Characterization of an Aldolase-binding Site in the Wiskott-Aldrich Syndrome Protein*8

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The thrombospondin-related anonymous protein (TRAP) is an essential transmembrane molecule in Plasmodium sporozoites. TRAP displays adhesive motifs on the extracellular portion, whereas its cytoplasmic tail connects to actin via aldolase, thus driving parasite motility and host cell invasion. The minimal requirements for the TRAP binding to aldolase were scanned here and found to be shared by different human proteins, including the Wiskott-Aldrich syndrome protein (WASp) family members. In vitro and in vivo binding of WASp members to aldolase was characterized by biochemical, deletion mapping, mutagenesis, and co-immunoprecipitation studies. As in the case of TRAP, the binding of WASp to aldolase is competitively inhibited by the enzyme substrate/products. Furthermore, TRAP and WASp, but not other unrelated aldolase binders, compete for the binding to the enzyme in vitro. Together, our results define a conserved aldolase binding motif in the WASp family members and suggest that aldolase modulates the motility and actin dynamics of mammalian cells. These findings along with the presence of similar aldolase binding motifs in additional human proteins, some of which indeed interact with aldolase in pull-down assays, suggest supplementary, non-glycolytic roles for this enzyme.

Aldolase catalyzes the cleavage of fructose 1,6-phosphate (F1,6P) into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in the glycolytic pathway or the reverse reaction during gluconeogenesis. Besides its glycolytic role, aldolase bundles microtubules by tethering the α subunit of tubulin (1, 2) and cross-links actin filaments (3–5). In fact, two different pools of aldolase are found in mammalian cells, one in the cytosolic fluid phase and one in the solid phase, most likely associated to cytoskeletal components (6). Similar features have been verified for other glycolytic enzymes (2), leading to the hypothesis that the cytoskeleton provides a template for the coordinated delivery of intermediate metabolites among sequential glycolytic enzymes, thus improving the efficiency of the pathway. This “matrix” would be further strengthened by cytoskeleton-independent interactions among different glycolytic enzymes.

In addition to cytoskeletal proteins, aldolase binds to the erythrocyte anion exchanger Band 3 (7, 8), different subunits of the vacuolar-type H+ -ATPase (9, 10), glucose transporter GLUT4 (11), Sorting-Nexin 9 (12), phospholipase D2 (13), Mycobacterium superoxide dismutase (14), fructose 1,6-bisphosphatase (15), calcium-signaling protein S100A12 (16), calcium/calmodulin-dependent protein kinase phosphate (17), and the light chain 8 of dynein (18). This complex network of aldolase associations might help in coupling the glycolytic pathway to ATP-hydrolyzing processes, as proposed for the vacuolar H+ pump (10). In most of cases, however, aldolase seems to be targeted for structural rather than enzymatic purposes. For instance, binding of the glucose transporter GLUT4 to aldolase is supposed to provide GLUT4 with an anchorage to F-actin, thus allowing its translocation from intracellular vesicles to the cell surface upon insulin stimulation (11, 19). Similarly, the cytoplasmic N-terminal domain of Band 3 tethers to the underlying cytoskeleton through an aldolase bridge (8, 20). A quite similar scaffolding activity has been recently shown in Plasmodium and other apicomplexan parasites. In this case, aldolase connects the actin filaments to the cytoplasmic tail of members from the TRAP family (21, 22). The parasite actin-myosin motor localized underneath the plasma membrane then promotes the antero-posterior redistribution of TRAP molecules, which is critical for parasite motility and host cell invasion (for review, see Ref. 23). The binding of TRAP molecules to aldolase is mediated by a short acidic sequence containing a sub-terminal tryptophan (Trp) conserved across the protein family and is competitively inhibited by aldolase substrate/products (21, 22). In the present work we defined the minimal aldolase binding requirements in the TRAP motif and used this information for the identification of novel aldolase binding molecules among human proteins.

EXPERIMENTAL PROCEDURES

Aldolases—Histidine-tagged Plasmodium falciparum aldolase was expressed in Escherichia coli and purified as described (24). Rabbit aldolase A was from Sigma-Aldrich. Both molecules were labeled with EZ-link Sulfo-NHS-Biotin (Pierce) as described (21).

