Interactions between CD36 and global intestinal alkaline phosphatase in mouse small intestine and effects of high-fat diet

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Running head: CD36 and gIAP in mouse intestine

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ABSTRACT
The mechanisms of the saturable component of long chain fatty acid (LCFA) transport across the small intestinal epithelium, and its regulation by a high-fat diet (HFD), are uncertain. It is hypothesized here that the putative fatty acid translocase/CD36 and intestinal alkaline phosphatases (IAPs) function together to optimize LCFA transport. Phosphorylated CD36 (pCD36) was expressed in mouse enterocytes, and was dephosphorylated by calf intestinal alkaline phosphatase (CIAP). Uptake of fluorescently-tagged LCFA into isolated enterocytes was increased when cells were treated with CIAP; this was blocked with a specific CD36 inhibitor. pCD36 co-localized in enterocytes with the global IAP (gIAP) isozyme, and specifically co-immunoprecipitated with gIAP but not the duodenal-specific isozyme (dIAP). Purified recombinant gIAP dephosphorylated immunoprecipitated pCD36, and antiserum to gIAP decreased initial LCFA uptake in enterocytes. Body weight, adiposity, plasma leptin and triglycerides were significantly increased in HFD mice compared to normal-fat diet controls. HFD significantly increased immunoreactive CD36 and gIAP but not dIAP, in jejunum but not duodenum. Uptake of LCFA was increased in a CD36-dependent manner in enterocytes from HFD mice. It is concluded that CD36 exists in its phosphorylated and dephosphorylated states in mouse enterocytes, that pCD36 is a substrate of gIAP, and that dephosphorylation by IAPs results in increased LCFA transport capability. HFD upregulates CD36 and gIAP in parallel, and enhances CD36-dependent fatty acid uptake. The interactions between these proteins may be important for efficient fat transport in mouse intestine, but whether the observed changes in gIAP and CD36 in enterocytes are contributing factors to HFD-induced obesity remains to be determined.

Keywords: intestinal plasticity; phosphorylation; enterocytes; adiposity; obesity
In addition to simple diffusion, long chain fatty acids (LCFA) cross plasma membranes by a saturable mechanism that requires one or more proteins (reviewed in (16, 35). One protein implicated in this process is the Class B scavenger receptor CD36 in humans, and its rodent homolog also known as fatty acid translocase (FAT). FAT/CD36 (called CD36 hereafter) is a cell surface glycoprotein found in many cell types, including platelets, endothelial cells, and monocytes (2); differentiated adipocytes (23); mammary epithelial cells (17), and intestinal enterocytes (13, 41). It is probably ubiquitous among vertebrates, including mice (12), rats (58), humans (53) and other mammals (7). CD36 binds a broad spectrum of extracellular ligands, including thrombospondin-1 (2), oxidized LDL (15), collagen (52) and LCFA (5, 29). Among numerous functions CD36 has been demonstrated or believed to play a role in is facilitation of the transport of LCFA into adipocytes (23), platelets (49), skeletal muscle cells (9), cardiomyocytes (6, 8, 28, 32, 43, 51, 59), and enterocytes (41).

The mechanism of action of CD36 in enterocytes is unknown. In all cell types in which it has been examined, however, CD36 is extensively post-translationally regulated, resulting in an apparent molecular weight between ~70-88 kDa. In stably transfected human embryonic kidney cells, for example, CD36 is palmitoylated on both putative cytoplasmic tails (54); palmitoylation of the protein and its glycosylation state are important in trafficking the protein to and from the plasma membrane (27, 55), and possibly in localizing it to lipid rafts in the cell membrane (55). In addition, the extracellular fragment of CD36 contains two consensus phosphorylation sites, including one at Thr92. This residue is recognized by protein kinase C and, to a lesser extent, protein kinase A in 3T3 cells that have been transfected with human CD36 (26). The mechanism and regulation of phosphorylation of CD36 in vivo is uncertain, but in platelets it depends upon the action of an unidentified ectokinase (19, 24). Phosphorylation of CD36 is important in
determining ligand specificity in platelets (3), increasing adherence of red blood cells to human
dermal microvascular endothelial cells (26), and of particular relevance to the present
investigation decreasing palmitate uptake in platelets (19). Whether CD36 is phosphorylated in
enterocytes, however, is currently unknown.

Dephosphorylation of CD36 is accomplished by alkaline phosphatases (26). COS cells
transfected with CD36 express the protein in lipid rafts, identified by cross-linking experiments
that revealed CD36 co-patching with placental alkaline phosphatase, a marker of these
membranous structures (14). The association of CD36 with lipid rafts is crucial for its ability to
increase LCFA uptake (14). Lipid rafts are also present on the brush-border of enterocytes (21)
where they are enriched in alkaline phosphatases (22); this suggests the possibility that
enterocyte CD36 and intestinal alkaline phosphatases may interact.

