromatography on anti-phosphotyrosine agarose. Details of the purification and characterization of phosphoALBP will be published elsewhere (Buelt et al., 1992).

Preparation of the 3T3-L1 acid phosphatase

The 3T3-L1 acid phosphatase was partially purified from a cytoplasmic extract of 3T3-L1 adipocytes. All steps of the purification were assayed using pNPP according to

the method of Taga and Van Etten (1982). Cultured 3T3-L1 cells were differentiated into adipocytes as previously described (Matarese & Bernlohr, 1988) and used within 10 days of acquiring the adipocyte phenotype. To prepare a cytoplasmic extract, monolayers of adipocytes were washed three times with 10 mM $NaPO_4$ buffer, pH 7.0, containing 150 mM NaCl and 1 mM EDTA. The monolayers were then incubated for 15 min at room temperature with wash buffer containing 50 μg/mL digitonin (2 mL/10-cm plate). The digitonin releasate was collected and the pH was immediately adjusted to 5.0 with 2 M sodium acetate pH 5.0. Following acidification, the sample was incubated overnight at 4 °C to allow insoluble proteins to precipitate. The soluble protein fraction was recovered by centrifugation (100,000 × *g*, 30 min, 4 °C), and soluble lipid was removed by chromatography at 37 °C on Lipidex 1000 resin. The delipidated soluble extract was concentrated by ultrafiltration (Amicon YM5 membrane) and applied to a calibrated 5 × 100-cm Sephadex G-75 column equilibrated at pH 4.85 with 25 mM sodium acetate. The column fractions containing active phosphatase were pooled and applied to a Beckman Spherogel TSK CM-3SW high performance liquid chromatography (HPLC) column equilibrated in 25 mM sodium acetate, pH 4.85. Elution of active phosphatase was accomplished using a linear NaCl gradient from 0 to 0.4 M in 25 mM sodium acetate, pH 4.85. Acid phosphatase activity eluted in 300 mM NaCl, and, when analyzed by SDS-PAGE, an 18-kDa band (phosphatase) was observed together with several lower molecular weight proteins. Phosphatase was stored in column elution buffer at 4 °C. The specific activity of partially purified 3T3-L1 adipocyte acid phosphatase was 1–5 units/mg. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μmol of pNPP per minute at 37 °C.

Phosphatase assays

The phosphatase activity was measured at 37 °C using either pNPP or phosphorylated ALBP as substrate. In assays using pNPP as substrate, the indicated amount of acid phosphatase was incubated with pNPP in 50 mM sodium acetate, pH 5.0, at 37 °C. The reactions were quenched at the given times by addition of 1 mL of 1 N NaOH and the amount of product *p*-nitrophenol released was measured from the absorbance at 405 nm using a molar extinction coefficient of 18,000 M^{-1} cm^{-1} (Zhang & Van Etten, 1990).

The phosphoALBP phosphatase activity was determined in a similar manner. Isolated phosphorylated ALBP was incubated at either pH 5.0 in sodium acetate, 0.15 M NaCl, or at pH 7.0 in 50 mM Tris-acetate, 0.15 M NaCl, with the indicated amounts of 3T3-L1 phosphatase at 37 °C. The concentration of phosphorylated protein in most reactions was 2 μM, which was determined to be 10-fold greater than the apparent K_m. The dephosphorylation was monitored by removing an aliquot of the reaction and immediately adding SDS to a final concentration of 1.0% followed by heating to 95 °C for 3 min. The extent of dephosphorylation was determined by SDS-PAGE and autoradiography. The dephosphorylation was quantitated by densitometric analysis of the corresponding autoradiogram using a Hoefer GS300 scanning densitometer and by direct scintillation counting of the radioactive protein band excised from the dried polyacrylamide gel.

Immunochemical methods

Total antiserum raised in rabbits against the bovine heart acid phosphatase was dialyzed into 50 mM sodium phosphate, pH 7.0, and applied to a 1.0-mL column of Protein A Sepharose (Pharmacia) equilibrated in the same buffer. The unbound material was discarded, and the bound immunoglobulin was eluted with 0.1 M glycine HCl, pH 3.0. The eluted antibody was neutralized with 1 M Tris-HCl, pH 8.0, dialyzed into 50 mM sodium phosphate, pH 7.0, and stored at a concentration of 1 mg/mL at −20 °C until use. Immunoblotting was performed as described by Baxa et al. (1989) using anti-BHAP sera at a dilution of 1:1,000 and detection with ^{125}I-labeled Protein A (Amersham).