Synthetic Peptides—Peptides were custom-synthesized by Genemed Synthesis Inc. (San Francisco, CA) and purified by high pressure liquid chromatography. Their sequences (from the N to C termini) are: TRAP, ETGEGKDLDELQEQFLPEENWN; Wiskott-Aldrich syndrome protein (WASp), EDAQGEDEDEDEWDD; Band3, MEQDDEDDYDMEENLEQEYED; CS, TCGGSRVRRKKNVVKQ.

Antibodies—Goat anti-rabbit aldolase and rabbit anti-Bloom Syndrome Protein antibodies were from Chemicon Int. (Temecula, CA). Rabbit anti-actin antibody was from Sigma-Aldrich. Rabbit antibodies anti-human cortactin, cytochrome P450 reductase, and Rho guanine nucleotide-disassociation inhibitor (Rho GD1) were from Santa Cruz.
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Biotechnology (Santa Cruz, CA). Rabbit antibodies anti-human neural WASp (N-WASp), WASp, and p34 were kindly provided by Protea Biosciences (Morgantown, WV), Dr. Katherine Siminovitch, and Dr. Matt Welch, respectively. Mouse antibody to the TRAP C terminus has been described (21).

Cloning and Expression Methods—The human WASp cDNA clone was purchased from American Type Culture Collection (Manassas, VA), and the full-length open reading frame or its truncated derivatives (as defined in Ref. 25) were cloned into pGEX4T-1 (Amersham Biosciences) or pcDNA3.1 (Invitrogen) by PCR. HeLa cells were transiently transfected using the Lipofectamine™ (Invitrogen) method following manufacturer’s guidelines and analyzed 36 h afterward. The GST fusion protein spanning the last 25 residues of Plasmodium berghei TRAP has been described (21), and mutants were generated by PCR using this clone as template. The aldolase binding regions of human GLUT4 (11) and human Band 3 (7) were constructed by fill-in of partially overlapping oligonucleotides in the presence of Taq DNA polymerase and dNTPs. Additional mutant clones derived from pGEX-WASp, pGEX-GLUT4, and pGEX-Band3 clones were generated by PCR and cloned into the EcoRI/SalI sites of pGEX4T-1. The GST fusion molecules containing the pleckstrin homology domain of the human phospholipase D2 and the E subunit of the peripheral domain (V1) of the human vacuolar-type H+ -ATPase have been described (9, 13). GST fusion proteins were expressed in E. coli, purified to apparent homogeneity as described elsewhere (26), and quantified using the Micro BCA™ protein assay kit (Pierce).

Binding and Competition Assays—Polystyrene ELISA microplates (Nunc, Roskilde, Denmark) were coated either with 4 pmol/well of GST fusion protein or 1 nmol/well of synthetic peptide and probed with the indicated biotin-labeled aldolase as described (21). For displacement assays, a fixed aldolase concentration able to render ~80% of maximal detection in standard binding assays was used for each coating molecule. Different concentrations of the indicated competitors were adjusted to a final volume of 20 μl in PBS and added to the plate. The percentage of inhibition (mean value for triplicate wells) was calculated taking as 0% the mean value recorded for the biotin-labeled protein percentage of inhibition (mean value for triplicate wells) was calculated required to achieve 50% inhibition under these conditions. The relative (2.5 mg of protein/ml) was labeled with 0.5 mM EZ-link sulfo-NHS-biotin and centrifuged 2 times at 14,000 rpm for 30 min. The supernatant (250 μl) was preadsorbed for 1 h with 200 μl of NHS-activated Sepharose 4B (Amersham Biosciences) equilibrated in RIPA buffer and centrifuged for 2 min at 500 rpm. The supernatant (200 μl) was further incubated for 4 h with 150 μl of NHS-activated Sepharose coupled to rabbit aldolase A (~6 mg of protein/ml Sepharose beads) following the manufacturer’s guidelines. In a parallel experiment, 50 μl of the supernatant were incubated with 50 μl of the same aldolase-coupled Sepharose beads but in the presence of 15 μl (0.3 mg/ml) of GST-TRAP to be used as the positive control. In both cases the unbound fractions were collected, and Sepharose beads were washed 5 times in 1 ml of RIPA buffer and stripped at 100 °C in 200 and 40 μl of denaturing loading buffer. The unbound and bound fractions were run on SDS-PAGE (4–20% gradient gels), transferred to Sequi-Blot™ polyvinylidene difluoride membranes, and analyzed by Western blotting using HRP-conjugated Neutravidin™ (1:25,000; Pierce). When looking for specific proteins, identical membranes were probed with the indicated antibodies followed by secondary antibodies coupled to alkaline phosphatase (Sigma-Aldrich). These blots were developed using 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt and nitro blue tetrazolium chloride (Sigma-Aldrich). Blocking of the membranes and antibody dilutions was done in Tris-buffered saline supplemented with 5% nonfat milk and 0.1% Tween 20.