Mammalian alkaline phosphatases comprise up to four distinct families, including
intestinal alkaline phosphatases (IAPs) (33, 36, 39, 41). Murine enterocytes express two
phosphatases called duodenal IAP (dIAP), and global IAP (gIAP), which are the products of the
_Akp3_ and _Akp6_ genes, respectively; they also express low levels of an embryonic isozyme called
EAP (37, 39). IAPs have been implicated in the innate immune responses of the mammalian
intestine, and in local pH homeostasis along the brush border (33). They also appear to play a
role in mucosal handling of fat. For example, mRNA of all three mouse intestinal phosphatases
is increased in the duodenum when the fat content of the diet is changed from very low to
moderate or moderately high (41). Interestingly, however, inactivation of the _Akp3_ gene results
in greater weight gain in HFD mice, suggesting that dIAP may negatively regulate fat absorption
(38); however, it is also possible that this may result from the concominant upregulation of _Akp6_
that is observed in distal regions of the intestine in these mice (41).
In mouse small intestine, CD36 is primarily expressed in the proximal region (duodenum); more distal regions including jejunum express CD36 but at lower levels (41). This is consistent with the relative contributions of proximal and distal regions of the mammalian small intestine in nutrient digestion and absorption. However, the expression of CD36 mRNA in mouse jejunum is increased when the fat content of the diet is increased from 3% to 40% of total calories; moreover, exteriorized jejunal loops from such animals demonstrate an increased ability to absorb LCFAs (45). Although proximal regions of the intestine were not examined in that study, nor were CD36 protein levels, the data nonetheless suggest that distal regions of the mouse small intestine adapt to increased dietary fat content, which may depend in part on changes in CD36 expression. In humans, a chronic dietary fat content of 3% would rarely if ever occur; a typical western diet may range from about 15-45% or greater energy from fat. Thus, an animal model with controlled fat consumption that approximates this range would be an advantageous model for understanding the role of CD36 and other proteins in the intestinal adaptations to HFD in humans.

Given the importance of CD36 for LCFA uptake in other cell types, its regulation by dephosphorylation, its expression in the intestinal epithelium, its location in cellular compartments that harbor alkaline phosphatases, and the ability of CD36 to act as a substrate for alkaline phosphatase, we asked the following questions. First, is CD36 phosphorylated in enterocytes and can it be dephosphorylated by IAPs? Second, does treatment of enterocytes with IAP increase uptake of LCFAs by the cells in a CD36-dependent manner? Third, does CD36 in enterocytes associate with one or more isozymes of mouse IAP and if so is that isozyme capable of dephosphorylating pCD36? Fourth, does chronic HFD induce changes in CD36 and IAP
isozyme protein expression in the murine small intestine, and are these changes reflected in increased cellular uptake of LCFA?

MATERIALS AND METHODS

Animals

Adult male C57Bl/6 mice (Taconic Breeding Laboratories, Hudson NY) were housed singly with lights on from 0700h-1900h, and fed standard rodent chow ad libitum unless otherwise noted. All procedures were approved by the Boston University Institute Animal Care and Use Committee.

Preparation and Purification of Recombinant Enzymes

CHO (Chinese hamster ovary) cells were transfected with pCMV Script expression vector containing a coding sequence of Akp6 in which the GPI anchoring site was replaced by a FLAG sequence as described (11) or a coding sequence of human TNAP (tissue non-specific alkaline phosphatase) D361V mutant. TNAP$_{D361V}$ enzyme is also FLAG tagged and has no detectable $p$-nitrophenol phosphatase activity. The mutation was introduced by PCR-based site directed mutagenesis as previously described (11). CHO cells stably expressing these secreted forms of gIAP and TNAP$_{D361V}$ were obtained after 3 weeks of selection under G418. The media were replaced with Opti MEM (Invitrogen, Carlsbad CA) for 48 hr prior to collection of the supernate. Expressed enzymes were further purified from the supernate with anti-FLAG M2 column (Sigma-Aldrich, St. Louis MO) according to the manufacturer's protocol and the protein concentration was determined with a micro BCA Assay kit (Thermo Fisher Scientific, Waltham
MA). The activity of recombinant gIAP in a para-nitrophenylphosphate assay was equivalent to 0.0046 units CIAP/mL.

Detection of pCD36

A modification of a procedure described for platelets by Ho et al. (26) was used to detect pCD36 from mouse intestinal tissue. Duodenal tissue (considered here as the first 5 cm distal to the pylorus) was homogenized in RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) for 30 seconds on ice. The cell lysate was immediately aliquoted into two microcentrifuge tubes. Ten µl of phosphatase inhibitor cocktails (PIC) 1 (for serine/threonine protein phosphatases and L-isozymes of alkaline phosphatase) and 2 (for tyrosine protein phosphatases, acid and alkaline phosphatases) (Sigma-Aldrich, St. Louis MO) were added to one aliquot. Both tubes (+/- phosphatase inhibitor cocktails) were incubated at 4°C for 1 hour. Lysates were centrifuged at 1000g for 5 minutes to pellet unlysed cells. The resulting supernate was added to an equal volume of 2x Laemmli buffer and boiled.

To examine effects of exogenous alkaline phosphatase treatment on CD36 phosphorylation, CD36 was immunoprecipitated from tissue homogenates solubilized in the presence of PIC 1 and 2. Following centrifugation to remove unlysed cells, 125 µl lysate was added to 500 µl of RIPA buffer and 5 µl of anti-CD36 (CD36-H-300 or CD36-N-15 antiserum, [Santa Cruz Biotechnology, Santa Cruz CA]). The antibodies were generated against human CD36 and cross-react with mouse CD36 (34, 60). In preliminary studies, we confirmed using western blots that the antisera primarily recognized a single protein of the expected molecular weight. In addition, following treatment with the deglycosylating enzyme PNGaseF (New
England Biolabs, Ipswich MA), a lower Mr immunoreactive band of approximately 55 kDa appeared (not shown); this is the expected size of the nascent, non-glycosylated form of CD36 in mice (17). We also confirmed in preliminary studies that the immunoreactive band reacted with antiserum to phosphothreonine but not to phosphoserine (not shown), as predicted for CD36 (3). Immunoprecipitations were incubated at 4°C overnight while shaking; 20 µl of Protein A/G PLUS agarose beads (Santa Cruz Biotechnology) were then added for 2 hours at 4°C while shaking. The beads were pelleted at 2000g for 3 minutes, washed three times in 1% NP-40 in PBS, then washed three additional times in a second buffer solution containing 0.01 M Tris, pH 7.5, 1 mM EDTA, and PBS with 0.1 M NaCl. The beads were then resuspended in alkaline phosphatase buffer (Promega, Madison WI), and incubated for 25 minutes at 37°C with or without 100 units of calf intestinal alkaline phosphatase (CIAP, Promega) or with 1 or 10 µl of recombinant enzymes. After incubation, the beads were pelleted by centrifugation at 2000g for 3 minutes and resuspended in an equal volume of Laemmli buffer before boiling for SDS-PAGE.