Inhibition of phosphatase activity by PAO and reactivation by 2,3-DMP

The acid phosphatase was incubated at pH 8.0 in 1 mM Tris-HCl with the indicated concentrations of PAO for 30 min. Following modification, either 1 M Tris-acetate, pH 7.0, or 1 M acetic acid, pH 5.0, was added to a final concentration of 50 mM. Activity was assayed as described previously using either phosphoALBP or pNPP as substrate. A time course of modification indicated that the reaction was complete after 20 min under all conditions utilized.

To demonstrate reactivity with 2,3-DMP, the 3T3-L1 phosphatase was first modified with PAO as described at pH 8.0. Following modification, the reaction mixture was applied to a 1.0 × 20-cm Sephadex G-50 column equilibrated in 10 mM Tris-HCl, pH 8.0. The phosphatase-containing fractions were identified by the absorbance at 280 nm, pooled, and concentrated using Centricon-10 centrifugation (Amicon). The modified protein was incubated with the indicated concentrations of either 2-mercaptoethanol or 2,3-DMP for 60 min. The pH of the reaction was adjusted to 5.0 with 1 M acetic acid buffer and the activity was assayed using pNPP as the substrate.

Kinetic measurements

Kinetic parameters were measured at either pH 5.0 or 7.0 in 50 mM Tris-acetate buffer using pNPP as substrate. Duplicate points at 8–12 substrate concentrations were utilized. Apparent K_m values were corrected for ionization of substrate by the method of Lawrence and Van Etten (1981). Inhibition by orthovanadate was measured at three concentrations, and K_i values were calculated as described by Taga and Van Etten (1982).

Isolation of HAAP cDNA clone

To identify the human adipocyte acid phosphatase, the 580-bp EcoR1–Sa11 fragment containing the bovine heart acid phosphatase coding region (Wo et al., 1992) was radiolabeled and used to screen a human adipocyte cDNA library. The hybridization was performed at 42 °C in 50% formamide, 0.05× BLOTTO, and 6× SSC (Maniatis et al., 1989). Following hybridization, the nitrocellulose filters were washed at 60 °C in 0.5× SSC and 0.1% SDS and exposed to film at −70 °C. From approximately 20,000 plaques screened, a single hybridizing plaque was obtained, and the clone, HAAPα, was sequenced by the dideoxy method (Sanger et al., 1977). The cDNA library was rescreened a second time using a 400-bp EcoR1–Pst1 fragment from HAAPα as the hybridization probe. The hybridization was carried out in 50% formamide, 0.2× BLOTTO, 6× SSC, and 10 μg/mL sonicated salmon sperm DNA at 42 °C. The filters were then washed in 0.1× SSC and 0.1% SDS at 65 °C and exposed to Kodak X-ray film at −70 °C. A second clone, HAAPβ, was isolated and also sequenced by the dideoxy method.

Southern analysis of human DNA

Human DNA (10 μg) was digested with the appropriate restriction endonuclease and subsequently separated on a 1.2% agarose gel. The DNA fragments were transferred to nitrocellulose and hybridized at 42 °C in 4× SSC, 50% formamide, and 0.1% SDS. The EcoR1 fragment from HAAPα or HAAPβ was labeled using the random-primer method of Feinberg and Vogelstein (1983) for use as the hybridization probe. The hybridized filter was washed two times at 65 °C with 0.1× SSC and 0.1% SDS and exposed to Kodak X-ray film at −70 °C.

Expression and purification of PTPase and HAAPβ

HAAPβ and the rat brain PTPase, PTPU323, were expressed in *E. coli* and purified as GST fusion proteins, GST-HAAPβ and GST-K-PTPU323, as described by Smith and Johnson (1988) and Guan and Dixon (1991). Briefly, HAAPβ was subcloned into the EcoR1 site of the expression vector pGEX-1, and the resulting plasmid was used to transform *E. coli* strain JM109. The recombinant plasmid pGEX-KG containing the rat brain PTPase cDNA, designated PTPU323, was used to transform competent *E. coli* strain JM109. Successful transformants were screened for expression of fusion proteins by SDS-PAGE.

To purify the GST fusion proteins, overnight cultures of *E. coli* harboring the recombinant plasmid were diluted 1:10 and allowed to grow for 1 h at 37 °C. Expression of the fusion protein was induced by addition of IPTG to a final concentration of 0.1 mM. The culture was allowed to grow 3–6 h at which time the cells were collected by centrifugation and resuspended in solubilization buffer containing 20 mM sodium phosphate, pH 7.3, 150 mM NaCl, and 1% Triton X-100. The cells were lysed by sonication, and the bacterial debris was removed by centrifugation. The supernatant was mixed with glutathione agarose, and the fusion protein was allowed to bind at 4 °C for 15 min. The resin was collected in a column and washed with solubilization buffer. The GST-HAAPβ fusion protein was eluted with 5 mM glutathione in 10 mM sodium acetate, pH 5, 5 mM EDTA, and 10% glycerol and the GST-K-PTPU323 fusion protein was eluted in the same buffer at pH 6. Protein concentration was determined by the method of Bradford (1976), and phosphatase activity was followed throughout the purification using pNPP as substrate as described before. The purity of the eluted fusion proteins was assessed by SDS-PAGE and Coomassie blue staining. The specific activity of the purified fusion GST-HAABβ was typically 10–20 units/mg and 20–30 units/mg for GST-K-PTPU323.