GST-WASp Affinity Purification and Aldolase Activity Assays—In a similar GST-WASp pull-down experiment performed on a non-biotinylated cell lysate, the GST-WASp-coupled Sepharose beads were subjected to 5 washings in RIPA buffer and then eluted with 200 μl of 10 mM F1,6P (Sigma-Aldrich) in the same buffer. The sample was extensively dialyzed to remove the F1,6P and assayed for protein concentration using the Micro BCA™ protein assay kit (Pierce). Aldolase activity was recorded on appropriate dilutions of the total lysate and the F1,6P-eluted fraction as described (21).

Aldolase Pull-down Assays—HeLa or 293T cells were resuspended at 5 × 107 cells/ml in RIPA buffer, kept on ice for 30 min, and centrifuged 2 times at 14,000 rpm for 30 min. The supernatant (250 μl) was preadsorbed for 1 h with 200 μl of NHS-activated Sepharose 4B (Amersham Biosciences) equilibrated in RIPA buffer and centrifuged for 2 min at 500 rpm. The supernatant (200 μl) was further incubated for 4 h with 150 μl of NHS-activated Sepharose coupled to rabbit aldolase A (~6 mg of protein/ml Sepharose beads) following the manufacturer’s guidelines. In a parallel experiment, 50 μl of the supernatant were incubated with 50 μl of the same aldolase-coupled Sepharose beads but in the presence of 15 μl (0.3 mg/ml) of GST-TRAP to be used as the positive control. In both cases the unbound fractions were collected, and Sepharose beads were washed 5 times in 1 ml of RIPA buffer and stripped at 100 °C in 200 and 40 μl of denaturing loading buffer. The unbound and bound fractions were run on SDS-PAGE (4–20% gradient gels), transferred to Sequi-Blot™ polyvinylidene difluoride membranes, and analyzed by Western blotting using HRP-conjugated secondary antibodies (Amersham Biosciences) and the SuperSignal West Pico chemiluminescent Substrate (Pierce).

Aldolase Immunoprecipitation Assays—HeLa cells were serum-starved for 24 h and then stimulated for the indicated time periods with 100 ng/ml human recombinant epidermal growth factor (EGF; Sigma-Aldrich) (27). Cells were washed with ice-cold PBS, mechanically detached, and resuspended in ice-cold immunoprecipitation buffer (20 mM Heps, pH 7.3, 75 mM NaCl, 1 mM EDTA, 1% Nonidet P-40). The cleavable cross-linking reagent diethio(bisuccinimidyl propionate) (Pierce) was added to the lysates (0.25 mM) for 30 min to stabilize the protein complexes and then quenched with 200 mM Tris-HCl, pH 7.5. After centrifugation at 14,000 rpm, supernatants were preadsorbed for 45 min with protein G-Sepharose 4 Fast Flow (Pierce) and incubated overnight at 4 °C with 100 μg of the indicated antibody and 25 μl of fresh protein G-Sepharose beads. After extensive washings, aliquots of the unbound and bound fractions were probed by Western blotting.
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TABLE 1

| Protein             | Modification          | Relative binding |
|---------------------|-----------------------|------------------|
| GST-TRAP<sup>+</sup> |                       | 1                |
| GST-TRAP-W25A       | Trp<sup>35</sup> to Ala | <0.01<sup>a</sup> |
| GST-TRAP-W25H       | Trp<sup>26</sup> to His | <0.01<sup>a</sup> |
| GST-TRAP-W25Y       | Trp<sup>26</sup> to Tyr | 0.01<sup>a</sup> |
| GST-TRAP-W25F       | Trp<sup>26</sup> to Phe | 0.02<sup>a</sup> |
| GST-TRAP<sub>Δac1d1</sub> | EDND<sup>24</sup> to AGNA<sup>24</sup> | <0.01<sup>a</sup> |
| GST-TRAP<sub>Δac1d2</sub> | ENE<sup>15</sup> to ANA<sup>15</sup> | 0.12<sup>c</sup> |
| GST-TRAP<sub>Δac1d3</sub> | DDE<sup>9</sup> to AAA<sup>9</sup> | 0.22<sup>c</sup> |
| GST-TRAP-F17A       | Phe<sup>17</sup> to Ala | 0.96<sup>a</sup> |
| GST-TRAP-FL/SS      | FKL<sup>19</sup> to SKS<sup>19</sup> | 0.92<sup>a</sup> |
| GST-TRAP<sub>Δhydroph1</sub> | QFKLP<sup>20</sup> deletion | 1.13<sup>c</sup> |
| GST-TRAP<sub>Δhydroph2</sub> | KGIV<sup>12</sup> deletion | 1.05<sup>c</sup> |
| GST-TRAP<sub>Δhydroph3</sub> | KGIV<sup>12</sup> deletion | 1.05<sup>c</sup> |