For immunoblots, 10 µl of sample was separated by 10% Tris-HCl SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% milk diluted in TBST overnight before detection with either anti-CD36 (H-300), anti-phospho-threonine (Clone 14B3, Santa Cruz Biotechnology) or anti-pCD36 (gift of Dr. M. Ho, University of Calgary) diluted with blocking buffer. The anti-pCD36 solution also contained a non-phosphorylated peptide (courtesy of Dr. M. Ho) to block nonspecific binding (26). Incubations were for 2 hours at room temperature. Membranes were then washed twice in TBST, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. Membranes were washed twice in PBS before detection with enhanced chemiluminescence. Where appropriate, band densities were quantified by densitometry (UN-SCAN-IT, Silk Scientific, Orem, UT).
**Co-immunoprecipitation**

Duodenal tissue was homogenized in buffer [20 mM HEPES, pH 7.4, 1 mM EDTA, 25 mM sucrose, 1% Nonidet P-40 substitute (NP-40), protease inhibitor cocktail (539134, Calbiochem, Gibbstown NJ)] and then rotated for 60 minutes at 4°C. Samples were centrifuged at 2000g for 5 minutes, and the protein concentration of the supernate [whole cell lysate (WCL)] was determined. Samples were immunoprecipitated with CD36 antibody. Non-immune goat serum (NGS) was used as negative control. WCL was rotated overnight at 4°C with 5 µg of CD36 antibody or 5 µl of a 1:10 dilution of NGS. The following morning, Protein G-Plus agarose beads were added and the solutions were rotated at 4°C. After 2 hours, the supernate was removed and boiled in an equal amount of 2x Laemmli buffer before separation using SDS-PAGE. The beads were washed three times in 1% NP-40 in PBS, and then washed three times in a second buffer solution containing 0.01 M Tris, pH 7.5, 1 mM EDTA, and PBS with 0.1 M NaCl. Antigens were eluted from the beads with 30 µl of 2x sample buffer and 30 µl of 2x Laemmli SDS buffer and were boiled for SDS-PAGE.

**Cell Isolation**

Intestinal epithelial cells were isolated according to the method of Bader et al. (4). Briefly, mice fasted overnight were euthanized by CO₂ exposure followed by decapitation, and the entire small intestine including the connection with the stomach was immediately removed. Fasting overnight was performed to prevent any potential acute effects of luminal fat on CD36 expression or internalization. The intestinal lumen was flushed thoroughly with Modified Eagle’s Medium containing PIC 1 & 2 at 37°C. The distal end of the intestinal tube was then tied closed.
with surgical thread and a syringe was inserted through the pyloric valve in order to fill the tube with approximately 2 ml physiological saline (37°C) containing PIC 1 & 2 and 1 mM 1,4-dithiothreitol (DTT) to facilitate removal of mucus. The pylorus was tied closed with surgical tubing and the intestinal tube was submerged in 37°C PBS and kneaded gently for 10 minutes. After incubation, the buffer in the intestinal tube was discarded and replaced with 2 ml PBS containing PIC 1&2, 1.5 mM EDTA and 0.5 mM DTT and submerged in 37°C PBS. The intestine was kneaded gently during incubation to liberate epithelial cells. Cells were collected by shaking the intestinal tube and washing with MEM into a 1.5 ml eppendorf tube pre-cooled to 4°C. All cells used for flow cytometry were pooled and counted by trypan blue exclusion to determine yield (~2.65x10^6 cells/mouse) and viability (~92%). Cells used for flow cytometry were washed 3 times in MEM containing 5% BSA; cells used for fatty acid uptake studies were washed 2 times in MEM. In some experiments, cells were isolated separately from duodenum and jejunum.

Fatty Acid Uptake

Isolated cells were treated with one or more concentrations of CIAP (New England Biolabs) or vehicle for 25 minutes at 37°C, after which they were treated with 0.5 mM of sulfosuccinimidyl oleate (SSO) or vehicle (DMSO) for 5 minutes at 37°C. SSO is a specific, irreversible CD36 inhibitor (23, 42, 46); viability of cells at the end of an experiment was similar regardless of treatment. In one experiment, approximately 10^6 cells were treated on ice with 10µl of either PBS or antisera to gIAP or dIAP for 30 minutes. The cells were then washed once in MEM before storage on ice. Fatty acid uptake was measured using the fluorescently-labeled long chain fatty acid, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic.
acid (BODIPY FL C16, Invitrogen, hereafter referred to as C16:bodipy), in combination with the quenching agent trypan blue, as described elsewhere (50). In some cases, a short-chain fatty acid analog, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid (BODIPY FL C5, or C5:bodipy) was used as a control because CD36 specifically binds medium to very long chain fatty acids (1, 20). Approximately 200,000 live cells were added to each well of optically transparent 96-well plates before addition of 1.25 µM bodipy fluor, 2.1 mM trypan blue (Sigma-Aldrich) and 2.5 µM fatty acid-free BSA (Sigma-Aldrich) as carrier. After excitation at 485 nm, fluorescence at 528 nm was determined using a Victor III plate reader (Perkin Elmer, Waltham MA) at room temperature. Because trypan blue cannot enter live cells, the only fluorescence detected was intracellular. Statistical significance was determined by two-way ANOVA or by paired t-tests for single timepoint determinations.