Acknowledgments

We thank Dr. Jack Dixon, University of Michigan, for kindly providing the pGEX-KG-PTPase plasmid and Dr. Leland Ellis, Dallas, for his gift of the soluble insulin receptor kinase. We appreciate the generosity of Dr. Brian Van Ness for providing the human DNA. We also thank Dr. Melissa Buelt for the preparation of phosphoALBP. This work was supported by NSF grant DMB8552942 (D.A.B.) and NIH grant GM27003 (R.L.V.E.).

References

Baxa, C.A., Sha, R.S., Buelt, M.K., Smith, A.J., Matarese, V., Chinander, L.L., Boundy, K.L., & Bernlohr, D.A. (1989). Human adipocyte lipid binding protein: Purification of the protein and cloning of its complementary DNA. *Biochemistry 28*, 8683–8690.

Bernier, M., Laird, D.M., & Lane, M.D. (1987). Insulin-activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA 84*, 1844–1848.

Bernier, M., Laird, D.M., & Lane, M.D. (1988). Effect of vanadate on the cellular accumulation of pp15, an apparent product of insulin receptor tyrosine kinase action. *J. Biol. Chem. 263*, 13626–13634.

Boivin, P., Galand, C., & Bertrand, O. (1987). Protein band 3 phosphotyrosyl phosphatase. Purification and characterization. *J. Biochem. 19*, 613–618.

Bradford, M. (1976). A rapid and sensitive method for the quantitation

of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.

Buelt, M.K., Shekels, L.L., Jarvis, B.W., & Bernlohr, D.A. (1991). In vitro phosphorylation of the adipocyte lipid binding protein (p15) by the insulin receptor: Effects of fatty acid on receptor kinase and substrate phosphorylation. *J. Biol. Chem. 266*, 12266–12271.

Buelt, M.K., Xu, Z., Banaszak, L., & Bernlohr, D.A. (1992). Structural and functional characterization of the phosphorylated adipocyte lipid-binding protein (pp15). *Biochemistry* (in press).

Camici, G., Manao, G., Cappugi, G., Modesti, A., Stefani, M., & Ramponi, G. (1989). The complete amino acid sequence of the low molecular weight cytosolic acid phosphatase. *J. Biol. Chem. 264*, 2560–2567.

Charbonneau, H., Tonks, N.K., Kumar, S., Diltz, C.D., Harrylock, M., Cool, D.E., Krebs, E.G., Fischer, E.H., & Walsh, K.A. (1989). Human placenta protein-tyrosine-phosphatase: Amino acid sequence and relationship to a family of receptor-like proteins. *Proc. Natl. Acad. Sci. USA 86*, 5252–5256.

Chernoff, J. & Li, H.-C. (1985). A major phosphotyrosyl-protein phosphatase from bovine heart is associated with a low molecular weight acid phosphatase. *Arch. Biochem. Biophys. 240*, 135–145.

Chinander, L.L. & Bernlohr, D.A. (1989). Cloning of murine adipocyte lipid binding protein in *Escherichia coli*: Its purification, ligand binding properties and phosphorylation by the adipocyte insulin receptor. *J. Biol. Chem. 264*, 19564–19572.

Cobb, M.H., Sang, B.-C., Gonzalez, R., Goldsmith, E., & Ellis, L. (1989). Autophosphorylation activates the soluble cytoplasmic domain of the insulin receptor in an intermolecular reaction. *J. Biol. Chem. 264*, 18701–18706.

Cuatrecasas, P. (1972). Isolation of the insulin receptor of liver and fat cell membranes. *Proc. Natl. Acad. Sci. USA 69*, 318–322.

Dissing, J., Johnsen, A.H., & Sensabaugh, G.F. (1991). Human red cell acid phosphatase (ACP1): The amino acid sequence of the two isozymes Bf and Bs encoded by the ACP1*B allele. *J. Biol. Chem. 266*, 20619–20625.

Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A., & Rutter, W.J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell 45*, 721–732.

Feinberg, A.P. & Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem. 132*, 6–13.

Frost, S.C. & Lane, M.D. (1985). Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. *J. Biol. Chem. 260*, 2646–2652.

Frost, S.C. & Schwalbe, M.S. (1990). Uptake and binding of radiolabelled phenylarsine oxide in 3T3-L1 adipocytes. *Biochem. J. 269*, 589–595.