<sup>a</sup> K<sub>c</sub> of the parental molecule; K<sub>c</sub> of the indicated molecule.
<sup>b</sup> Parental molecule (sequence EDVMADEDEKIEWGKLIPEDNDWNT).<sup>26</sup>
<sup>c</sup> K<sub>c</sub> significantly different (p < 0.01) from that of the parental molecule.

RESULTS

Mapping of the Aldolase-binding Site on TRAP—In a previous paper we showed that the last ~25 residues of the cytoplasmic tail of TRAP family proteins are sufficient to promote its binding to aldolase (21). To further pinpoint the relevant residues into the aldolase binding region in TRAP, we generated a broad panel of molecules bearing different mutations/deletions throughout this motif. The variants were expressed as GST fusion molecules, purified and tested for Plasmodium aldolase by competitive ELISA. Exchange of the sub-terminal Trp<sup>25</sup> to Ala led to a complete inhibition of the binding (Table 1), as previously demonstrated (21, 22). Notably, Trp<sup>25</sup> replacement by structurally related residues such as Phe, Tyr, or His still caused a dramatic decrease in the aldolase affinity. In fact, only the mutant molecules containing either Phe or Tyr were able to compete with the parental molecule, although their relative affinity was decreased by ~50-fold. On the other hand, the apparent aldolase affinity of TRAP molecules in which several acidic residues were exchanged to Ala (GST-TRAP<sub>Δac1d2</sub> and GST-TRAP<sub>Δac1d3</sub>) molecules was reduced by ~9-~5-fold, respectively, when compared with the parental molecule. The effect of altering either of these acidic patches, however, was not as drastic as mutating the row of acidic residues adjacent to Trp<sup>25</sup> (GST-TRAP<sub>Δac1d1</sub> protein). Therefore, multiple TRAP tail acidic residues mediate the binding to aldolase, although their relative contribution is hierarchical toward the Trp<sup>25</sup>. Finally, mutations in non-acidic residues were analyzed. Substitution of the Phe<sup>17</sup> residue alone (GST-TRAP-F17A protein) or in combination with Leu<sup>19</sup> (GST-TRAP-FL/SS protein) did not affect the affinity for aldolase. Similar results were obtained with mutant molecules in which two slightly hydrophobic stretches of residues (GST-TRAP<sub>Δhydroph1</sub> and GST-TRAP<sub>Δhydroph2</sub>) proteins) were deleted. Jointly, these data indicate that the aldolase binding properties in the TRAP tail rely only in the sub-terminal Trp and the acidic residues.

Identification of Novel Aldolase Binders—The simpler than expected aldolase binding motif revealed above prompted us to look for homologous sequences in the databases. For searching purposes, we used variations of peptides containing at least one Trp and eight acidic residues (either Asp or Glu) (see the details in the footnote to Supplemental Table 1). We found matches in a variety of transcription factors, importins, Bloom syndrome protein, zinc finger proteins, ubiquitin-modifying enzymes, translation initiation factors, and cortactin, some of which might indeed constitute novel aldolase binders (Supplemental Table 1). Several retrieved matches were found in the C terminus of different members of the WASp family, including WASp, N-WASp, WASp family member 1 (WAVE-1), WAVE-2, and WAVE-3. To get a first glimpse on the predictive value of our proposed aldolase binding motif, immobilized rabbit aldolase was incubated with a total HeLa cells lysate (293T cells were used to search for aldolase-Bloom syndrome protein (BSP) interaction). A sample of the lysate was added with GST-TRAP to be used as positive binding control. Equivalent aliquots (30 μl) of the unbound and bound fractions were evaluated by Western blotting using the indicated antibodies. The putative aldolase binding sequences are indicated. Rho GDP, Rho guanine nucleotide dissociation inhibitor; cytP450, cytochrome P450 reductase.