Flow Cytometry

Approximately 800,000 cells were used for each flow cytometry assay. In some cases, cells were incubated with 100 units CIAP for 25 minutes at 37°C before blocking. The cells were blocked in 10% horse serum and labeled with primary antibody in MEM containing 5% BSA. Cells were labeled with antisera to glAP, CD36 or pCD36, except for a control group in which cells were incubated without primary antibody. All cells, including controls, were then labeled with species-appropriate fluorescent secondary antibodies (sc-2090, sc-3751, Santa Cruz Biotechnology), then resuspended in 0.5 ml MEM and fixed on ice by addition of an equal volume of 2% paraformaldehyde in the dark. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Franklin Lakes NJ). Data analyses were performed with FlowJo (Tree Star Inc, Ashland OR).
Effects of High-Fat Diet on Body Weight, Adiposity and Protein Levels

Mice were randomly assigned to one of two dietary groups. HFD animals were fed a diet composed of 45% kilocalories from fat, of which 77% was saturated fat (Research Diets, New Brunswick NJ). Normal fat diet (NFD) controls were fed a diet composed of 15% kilocalories from fat, of which 77% was saturated. The amount of protein was identical between the diets (20% of total calories). The complete compositions of the diets are available through the supplier's website, http://www.researchdiets.com (Catalog #s D06061301 and D06061303). Animals were fed ad libitum.

Body weights were determined approximately twice per week. Food intake was measured weekly as previously described (56). Energy consumption was determined using the known caloric content of the diets. Statistical significance of body weights and energy consumption was determined by two-way ANOVA followed by Bonferroni multiple comparison method for pairwise comparisons.

One, two, five or nine weeks after beginning their respective diets, fasted mice were euthanized between 1000-1100h by CO₂ followed by decapitation. Food was removed from the cages between 1800-1900h the night before sacrifice to eliminate the possibility of acute nutrient-induced changes in enterocyte protein levels. Trunk blood was collected and the plasma was frozen for triglyceride analysis using the glycerol phosphate oxidase method (Pointe Scientific, Canton MI). Perirenal and inguinal fat pads were removed and weighed to determine the adiposity index as the ratio of the combined weight of both fat pads to the residual carcass mass, as previously described (18, 56). Statistical significance between dietary groups was determined by student's unpaired t-test.
Small intestines were removed in two sections. The first 5 cm segment beyond the pyloric valve was considered the proximal segment, or duodenum, and the next 5 cm segment was considered the distal segment, or a portion of the jejunum. All tissue was immediately frozen on dry ice and stored at -80°C for subsequent analysis of CD36 and IAP proteins.

Protein extracts of intestines were boiled for 5 minutes before separation using SDS-PAGE. After transferring to PVDF, membranes were blocked with 5% milk and then probed with either gIAP antiserum or dIAP antiserum. Anti-dIAP is highly specific for dIAP; anti-gIAP recognizes gIAP but also dIAP. Membranes were washed, incubated in the appropriate secondary antibody for 60 minutes, and washed again. Membranes were developed with enhanced chemiluminescence. Membranes that were subsequently probed for beta actin were first stripped in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) before probing with a beta actin antibody (Sigma-Aldrich) and appropriate secondary antibody, followed by detection by ECL.

RESULTS

In preliminary experiments with CD36 immunoprecipitated from whole homogenates of mouse intestinal tissue, we observed that antisera to CD36 and to pThr recognized a 75 kilodalton band on western blots, and that the intensity of the pThr band was decreased after treatment of homogenates with CIAP (Figure 1a) and increased by pretreatment with PIC 1&2 (not shown). This suggested that mouse intestine normally expresses CD36 in both the phosphorylated and non-phosphorylated forms. Because tissue homogenates may contain lysates of both enterocytes and vascular endothelial cells, however, and CD36 is constitutively phosphorylated in human dermal microvascular endothelial cells (26), it was necessary to
determine if the immunoreactive pCD36 observed in mouse intestinal lysates was expressed by enterocytes or contaminating endothelial cells. Flow cytometry was used to label cells with antisera that reacted with gIAP, a specific marker of enterocytes in intestinal tissue (40), and with antisera against pCD36 or CD36. Neither the number of gIAP-positive cells nor CD36-positive cells changed. The number of gIAP-positive cells did not change after treatment with CIAP, as expected; however pCD36 labeling of all cells was decreased after treatment with CIAP. When the data were gated to count only cells that expressed the enterocytic marker gIAP, phospho-specific CD36 antiserum co-labeled 80.2% of cells that stained with anti-gIAP (Figure 1b). Co-labeling was reduced to 52.2% when cells were pre-treated with CIAP (Figure 1b). Control cells labeled only with fluorescently-tagged secondary antibodies did not show significant staining and are shown overlaid with each experiment.