Guan, K. & Dixon, J.E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. *Anal. Biochem. 192*, 262–267.

Guan, K., Haun, R.S., Watson, S.J., Geahlen, R.L., & Dixon, J.E. (1990). Cloning and expression of a protein-tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA 87*, 1501–1505.

Hresko, R.C., Bernier, M., Hoffman, R.D., Flores-Riveros, J.R., Liao, K., Laird, D.M., & Lane, M.D. (1988). Identification of phosphorylated 422 (aP2) protein as pp15, the 15-kilodalton target of the insulin receptor tyrosine kinase in 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA 85*, 8835–8839.

Hresko, R.C., Hoffman, R.D., Flores-Riveros, J.R., & Lane, M.D. (1990). Insulin receptor tyrosine kinase-catalyzed phosphorylation of 422 (aP2) protein: Substrate activation by long chain fatty acid. *J. Biol. Chem. 265*, 21075–21085.

Hunter, T. (1989). Protein-tyrosine phosphatases: The other side of the coin. *Cell 58*, 1013–1016.

Kohanski, R.A. & Lane, M.D. (1983). Homogeneous functional insulin receptor from 3T3-L1 adipocytes: Purification using $N^{\alpha B1}$-(biotinyl-ϵ-aminocaproyl) insulin and avidin-Sepharose. *J. Biol. Chem. 258*, 7460–7468.

Laidler, P.M., Taga, E.M., & Van Etten, R.L. (1982). Human liver acid phosphatases: Cysteine residues of the low molecular weight enzyme. *Arch. Biochem. Biophys. 216*, 512–521.

Lawrence, G.L. & Van Etten, R.L. (1981). The low molecular weight acid phosphatase from bovine liver: Isolation, amino acid composition and chemical modification studies. *Arch. Biochem. Biophys. 206*, 122–131.

Liao, K., Hoffman, R.D., & Lane, M.D. (1991). Phosphotyrosyl turnover in insulin signaling: Characterization of two membrane-bound pp15 protein tyrosine phosphatases from 3T3-L1 adipocytes. *J. Biol. Chem. 266*, 6544–6553.

Maniatis, T., Fritsch, G.F., & Sambrook, J. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Matarese, V. & Bernlohr, D.A. (1988). Purification of murine adipocyte lipid binding protein: Characterization as a fatty acid and retinoic acid binding protein. *J. Biol. Chem. 263*, 14544–14551.

Pigiet, V. & Conley, R.R. (1978). Isolation and characterization of phosphothioredoxin from *Escherichia coli*. *J. Biol. Chem. 253*, 1910–1920.

Proudfoot, N.J. & Brownlee, G.G. (1976). 3′ Non-coding region sequences in eukaryotic messenger RNA. *Nature 263*, 211–214.

Rosen, O.M. (1987). After insulin binds. *Science 237*, 1452–1458.

Sanger, F., Nicklen, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74*, 5463–5467.

Smith, D.B. & Johnson, K.S. (1988). Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene 67*, 31–40.

Taga, E.M. & Van Etten, R.L. (1982). Human liver acid phosphatases: Purification and properties of a low molecular weight isoenzyme. *Arch. Biochem. Biophys. 214*, 505–515.

Tonks, N.K., Diltz, C.D., & Fischer, E.H. (1990). CD45, an integral membrane protein tyrosine phosphatase: Characterization of enzyme activity. *J. Biol. Chem. 265*, 10674–10680.

Waggoner, D.W. & Bernlohr, D.A. (1990). In situ labeling of the adipocyte lipid binding protein with 3-[^{125}I] iodo-4-azido-*N*-hexadecylsalicylamide: Evidence for a role of fatty acid binding proteins in lipid uptake. *J. Biol. Chem. 265*, 11417–11420.

Waheed, A., Laidler, P.M., Wo, Y.-Y.P., & Van Etten, R.L. (1988). Purification and physicochemical characterization of a human placental acid phosphatase possessing phosphotyrosyl protein phosphatase activity. *Biochemistry 27*, 4265–4273.

Wo, Y.-Y.P., Zhou, M.-M., Stevis, P., Davis, J.P., Zhang, Z.-Y., & Van Etten, R.L. (1992). Cloning and expression of the low molecular weight phosphotyrosyl protein phosphatase from bovine heart. *Biochemistry* (in press).

Xu, Z., Buelt, M.K., Banaszak, L., & Bernlohr, D.A. (1991). Expression purification and crystallization of the adipocyte lipid binding protein. *J. Biol. Chem. 266*, 14367–14370.

Zhang, Z.-Y. & Van Etten, R.L. (1990). Purification and characterization of a low molecular weight acid phosphatase-A phosphotyrosyl protein phosphatase from bovine heart. *Arch. Biochem. Biophys. 282*, 39–49.