FIGURE 1. Identification of aldolase binders. Immobilized rabbit aldolase was incubated with a total HeLa cells lysate (293T cells were used to search for aldolase-Bloom syndrome protein (BSP) interaction). A sample of the lysate was added with GST-TRAP to be used as positive binding control. Equivalent aliquots (30 μl) of the unbound and bound fractions were evaluated by Western blotting using the indicated antibodies. The putative aldolase binding sequences are indicated. Rho GDP, Rho guanine nucleotide dissociation inhibitor; cytP450, cytochrome P450 reductase.
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In Vivo Interaction of WASp Family Members and Aldolase—To analyze if aldolase and WASp family members can also interact in vivo, two approaches were undertaken. First, HeLa cells, which do not express WASp, were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). WASp family members were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). WASp family members were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). WASp family members were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). WASp family members were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A).

were differentially retained in the GST–WASp-coupled Sepharose beads, the major being an ~40-kDa protein identified as aldolase by Western blotting (Fig. 3, lower panel). In a similar experiment, the GST–WASp-coupled Sepharose beads were eluted with 10 mM F1,6P, and the degree of aldolase purification was calculated by measuring both protein concentration and enzymatic activity on the total lysate and the F1,6P-eluted fraction. Although the recovery of aldolase activity on the latter fraction was about 49% of the total input activity, the enzyme was purified by 904-fold (as a result of 1846-fold reduction in the total protein content).

The GST–WASp-coupled Sepharose beads, but not those GST–WASp-W13F-coupled, did also retain p34, one of the subunits of the Arp2/3 complex, which is the primary target of the WASp acidic tail in vivo (28). Actin, however, was only detected in the unbound fraction, indicating that even in these conditions aldolase interacted with the GST–WASp itself and not indirectly with actin filaments attached to this molecule via the Arp2/3 complex (Fig. 3, lower panel).

In Vivo Interaction of WASp Family Members and Aldolase—To analyze if aldolase and WASp family members can also interact in vivo, two approaches were undertaken. First, HeLa cells, which do not express WASp, were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). Lysates obtained from transfected cells were immunoprecipitated using either an anti-aldolase antibody or control immunoglobulins (indicated by arrowheads). WASp family members were transiently transfected either with the full-length human WASp cDNA or with a truncated construct lacking the A motif (Fig. 2A). WASp expression in transfected cells was monitored by Western blotting and indirect immunofluorescence (Fig. 4A). Lysates obtained from transfected cells were immunoprecipitated using either an anti-aldolase antibody or control immunoglobulins (indicated by arrowheads).

We next followed the association of aldolase with endogenous N-WASp in EGF-stimulated HeLa cells. It has been documented that after EGF treatment N-WASp undergoes a transient conformational change resulting in the exposure of the C-terminal region. This structural modification enables its interaction with the Arp2/3 complex, thus leading to actin remodeling in the cell cortex, extension of lamellipodia, and membrane ruffling (27, 29). The “activation” of N-WASp is maximal at 1–5 min and diminished to almost basal level by 20–30 min after
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![Image](An Aldolase-binding Site in WASp)

**FIGURE 4. In vivo interaction between aldolase and WASp family proteins.** A, lysates obtained from HeLa cells previously transfected with the full-length human WASp cDNA (FL), a construct lacking the acidic tail (ΔC-t), or with the plasmid alone (mock) were probed with a rabbit anti-WASp antibody (1:1000). Molecular markers (in kDa) are indicated. B, total lysates obtained from 10^6 cells transfected with the indicated construct were immunoprecipitated either with anti-aldolase (αaldo) antibody or control immunoglobulin (C). Aliquots were probed as before with the anti-WASp antibody. A nonspecific interaction with both WASp constructs was verified for our control immunoglobulin (C). C, HeLa cells were stimulated or not with EGF (100 ng/ml) for the indicated time periods and immunoprecipitated as in B using the anti-aldolase antibody. The precipitated fraction was probed with an anti-N-WASP (1:3000, upper panel) and, for normalization purposes, with the same anti-aldolase antibody used for immunoprecipitation followed by HRP-conjugated protein G (lower panel).