Enterocytes were then isolated for assessment of CD36-dependent LCFA uptake and its sensitivity to CIAP treatment. Cells were first pretreated with CIAP and then subsequently incubated with the irreversible CD36 inhibitor SSO or vehicle. All cells were then incubated with the C16:bodipy-labeled LCFA in the presence of the quenching agent trypan blue. Fatty acid uptake was monitored by emission at 528 nm beginning 10 seconds after addition of the fatty acid analog and trypan blue. Treatment of cells with CIAP significantly increased fatty acid uptake compared to untreated cells; this effect was eliminated by incubation with the CD36 inhibitor SSO (Figure 2a). At later timepoints, some of the intracellular fluorescence shifts to a different wavelength when the analog gets incorporated into triglycerides, and some of the analog may diffuse out of the cell. In order to focus strictly on the saturable component of fatty acid uptake, therefore, in subsequent experiments only the initial rate of uptake (i.e., the initial timepoint) was quantified, as previously described by others (50). There was no effect of CIAP
or SSO on the initial uptake of a short chain analog (C5:bodipy, Figure 2b). The effect of CIAP on the initial uptake of C16:bodipy was dose-dependent (Figure 2c).

Based on the colocalization of pCD36 and gIAP in enterocytes, and the effects of CIAP and SSO on fat uptake by these cells, we next determined whether CD36 physically interacted in vivo with a murine intestinal alkaline phosphatase and if so, which one(s). As shown in Figure 3, gIAP but not dIAP specifically co-immunoprecipitated with CD36. The antiserum used to detect gIAP crossreacts with dIAP; however, the lack of any staining with the dIAP antiserum (which is specific for dIAP) indicated that all of the observed signal was accounted for by gIAP. The molecular weight range of the immunoreactive protein in the supernate was broader than that of the pellet. This was expected because of the varying amounts of post-translational glycosylation of the protein as it matures through the secretory pathway. The single high Mr band in the pellet likely corresponds to the fully glycosylated mature protein, not recently translated protein that is still undergoing modification.

To determine if gIAP could dephosphorylate mouse intestinal CD36, CD36 was immunoprecipitated from mouse small intestine in the presence of PIC 1 and 2. After immunoprecipitation and washing, the beads and their attached CD36 were incubated with purified recombinant gIAP (r-gIAP) or a recombinant catalytically inactive mutant of tissue-nonspecific alkaline phosphatase (r-TNAP<sup>D361V</sup>) that was prepared in parallel with r-gIAP. Samples were then immunoblotted for phospho-threonine. r-gIAP decreased the amount of a Thr-phosphorylated protein of 75 kilodaltons compared to beads treated with the catalytically inactive r-TNAP<sup>D361V</sup> mutant (Figure 4).

It was next determined whether a chronic HFD altered the expression of CD36, dIAP and/or gIAP. As expected, HFD mice gained significantly more weight than did NFD controls,
with the first significant difference at five weeks (Figure 5). HFD animals consumed slightly but significantly more energy over the course of the experiment, but there was no individual time point when the two groups differed significantly from each other (not shown). Plasma triglycerides and adiposity index were significantly increased by 2 weeks of HFD (Table 1). Protein levels were examined by western blot only at five weeks, the first time when all three phenotypic markers of HFD-induced adiposity were significantly increased. HFD significantly increased jejunal but not duodenal levels of CD36 and gIAP proteins at five weeks (Figure 6). By contrast, there was no effect of diet on the amount of dIAP protein in duodenum or jejunum; dIAP was not detectable under any conditions in jejunum.

The initial rate of fatty acid uptake was measured in enterocytes isolated from the jejunums of another group of mice fed either HFD or NFD as above. The body weights of the mice after 5 weeks on the diet (NFD: 25.5 ± 1.4 g, HFD: 33.3 ± 1.9 g) were significantly different and roughly similar to those of the mice in the previous experiment (Figures 5 and 6). Plasma triglycerides and adiposity index were not determined in these mice, but plasma leptin concentrations, another marker of adiposity, were significantly higher in the HFD mice as expected (NFD: 5.2 ng/mL ± 2.2 ng/mL, HFD: 19.5 ng/mL ± 3.4 ng/mL). HFD significantly increased uptake of the analog in enterocytes compared to NFD; the addition of SSO to cells from HFD animals restored uptake to levels not significantly different from untreated NFD cells (Figure 7).

To confirm whether endogenous gIAP participates in the control of the initial rate of fatty acid uptake, enterocytes isolated from duodenums and jejunums were treated with anti-gIAP, anti-dIAP or left untreated, washed to remove serum, and then incubated with C16:bodipy for uptake measurements. Enterocytes treated with antiserum that reacted with gIAP showed
decreased uptake of the analog compared to cells treated with anti-dIAP or cells left untreated (Figure 8).

**DISCUSSION**

We demonstrate that murine small intestine expresses fatty acid translocase/CD36 in its phosphorylated and dephosphorylated states, and that both forms of the protein are present in enterocytes. Treatment of isolated enterocytes with calf intestinal alkaline phosphatase results in dephosphorylation of pCD36, and is associated with an increase in fatty acid uptake; this effect is prevented by treatment with a specific inhibitor of CD36. In addition, CD36 specifically interacts with the global (g)- but not duodenal (d)- isoform of murine IAP under the conditions tested, and is dephosphorylated by recombinant gIAP. HFD specifically increases immunoreactive CD36 and gIAP protein levels, but not dIAP, in jejunum but not duodenum. Finally, HFD also increases the initial rate of fatty acid uptake in enterocytes isolated from the small intestine in a CD36- and gIAP-dependent manner. Thus, pCD36 appears to be a physiological substrate for the g-isozyme of IAP in mice. Intestinal expression of both CD36 and gIAP proteins are regulated by diet in parallel, region-specific manners and are associated with diet-driven alterations in the pattern of intestinal fatty acid uptake.

The physiological functions of CD36 in the small intestine are likely to be related to the facilitation of vectorial fat absorption. CD36 expression correlates positively with fatty acid absorption in cultured Ob17PY fibroblasts (29), and uptake of fatty acids appears to be reduced in the proximal intestines of CD36 null mice (42). Whether CD36 directly facilitates LCFA transport is unknown, although the protein can bind LCFAs (5). One model suggests that CD36 acts in concert with other plasma membrane and cytosolic fatty acid-binding proteins to bring
LCFAs into close proximity with the plasma membrane. Once there, the LCFAs may be shuttled to intracellular sites, thereby maintaining a steep inwardly-directed diffusion gradient (16). However, CD36 may also be significant in the coordination of the intracellular events required for proper chylomicron synthesis (57). The binding of LCFA to membrane CD36 has recently been demonstrated to result in internalization of the protein during the earliest phase of LCFA absorption; this event appears to be critical for initiating the events that regulate the subsequent processing of intracellular triglycerides into properly formed chylomicrons (57). The mechanism of this action is uncertain; however, LCFA activation of ERK1/2 has been show to be dependent on CD36 and is associated with the expression of proteins required for chylomicron assembly and secretion (57). Our data show that phosphorylation of CD36 inhibits LCFA uptake in the early stage of uptake, providing a possible link between gIAP, CD36, the initial phase of fat transport, and its subsequent processing.

Regardless of the precise mechanisms, the importance of ectophosphorylation in determining CD36 ligand specificity and its ability to mediate LCFA uptake has been established in other systems (3, 19, 26). Specifically, dephosphorylation of an extracellular residue of CD36 has been shown to increase palmitate uptake in platelets (19). In this study, we demonstrate that CD36 is phosphorylated in small intestinal epithelial cells, and that pCD36 is subject to dephosphorylation with CIAP and recombinant gIAP. Homogenates of small intestine include the microvasculature and, consequently, endothelial cells (44). Human dermal microvascular endothelial cells constitutively express pCD36 (26), highlighting the possibility that at least a portion of the pCD36 that we observed in preliminary immunoblots of whole tissue homogenates originated from endothelial cells. Using flow cytometry, however, we confirmed that a population of cells that stained for gIAP, which is present only in epithelial cells (25, 39, 41),
also stained for pCD36. Treatment of the cells with CIAP had no effect on the number of gIAP+ cells, as expected, but decreased the proportion of gIAP+ cells that co-labeled with pCD36. These data demonstrate that the phosphorylated ectodomain of CD36 is available as a substrate for an intestinal alkaline phosphatase.

Although the proportion of gIAP+ cells that costained with pCD36 decreased from 80.2% to 52.2%, it is possible that the decrease in CD36 phosphorylation is actually more substantial. Our preliminary western blots using pCD36 antisera were performed in conjunction with a non-phospho-peptide to reduce nonspecific binding to the non-phosphorylated form of CD36; however this peptide was not included when intestinal cells were stained for flow cytometry. The absence of this peptide might explain the modest reduction in staining observed by flow cytometry compared to the more robust change in pCD36 staining observed using anti-pThr (Figure 1A) or anti-pCD36 (plus blocking peptide) in preliminary western blots (not shown).

After determining that pCD36 was present on intestinal epithelial cells and that its phosphorylation state could be controlled by CIAP, we assayed fatty acid uptake in cells that were treated with CIAP or left untreated. CIAP-treated cells took up significantly greater amounts of the LCFA analog than did untreated cells in a dose-dependent manner, supporting the hypothesis that dephosphorylation of pCD36 enhances uptake of LCFAs in mouse enterocytes. CIAP may have dephosphorylated other membrane proteins in addition to CD36; however, SSO, a specific and irreversible inhibitor of CD36, completely eliminated the effect of CIAP on fatty acid uptake. Moreover, the effect of CIAP was specific for the long chain analog, consistent with the known ligand specificity of CD36 (5). Pretreatment of enterocytes with antisera to gIAP but not anti-dIAP decreased initial LCFA uptake, presumably by specifically immunoneutralizing endogenous gIAP and inhibiting CD36 dephosphorylation. It is also
interesting that the untreated cells took up more fatty acid than did the SSO-treated cells, suggesting some degree of constitutive CD36-dependent uptake. However, the possibility that SSO may have also inhibited additional proteins or transporters that contribute to LCFA transport cannot be discounted, although SSO has been shown to interact with CD36 but not with other proteins in adipocytes (46), which express some of the same fat-binding and transport proteins as are found on enterocytes.

The uptake assay was performed using cells in suspension rather than cells cultured as monolayers, raising the possibility that the loss of cell-cell contacts as well as apical-basolateral polarity may influence uptake kinetics or integral membrane protein location. An advantage of the cell suspension protocol, however, is that it lacks enzymatic digestions that could perturb membrane-bound protein structure. Cultured enterocytes have also been shown to dedifferentiate in culture, specifically decreasing the activity of alkaline phosphatase (48). Nonetheless, we have observed CIAP- and SSO-dependent uptake of the long chain analog in acutely plated primary cultures of mouse enterocytes (Lynes and Widmaier, unpublished observations).