EGF addition, as revealed by using a conformationally sensitive antibody (29). As shown in Fig. 4C, upper panel, N-WASP co-immunoprecipitated with aldolase roughly following the kinetics of N-WASP activation. As immunoprecipitation efficiency/loading control, we probed a duplicate membrane with an anti-aldolase antibody (Fig. 4C, lower panel).

**Competition among Aldolase Binding Molecules**—Even though a variety of aldolase binders was identified to date, detailed information on their binding features is only available for a few of them (21). Sorting-Nexin 9 displays a canonical Trp-containing, acidic motif (Supplemental Table 1), and its binding is inhibited by the enzyme substrate/products (12) and was, therefore, grouped with TRAP and WASp. A second subset of molecules (Band 3, α-tubulin, GLUT4) are related to TRAP/WASp in the sense that their binding to aldolase is also inhibited by enzyme turnover (11, 30) and mediated by a short sequence composed of one or two Tyr residues surrounded by acidic amino acids (21). Even though replacement of Trp by Tyr abolished the binding of TRAP/WASp to aldolase (Table 1 and Fig. 2E), we put forward the hypothesis that sequences from both groups represent variations on a common/analogous binding motif targeting to a specific region in aldolase. A third subset of aldolase binders includes the human phospholipase D2 and the E subunit of the human vacuolar H^+-ATPase, which do not bear a Trp/Tyr-containing, acidic region and that are not competed out by aldolase substrate/products. To test the above-mentioned hypothesis, GST fusion molecules from the different groups were tested for their ability to compete in aldolase binding assays. As shown in Fig. 5, GST-Band3, GST-TRAP, and GST-WASp were able to displace each other in a dose-dependent manner. The binding of GST-GLUT4 to aldolase was barely measurable by our method and, accordingly, did not displace any of the above molecules (Fig. 5B). It was, however, specifically competed by GST-Band3, GST-TRAP, and GST-WASp (data not shown). Importantly, GST fusion proteins containing the pleckstrin homology domain of human phospholipase D2 or the entire E subunit of the human vacuolar H^+-ATPase (GST-V-ATPase) were unable to displace any of the above molecules, including GST-GLUT4 (Fig. 5A and data not shown).

No significant displacement was observed when using the control GST-TRAP-W25A molecule, and more important, similar results were obtained when synthetic peptides spanning the TRAP, Band 3, and WASp aldolase binding sequences were used as competitors (data not shown), further demonstrating the specificity of the results. The TRAP-displacing ability of Band3 molecules mutated either in ‘Tyr’ or Tyr20 was drastically diminished, suggesting that both residues are critical for its interaction with aldolase (Fig. 5B).

Finally, while doing these assays we noticed that the apparent affinity of WASp for aldolase was significantly higher than that of TRAP or Band 3 (Fig. 5A). One salient feature of the WASp motif is that is the most acidic overall, i.e. containing the highest acidic/non-acidic residues ratio. To evaluate if this might be related to its enhanced aldolase affinity, we constructed a novel GST-WASp recombinant mutant in which a non-charged tripeptide (QNQ) was inserted into the acidic row (GST-WASPins). As shown in Fig. 2E, the apparent affinity of this mutant was diminished by ∼3-fold when compared with the parental molecule.

**DISCUSSION**

The members of the WASp family regulate actin dynamics and ultimately cell motility through their concerted binding to actin monomers, the Arp2/3 complex, and different regulatory molecules triggered upon extracellular stimuli (28). All of them display a characteristic C-terminal signature that is involved in their binding to and activation of the Arp2/3 complex. This motif is basically a short sequence (18–20 residues) enriched in acidic amino acids and bearing a highly conserved Trp residue (28) and is, thus, very similar to the core structure of the aldolase binding motif present in Plasmodium TRAP, as evidenced by mutagenesis studies (Table 1). This structural similarity prompted us to analyze whether the acidic tail of WASp would also function as an aldolase binding motif. Even though the C terminus of WASp binds readily to aldolase (Figs. 2 and 3), a fusion construct expressed in bacteria and containing the full-length molecule failed to do so (Fig. 2A). This is likely due to the burrowing of the central C-terminal and acidic region over the Rho GTPase binding region, thus concealing the terminal TRAP-like sequence. This is consistent with the well known “auto-inhibited” status to the WASp family proteins in the absence of activating stimuli (31). In keeping with this, we show that co-immunoprecipitation of N-WASP and aldolase only takes place after incubation of the cells with EGF (Fig. 4C), a treatment that switches N-WASP into an “activated” state (29). In WASp-transfected cells this activation step is not required to detect the WASp-aldolase interaction, likely because of the large