The identity of the kinase responsible for phosphorylating CD36 remains unresolved, but preliminary studies of whole tissue lysates revealed an increase in pCD36 staining of lysates treated with inhibitors of alkaline phosphatases and other phosphatases. This observation led us to isolate epithelial cells in the presence of these inhibitors and suggests that kinases capable of phosphorylating CD36 are present in tissue lysates. CD36 also has ecto-NTPase activity, but no studies have demonstrated autophosphorylation of CD36 (30, 31). Identifying this kinase would help determine if a high-fat diet may also alter the rate of constitutive CD36 phosphorylation.
Given our finding that a population of enterocytes co-expressed pCD36 and gIAP, and the close proximity of these two proteins in kidney epithelial cell lipid rafts (14), we predicted that intestinal CD36 would co-immunoprecipitate with one or more of the isozymes of IAP in the murine small intestine. gIAP, but not dIAP, co-immunoprecipitated with CD36. This suggests that pCD36 could be an *in vivo* substrate for gIAP but possibly not for dIAP. That the two isozymes might have different substrates is not unexpected. The highest degree of dissimilarity between the two isozymes is in the crown region (41), a region known to determine a significant portion of substrate specificity between alkaline phosphatase isozymes and also to potentially mediate interactions between alkaline phosphatases and other protein ligands (10). If pCD36 is in fact a specific substrate of gIAP and not dIAP, the *Akp3*−/− mouse that expresses constitutively higher levels of gIAP could potentially have a higher rate of gIAP-mediated dephosphorylation of pCD36. This, in turn, may either increase the activity of CD36 as a translocase or alter its ligand specificity; in either case, it is tempting to speculate that this might partly contribute to the higher rate of fatty acid absorption observed in *Akp3*−/− mice (41). It is also worth noting that a distinct glycosylation state of gIAP co-immunoprecipitated with CD36. The molecular weight of gIAP ranges between 75 and 110 kilodaltons, increasing when the nascent protein is glycosylated. The molecular weight of the gIAP that co-immunoprecipitated with CD36 in the present experiments suggests that enterocyte CD36 physically interacts with a mature form of gIAP. This is consistent with gIAP that is expressed at the brush border membrane and not protein that has just been translated. Our results also indicate that recombinant gIAP acts to dephosphorylate a threonine residue in immunoprecipitated CD36, presumably Thr92, supporting the role of this residue in allosterically regulating the activity of CD36.
In the presence of normal biliary flow, fat absorption in the small intestine decreases as a function of the distance from the pylorus (61). However, the absorptive capacity of the distal region of the intestine increases in response to an increase in the proportion of dietary calories from fat (45). This is an example of the well-recognized plasticity of the vertebrate intestine to changes in nutrient consumption. We tested the hypothesis, therefore, that if CD36 and IAPs are part of the coordinated mechanism for maximizing fat transport across the intestinal mucosa, then expression of CD36 and IAPs in the distal regions of the small intestine should increase when mice are chronically fed an HFD. Previous studies have suggested that CD36 expression throughout the intestine is sensitive to both the total fat content of the diet (45) and to the specific fats in the diet (47). Those studies compared higher-fat diets to standard lab chows that contained less than or approximately 5% fat. In the present study, we examined changes in fat content from a low of 15% to a high of 45%; this represents the approximate range of diets prevalent in western societies today and extends beyond the currently recommended daily intake of 30%. Our data indicate that in the transition from a 15% to a 45% fat diet, the amounts of CD36 and gIAP proteins, but not dIAP protein, were significantly and specifically increased in the jejunums of mice. Therefore, regional, parallel changes in CD36 and gIAP proteins may be key events in the adaptation of the intestinal mucosa to high-fat diets in mice. This is supported by the observation of increased transport of the LCFA analog into isolated enterocytes from HFD mice. These observations may explain in part our previous observation that mice consuming a high-fat diet absorb the same percentage of ingested energy as mice fed a regular diet [25]. Whether this contributes to the advent of obesity that occurs in response to HFD remains to be determined; notwithstanding, any adaptation that promotes an increase in the absorptive capacity of the distal regions of the intestine may be acutely adaptive, but maladaptive in situations of
chronic high-energy intake. Whether the ratio of phosphorylated to dephosphorylated CD36 changes \textit{in vivo} under these conditions is uncertain. However, because the absolute amounts of both CD36 and gIAP proteins are increased with HFD, it is likely that even in the presence of an unchanged relative amount of pCD36/CD36, the absolute amount of dephosphorylated protein would be increased, accounting in part for the observed increase in LCFA uptake capacity.

\textbf{Perspectives and Significance}

These observations may explain in part our previous observation that mice chronically consuming a high-fat diet absorb the same percentage of ingested energy as mice fed a regular diet (56). Whether this contributes to the advent of obesity that occurs in response to HFD remains to be determined; notwithstanding, any adaptation that promotes an increase in the absorptive capacity of the distal regions of the intestine may be acutely adaptive, but maladaptive in situations of chronic high-energy intake.
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DISCLOSURES
The authors have nothing to disclose.
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**Table 1.** Effect of high-fat diet (HFD) on plasma triglycerides and adiposity index (combined weight of perirenal and inguinal fat pads divided by residual carcass mass x 100). Values are means and SE of 6 animals per group. Asterisks represent values that were significantly different (at least p<0.05) compared to normal-fat diet (NFD) controls at that timepoint.
REFERENCES