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amount and/or the partially aberrant folding of the expressed protein (Fig. 4B). A WASp construct solely lacking the acidic tail, however, is unable to bind to aldolase in vitro (Fig. 2A) and in vivo after being ectopically expressed (Fig. 4B). As mentioned above, the same aldolase binding motif defined here is involved in anchoring of WASp to the Arp2/3 complex, and even more compelling, site-specific replacement of the antepenultimate Trp residue also impairs the Arp2/3 binding/activation (Fig. 3 and Refs. 25 and 31). Further experiments are currently under way to ascertain the in vivo physiological role of WASp-aldolase interaction, but one interesting possibility is that aldolase modulates WASp-mediated responses by its competition with the Arp2/3 complex and, therefore, influences the actin dynamics of mammalian cells. Identification of an aldolase mutant displaying full enzymatic and F-actin binding activity but impaired binding to the WASp acidic tail would be particularly helpful to study this hypothesis. The putative binding of aldolase to cortactin (Fig. 1), which is another protein involved in the regulation of actin dynamics (32), suggests an even broader role for this glycolytic enzyme in cytoskeleton remodeling.

In recent years different studies have shown that aldolase binds to and affects the function and/or subcellular distribution of a plethora of functionally and evolutionarily unrelated proteins. In addition to its established role in carbohydrate metabolism, aldolase might be involved in different aspects of cellular physiology including vesicular and molecular trafficking and, as proposed here, actin remodeling. These “moonlighting” activities, i.e. supplementary functions unrelated to catalysis (33), are consistent with the huge aldolase concentration in eukaryotic cells.

**FIGURE 5. Competition among aldolase binding molecules.** A. ELISA wells coated with the indicated GST fusion molecule were added with a fixed concentration of biotin-labeled *Plasmodium* aldolase followed by increasing amounts of the indicated competitors. Aldolase binding was recorded as in legend to Fig. 2B. Mean absorbance values (S.D. values did not exceed 10% of the mean values, not shown) are indicated. B. GST-TRAP-coated wells were added with 15 nM of biotin-labeled *Plasmodium* aldolase followed by the indicated competitor (1 μM final), and the extent of displacement was compared with wells supplemented with PBS (taken as 0% displacement). Mutated/added residues are denoted in bold italics.
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cells (μM range), largely exceeding the glycolytic requirements (34). The broad distribution of sequences resembling the TRAP acidic tail among human proteins (Supplemental Table 1) further supports this idea. Moreover, all of the tested putative aldolase binders displaying this molecular signature indeed recognized the enzyme in pull-down assays (Fig. 1). It should be stated, however, that the presence of this motif only provides the first step toward the identification of a bona fide aldolase binder. Subcellular localization (many of the retrieved sequences correspond to intranuclear proteins), post-translational modifications (as the phosphorylation events described in Sorting-Nexin 9 and Band 3 (12, 30)), or structural constraints might preclude the binding of these sequences to the enzyme. Another important parameter affecting the overall aldolase affinity and, thus, the in vivo functionality of this motif in different proteins would be the ratio and distribution of acidic amino acids surrounding the conserved Trp residue. As shown in Fig. 5A, the WASp sequence, which is more acidic than that of TRAP, displays a higher apparent affinity for aldolase in competition assays, and this affinity is reduced by 3-fold after insertion of a non-charged tripeptide (Fig. 2E). Deletion of non-acidic residues in TRAP also leads to an increase, albeit not significant, in its relative binding to aldolase (Table 1).

The C terminus of WASp and perhaps all of the molecules bearing a similar sequence (Supplemental Table 1) display the same aldolase binding features as those described for TRAP; that is, inhibition by similar sequence (Supplemental Table 1) display the same aldolase binding motif that is present in the WASp family members. This fact provides the first step toward the identification of a functional ternary complex (TRAP/Band3/GLUT4-aldolase-actin filaments). This kind of regulation might have been selected for to prevent the formation of nonproductive associations of these molecules with "soluble" (cytoskeleton-detached) aldolase.

Together, our results define and characterize a conserved aldolase binding motif that is present in the WASp family members. This fact suggests a novel way (besides its actin filament cross-linking capacity (3–5)) through which aldolase might modulate the actin dynamics of mammalian cells. The presence of similar sequences in additional human proteins suggests an even broader range of supplementary, non-glycolytic roles for aldolase.

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