Figure legends

**Figure 1: Immunoreactive CD36, its sensitivity to dephosphorylation, and its localization to enterocytes in mouse small intestine.** a.) Small intestines from adult male mice were homogenized in the presence of PIC 1 and 2 and then washed to remove the inhibitors. CD36 was immunoprecipitated from the homogenates, and then the immunoprecipitates were incubated for 25 minutes at 37°C with or without 100 units calf intestinal alkaline phosphatase (CIAP); samples were immunoblotted for phosphothreonine, stripped and then reprobed for total CD36. The molecular weights of the CD36 and p-Thr bands were identical. b.) Acutely isolated intestinal epithelial cells pooled from the first 10 cm of the intestine were incubated for 25 minutes at 37°C in the presence or absence of 100 units of CIAP, then incubated with antisera to the enterocyte marker gIAP and to pCD36. After gating for gIAP-positive cells, pCD36 staining in the absence or presence of CIAP treatment is shown. In both histograms, a gate was set using unstained controls (dark horizontal line) and the percentage of gIAP+ cells that also stained positive for pCD36 above this control is indicated (80.2 and 52.2 percent, respectively). The upward spike in both panels represents the no primary antibody control for pCD36 staining. The y axis represents the percentage of maximum staining for pCD36.

**Figure 2: Uptake of a fluorescently-labeled LCFA analog into isolated mouse enterocytes.** a.) Cells prepared from pools of combined duodenums and jejunums were incubated with vehicle, CIAP (100 units), or CIAP plus the CD36 inhibitor SSO (0.5 mM) followed by a C16:bodipy-labeled LCFA analog. Intracellular fluorescence is given in arbitrary fluorescence units (AFU) and is an indicator of uptake of the analog. Each point is the mean and SE from three pools of mice run separately, each with three technical replicates per experiment. The CIAP-treated cells had significantly greater uptake than vehicle or CIAP/SSO-treated cells. b.) There was no effect of CIAP on the initial rate of uptake of a short chain C5:bodipy analog; the data for the C16 analog are reproduced from the initial timepoint in part (a) and are normalized to the untreated values for each analog. Asterisks indicate values significantly different (p<0.001) compared to the respective untreated group. c.) The effect of CIAP on uptake of the
C16:bodipy analog was concentration-dependent. Asterisks signify values significantly different each other (p<0.001).

**Figure 3: CD36 co-immunoprecipitates with gIAP but not dIAP.** CD36 was immunoprecipitated from duodenal tissue homogenates. Nonimmune normal goat serum was used as negative control. The resulting pellets and supernates were prepared for western blotting. Membranes were stained for either gIAP or dIAP. Both phosphatases are extensively post-translationally modified and show an apparent molecular weight range between 75 and 110 kDa. The most mature form (110 kDa) of gIAP coimmunoprecipitated with anti-CD36. The bands in the supernate lanes represent IAP that did not immunoprecipitate and were therefore not associated with CD36; the broad spectrum of Mr of those bands presumably reflects IAP of varying glycosylation states. Loading amounts between supernate and pellet are not quantitative with respect to each other. The blot shown is representative of three separate experiments.

**Figure 4: Recombinant gIAP can dephosphorylate CD36 at a threonine residue.** CD36 was immunoprecipitated from duodenal tissue homogenates. The resulting pellets were treated with either a recombinant catalytically inactive tissue-nonspecific alkaline phosphatase (r-TNAPD361V) or recombinant gIAP (r-gIAP), and prepared for western blotting. Membranes were stained with antiserum that recognizes phosphothreonine residues, then stripped and re-stained for total CD36 as a loading control. The blot shown is representative of three separate experiments.

**Figure 5: Body weights of C57Bl/6 mice fed either a normal-fat diet (NFD) or a high-fat diet (HFD).** Twenty four mice began the experiment in each diet group; the mice were randomly assigned into one of four groups to be sacrificed at 1, 2, 5 and 9 weeks after beginning the diets. Body weights significantly diverged by 5 weeks of treatment. Values are the mean and SE of 24, 18, 12 and 6 animals at each successive timepoint.

**Figure 6: Duodenal and jejunal expression of CD36 (A and B), gIAP (C and D) and dIAP (E and F) in mice fed either a normal-fat diet (NFD) or a high-fat diet (HFD) for 5 weeks.** (Animals are those that were sacrificed at the 5 week timepoint in Figure 5). Mice were fasted
overnight prior to sacrifice. Duodenal (A, C and E) and jejunal (B, D and F) tissue homogenates were prepared for western blotting. Membranes were stained for CD36, gIAP or dIAP, then stripped and reprobed for β-actin. Densitometry of 6 samples is shown as the ratio of each target to β-actin (*, p < 0.01). dIAP was not detectable (ND) in jejunal samples from mice fed either diet.

Figure 7: Effect of HFD on uptake of C16:bodipy fatty acid analog in isolated enterocytes. Following five weeks on either NFD or HFD, mice were euthanized and the jejunums were dissected for epithelial cell isolation and fatty acid uptake experiments (AFU, arbitrary fluorescence units). The bars represent data from three replicate experiments. Cells were not treated at any time with IAP, but were treated with the CD36 inhibitor SSO or its vehicle control (DMSO). The effect of HFD was significant (asterisk); SSO treatment of HFD cells reduced uptake to levels that were no longer significantly different from NFD controls (no SSO).

Figure 8: Effect of anti-alkaline phosphatase serum on uptake of C16:bodipy fatty acid analog in isolated enterocytes. Mice were euthanized and the duodenums and jejunums were dissected for epithelial cell isolation and fatty acid uptake experiments. The bars represent data from three replicate experiments, with the untreated control group normalized to 1.0 arbitrary fluorescence units for comparative purposes across experiments (*, p < 0.05).
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**Size:**
- 110 kDa
- 75 kDa
Relative AFU

Untreated (PBS)  Anti-dIAP  Anti-gIAP

0.75  0.5  1  1.25